DNA PROFILING AND POPULATION HISTORY IN CONSERVATION

ASSESSMENT OF DNA PROFILING IN RECONSTRUCTING THE HISTORY OF NATURAL POPULATIONS AND IDENTIFYING CONSERVATION UNITS

By

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ABSTRACT

The fundamental objective of conservation genetics is the identification of the basic units of conservation. Central to this objective is the reconstruction of the adaptive and evolutionary history of populations to evaluate their conservation status. Evolutionary history involves both microevolutionary and macroevolutionary processes and adaptive history is the evolution of specific characters to selective ecological processes in differential heterogeneous environments. Neutral DNA markers such as mitochondrial DNA, minisatellites and microsatellites are most often used for reconstructing history and identifying conservation units. This thesis examined three biological systems: 1) an African cichlid, 2) Canadian moose populations and 3) eastern North American wolves and coyotes to test two hypotheses. Firstly, neutral DNA markers can be used to accurately reconstruct the evolutionary history of populations. Secondly, neutral DNA markers are concordant with adaptive distinctiveness in reconstructing the adaptive history of populations. Few studies have examined these relationships. Lake Magadi tilapia showed discordant patterns between adaptive morphological, physiological and behavioural characters and genetic structure assessed with mitochondrial DNA. I propose this discordance has resulted from selection acting on mitochondrial DNA that has often been assumed to be "neutral". Neutral DNA markers accurately reflected the known history of the moose populations but discordant patterns were observed between neutral and functional loci indicating the former may not accurately reflect adaptive variation. DNA profiles of eastern wolves and coyotes showed a significant conflict in the interpretation of mtDNA and microsatellite data compared to previous genetic studies that examined wolf taxonomy. The data were consistent with the hypothesis of a North American-evolved wolf. Coyote-like mtDNA was not of coyote origin but represented divergent but related sequences of a North American wolf lineage independent of the gray wolf (*C. lupus*). Under this new model of eastern wolf evolution, we also identified the hybrid origin of eastern coyotes, contrary to previous interpretations, and genetically characterised different wolf "types" within Ontario. These findings could not reject the first hypothesis as neutral markers were used to reconstruct the histories of the three biological systems. However, the findings identified that it is

important to ensure the neutrality of DNA markers and that samples are representative of the taxa under investigation. The findings in this thesis did not support the second hypothesis, as neutral DNA markers were not concordant with adaptive characters, i.e. morphology, physiology and functional genetic markers.

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Preface

This thesis is organised into nine chapters. The first and ninth chapters are a general introduction and general discussion, respectively, providing an overview of the thesis and a synthesis of the results. Chapters two through eight have been written as manuscripts for submission to peer-reviewed journals.

Chapter 2: Genetic structure of Lake Magadi tilapia (*Oreochromis alcalicus grahami*) populations

Authors: P.J. Wilson, C.M. Wood, J.N. Maina and B.N. White

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Chapter 3: Discordance between genetic structure and morphological and physiological adaptation in Lake Magadi tilapia

Authors: P.J. Wilson, C.M. Wood, P.J. Walsh, A.L. Narahara-Bergman, H.L. Bergman, P. Laurent and B.N. White

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Contribution: Specimens were collected by PJW, CMW, PJW, AB, HB and PL. DNA profiles and a number of physiological experiments were performed by PJW. Data analysis and the writing of this paper were performed by PJ Wilson (genetics), CMW and PJ Walsh (physiology), AB and PL (morphology). BNW and CMW provided logistic and financial support. The research was conducted under the supervision and guidance of BNW and CMW

Chapter 4: Genetic variation and population structure of moose (*Alces alces*) at neutral and functional DNA loci

Authors: P.J. Wilson, S. Grewal, A. Sipak, A. Rodgers, R. Rempel, J. Saquet, H. Hristienko, F. Burrows, R. Peterson and B.N. White.

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Chapter 5: DNA profiles of the eastern Canadian wolf and the red wolf provide evidence for a common evolutionary history independent of the gray wolf.

Authors: P.J. Wilson, S. Grewal, I.D. Lawford, J.N.M. Heal, A.G. Granacki, D. Pennock, J.B. Theberge, MT Theberge, D.R. Voigt, W. Waddell, R.E. Chambers, P.C. Paquet, G. Goulet, D. Cluff and B.N. White

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Chapter 6: Mitochondrial DNA extracted from eastern North American wolves killed in the 1800s is not of grey wolf origin.

Authors: P.J. Wilson, S. Grewal, T. McFadden, R.C. Chambers and B.N. White.

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Chapter 7: Genetic evidence for the origin of the eastern coyote by hybridisation between western coyotes and the eastern timber wolf.

Authors: P.J. Wilson, S. Grewal, A. Granacki, K. Shami, R.C. Chambers, P.C. Paquet, J. B. Theberge, M.T. Theberge, B. Kelley, W. Waddell, M. Dumond and B.N. White.

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Chapter 8: Characterization of wolves across Ontario using mitochondrial and microsatellite DNA profiles.

Authors: S.K. Grewal, P.J. Wilson, A.D. Granacki, J.N. Heal, I. Lawford, T.L. Hillis, F.F. Mallory, H. Sears, M.T. Theberge, J.B. Theberge, D.R. Voigt, F. Burrows and B.N. White

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Chapter 1

General Introduction

The global loss of biological diversity is occuring at an unprecedented rate primarily as a result of human expansion and exploitation. As a result there is a recognised need to conserve biodiversity at three biological levels: ecosystems, species and genes. Population genetics is directly related to the latter two levels, and both are inextricably linked to the third. Anthropogenic impacts on ecosystems and species through development and overexploitation of biota further alter ecological and genetic systems and thereby increase the risk of extinction and the decline in the number of species (O'Brien 1995b, Frankham 1995, Lande 1998). Human activities that result in habitat destruction and fragmentation intensify the risk of environmental stochastic processes and genetic processes such as hybridisation and inbreeding (Lande 1998). The importance of genetic analyses to assessing biodiversity has resulted in the application of molecular and population genetics to conservation biology (O'Brien 1994a).

The initial applications of DNA markers to address questions in natural non-human populations using mitochondrial DNA (Avise et al. 1986), single-locus nuclear loci (Quinn and White 1987), and multi-locus DNA fingerprinting (Burke and Jeffreys 1986) identified the potential for molecular genetics to answer a wide range of ecological questions (Avise 1994, Ferris and Palumbi 1996). One significant application was the early use of DNA markers to assess the genetic variation of endangered species including the cheetah (Yuhki and O'Brien 1990, Menotti-Raymond and O'Brien 1993), the Puerto Rican parrot (Brock and White 1991), the North Atlantic right whale (Schaeff et al. 1997) and the St. Lawrence beluga whale (Patenaude et al. 1994).

The application of DNA markers to address ecological and conservation questions has emerged into the fields of **molecular ecology** and **conservation genetics**. The field of molecular ecology embodies the application of DNA markers to a range of ecological and evolutionary questions (Carvalho 1998). The

term "molecular ecology" has a wide usage as described in the aims and scopes of the journal ***Molecular Ecology*** (Est. 1992) – "directed at the interface of molecular biology with ecology, evolution, and population biology including [population structure and phylogeography, population genetic theory, conservation genetics, ecological interactions and molecular adaptation]".

Although presented as a component of molecular ecology, conservation genetics has been considered an applied science in its own right (O'Brien 1994a, Frankham 1995, Carvalho 1998). This is reflected in the recent expansion of a number of disciplines and journals focusing on the role of biology in conservation biology. ***Animal Conservation*** (Est. 1998) – "factors which influence the conservation of animal species and their habitats...ecology, behavioural ecology and wildlife biology; wildlife disease and epidemiology; evolutionary ecology and genetics; population biology; systematic biology and phylogenetics; biodiversity and biogeography". ***Conservation Ecology*** (Est. 1999) – "the conservation of ecosystems, landscapes, species, populations, and genetic diversity; the restoration of ecosystems and habitats; and the management of resources." ***Conservation Genetics*** (Est. 1999) – "Conservation issues are multifaceted and incorporate diverse disciplines [population genetics, molecular ecology and biology, evolutionary biology, and systematics]". The mandate of these journals includes a multifaceted approach incorporating multiple disciplines with genetic profiles for the purpose of conserving biodiversity.

The theory of applying molecular genetic markers to ecological based questions, including conservation biology, is rooted in the Ecological Genetics School (1950s-1970s) that emphasised studies on "adjustments in adaptations of wild populations to their environment through field and laboratory work" (Carvalho 1998). The fundamental approach of ecological genetics is the integration of interactive studies on genotype-environment studies to examine the dynamic nature of genetic structure in relation to environmental heterogeneity with a focus on populations as evolutionary units. This approach has been adopted by conservation genetics in the incorporation of a number of disciplines – ecological, physiological, morphological, behavioural – with genetic data (O'Brien 1994a, O'Brien 1994b, Carvalho 1998, Bowen 1999, Crandall et al. 2000) to identify units of conservation.

Ultimately, it is this identification of the smallest units of conservation that is the fundamental objective of conservation genetics. Basic to this objective is the reconstruction of the adaptive and

evolutionary history of populations to determine their conservation status (O'Brien 1994a, Volger and DeSalle 1994). The integration of multiple aspects of biology reflecting adaptive variation, ecology, morphology, physiology and behavior, with genetics is recommended to make realistic approximations of population history (O'Brien 1994a).

In the context of this thesis, evolutionary history involves both microevolutionary and macroevolutionary processes. Microevolutionary processes involve changes in genetic structure as a result of population demographic events including geographic separation, migration and population bottlenecks or expansions (O'Brien 1994a, Carvalho 1998). Macroevolutionary processes involve reproductive isolation and speciation among groups of organisms (Carvalho 1998).

Adaptive history is the evolution of specific characters to selective ecological processes in differential heterogeneous environments. Adaptive and ecological divergence can represent the evolution of ecological roles and associated adaptations in different species or among populations within a species (Givnish 1997). Adaptive history reflects natural history and is measured in terms of ecology, morphology, physiology and behaviour (O'Brien 1994a).

Consistent with a multidisciplinary approach are the criteria for the most commonly used operational term or definition of a conservation unit, the **evolutionary significant unit** (ESU). The need for an ESU concept stemmed from the need to describe evolutionary distinct groups when information about taxonomy was inadequate or too controversial based on evolutionary "distinctiveness" or "significance" (Ryder 1986, Volger and DeSalle 1994). The objective of the ESU concept is the preservation of unique ecological adaptations and the maintenance of evolutionary potential, although since its inception, the concept has evolved a number of different approaches (Ryder 1986, Waples 1991, Dizon et al. 1992, Volger and DeSalle 1994, Moritz 1994a).

The "Evolutionary Significant Unit" was first introduced based on concerns of what to preserve within zoos and to further address issues of potential hybridisation within captive breeding programs (Ryder 1986). The ESU represented an intra-specific conservation unit that required information on natural history, morphology, geographic distribution and genetic information. Furthermore, the criterion for the original ESU classification was that these multiple data sets be concordant.

Waples (1991) applied the ESU concept to salmonid stocks, defining the ESU as a population that is distinct based on its "evolutionary legacy". While the ESU again represented an intra-specific measure of adaptive distinctiveness, isolation was added as a prerequisite for biological uniqueness. Specifically, an ESU was defined as a "population (or group of populations) that (1) is substantially reproductively isolated from other conspecific population units, and (2) represents an important component in the evolutionary legacy of the species". Isolation acknowledged local population adaptations as reservoirs for unique genetic variation and both genetic and phenotypic data (morphology, physiology, behaviour and life history traits) were used to identify isolation and adaptive significance.

Dizon et al. (1992) expanded on Waples (1991) ESU criteria by identifying proxies required to establish adaptive uniqueness. The criteria remained similar with respect to reproductive isolation and differential selective pressures resulting in adaptive significance being required for ESU status. Four categories or proxies for differential selection were proposed: 1) distribution data, e.g. geographic barriers; 2) population response data, i.e. demography; 3) phenotypic data, e.g. morphology and physiology; and 4) genotypic data, i.e. neutral genetic markers indicating isolation or evolutionary divergence. Following this categorisation, a decision is based on the phylogeographic classification incorporating evolutionary uniqueness based on phenotypic or genotypic data (phylogeny) and distribution data (geography). This approach was based on phenetic similarity or dissimilarity to imply reproductive isolation.

Volger and DeSalle (1994) incorporated the phylogenetic species concept into identifying conservation units. Instead of reproductive isolation or phenetic similarity, the definition of an ESU was proposed to utilise shared diagnosable characters, similar to a cladistic approach to phylogenetic species classification. Volger and DeSalle (1994) made a distinction between "phylogenetic species" and "evolutionary significant units" classifying ESUs based on evolutionary distinctiveness that warrants separate protection. Diagnosable characters were required to be shared among all individuals of a potential conservation unit and this criterion did not require the reconstruction of a phylogeny to classify a population or set of populations as an ESU, particularly when using ecological, morphological or behavioural characters.

Moritz (1994a, 1994b, 1995, 1999) proposed a similar cladistic approach, where a criterion of reciprocal monophyly at mitochondrial DNA and significant allele frequency differences at nuclear loci were used to establish ESU status. This definition was based on the premise that populations have been separated for a long time and have a high probability of maintaining evolutionary uniqueness based on adaptation to differential environmental conditions. However, Moritz's (1994a, 1994b) criterion focused on historic isolation but did not include any non-genetic characters to infer adaptive distinctiveness. For populations that do not demonstrate reciprocal monophyly of mtDNA haplotypes, significant haplotype or allele frequency differences warranted a lower classification of Management Units (MU).

An ESU concept based on historic isolation and divergence as a requirement for evolutionary and adaptive divergence (Moritz 1994a) has tended to shift this unit of conservation away from intra-specific comparisons to higher taxonomic classifications of sub-species or species level classification. This is reflected in the divergence times required for reciprocal monophyly to evolve. These studies represent a shift from intra-specific micro-evolutionary processes to speciation events or macro-evolutionary processes, corresponding to a shift in populations to (sub)species as the minimal units of conservation.

Furthermore, despite the original objective of the ESU concept of concordance among multiple data sets (Ryder et al. 1986) and the modified version of this objective in subsequent ESU criteria (Dizon et al. 1992, Vogler and DeSalle 1994), few studies have made such comparisons (Carvalho 1998, Crandall et al. 2000), with some exceptions (e.g. Wang 1999). In fact, the majority of molecular ecology or conservation genetic studies examining ESU status have applied the Moritz (1994a) criterion to establish conservation priorities solely on neutral genetic markers (Legge et al. 1996, Duvernall and Turner 1998, Barratt et al. 1999, Bennetts et al. 1999, McLean et al. 1999, Sherwin et al. 2000). Future priorities in molecular genetic analysis of adaptation have been identified in a recent review as the ultimate aim of molecular ecology to promote an understanding of the origins and significance of adaptive variation in natural populations (Carvalho 1998).

Not strictly limited to studies examining ESU status, the exclusive use of DNA markers in conservation studies is widespread. Neutral DNA marker systems have been used to assess population structure and levels of gene flow and genetic variation within populations to assess population history

(O'Brien 1994a, Frankham 1995, Lacy 1997). Genetic variation can be reduced due to population events such as bottlenecks and isolation and low levels of genetic variation have been used to identify intra-specific conservation units (Patenaude et al. 1994, Lacy 1997), independent of assigning ESU status. Genetic variation, specifically heterozygosity, can also be depleted by inbreeding (mating between relatives) resulting in reduced fitness including effects such as lower reproduction potential and increase disease susceptibility (Lacy 1987, Frankham 1995, Lacy 1997). Furthermore, low levels of genetic diversity can decrease the short- and long-term potential for adaptation to environmental change (Lande and Shannon 1996, Lacy 1997). The majority of studies examining genetic variation have utilised neutral genetic markers as representative of overall genome-wide variation.

The importance of reconstructing population history has been valuable in conservation genetics, particularly in phylogeography. Phylogeography is a sub-discipline of biogeography that examines geographic distributions of genealogical lineages in a historical and spatial framework (Avise 1998, Avise 2000). Reconstruction of lineage distributions, similar to conservation genetics, relies on incorporating molecular and population genetics, demography, paleontology and historical geography. This approach provides a link between population genetics and systematics integrating microevolutionary and macroevolutionary processes, respectively (Bermingham ad Moritz 1998).

A recent opinion article by Bowen (1999) suggests a trichotomy has emerged within conservation genetics regarding the priorities that are used to determine conservation strategies. Conservation genetics was been identified to serve one of three goals corresponding to systematics, ecology and evolution (Bowen 1999). Systemitists would conserve distinct taxa, ecologists would focus on ecosystem protection and evolutionists focus on protecting the genetic diversity that allows biota to adapt to new conditions. Systematics, ecological genetics and evolution further relate to adaptive radiation, ecological adaptations and adaptive potential, respectively. The conclusion of this opinion article was that molecular genetics served all three goals equally and all are relevant criteria for assigning conservation status (Bowen 1999).

At present, the application of neutral DNA markers to defining conservation units, assessing genetic variation and reconstructing population history is based on three assumptions: 1) Independent neutral DNA marker systems consistently reflect population history (King and Burke 1999). 2) Neutral

genetic variation reflects adaptive genetic variation (King and Burke 1999, Hedrick 1999). As previously stated few studies have examined this relationship (Carvalho 1998, Crandall et al. 2000). 3) Isolation, reproductive or geograhic, is an essential criterion for evolutionary divergence and subsequent adaptive distinctiveness (Waples 1991, Dizon et al. 1992, Moritz 1994a).

The general objective of this thesis is to assess DNA markers in reconstructing population history and identifying units of conservation. Two specific hypotheses will be tested:

1. Neutral DNA markers can be used to accurately reconstruct the evolutionary history of populations.

2. Neutral DNA markers are concordant with adaptive distinctiveness in reconstructing the adaptive history of populations.

In testing the above hypotheses, this thesis will further assess the criteria for assigning conservation priorities or conservation units using specific criteria such as isolation, amount of divergence, adaptive history or adaptive potential.

Three species groups have been analysed to address the above objectives: 1) an African Rift Valley cichlid, specifically the Lake Magadi tilapia (*Alcolapia grahami*, formally *Oreochromis alcalicus grahami*); 2) Moose (*Alces alces*) with emphasis on Canadian populations; and 3) Eastern North American wolves and coyotes (*Canis* sp.).

Relevance of Lake Magadi Tilapia

African cichlids inhabiting the Rift Valley Lakes of Malawi, Victoria and Tanganyika demonstrate extraordinary rates of adaptive radiation (Meyer 1993). Consistent with these findings are the Magadi tilapia that represent a remarkable example of adaption in an extreme environment. Lake Magadi in Kenya is a soda lake with typical conditions including water pH 10, titration alkalinity > 300 mM, osmolality 525 mOsm, temperatures ranging from 32-42^{O}C, and O_2 levels fluctuating diurnally between extreme hyperoxia and anoxia. Adaptations to these environmental conditions may have evolved within the last 5,000-10,000 years based on the geological evidence of the Magadi lake basin (Butzer et al. 1972, Goetz and Hillaire-Marcel 1992). These adapations include the excretion of urea instead of ammonia

(Randall et al. 1989, Wood et al. 1989, Wood et al. 1994), the use of the swim bladder as an accessory air-breathing organ (Maina et al. 1995) and a modified gill structure (Laurent et al. 1995, Maina et al. 1996). Lake Magadi is separated into lagoon systems by precipitated bicarbonate called 'trona'.

A number of tilapia populations exist in various thermal spring lagoons around the margin of the lake, in apparent isolation from one another, separated by kilometers of solid trona crust (floating Na_2CO_3) underlain by anoxic water. Despite the apparent isolation of different populations, annual floods may provide opportunities for exchange of fish across the surface of the trona, and subsequent gene flow. The proposed history of these lagoon populations states 'if it were not for the periodic gene flow allowed by flood connections, between otherwise isolated fish communities, no doubt sub-specific differences might well have arisen in the area' (Coe 1966). However, morphological gill adaptations observed between two of the lagoon populations (Maina et al. 1996) strongly support population differentiation in the absence of gene flow. Furthermore the majority of adaptations described for *A. grahami* have been based on the examination of one lagoon population, the Fish Springs Lagoon population demonstrating the potential for additional adaptations in other lagoon systems.

I tested the hypothesis there is extensive migration and gene flow among *A. grahami* following annual flooding events during the rainy seasons resulting in no differentiation in phenotypic characters among lagoon populations.

The objective of this study was to characterise *Alcolapia grahami* lagoon populations using DNA markers and morphological and physiological characteristics. The first study examined fish from two Magadi lagoon populations, the Fish Springs Lagoon and the South East Lagoon, and fish representing *Alcolapia alcalicus* from Lake Natron, Tanzania (Chapter 2). The second study examined six Lake Magadi lagoon populations, fish from Little Magadi, a small lake north of Lake Magadi and *Alcolapia alcalicus* from Lake Natron (Chapter 3). The relevance of these studies to the objectives of the thesis include:

1. The question of isolation vs. gene flow among Lake Magadi lagoon populations allows the reconstruction of population history in an essentially non-disturbed biological system with a well-established geologic time line for the history of the lake ecosystem.

2. Lake Magadi tilapia provides an opportunity for comparison of neutral DNA markers to adaptive traits.

3. African cichlids in general represent an important speciose taxonomic group to assess both adaptive history and adaptive potential.

Relevance of Canadian Moose Populations

Conservation genetics has often focused on endangered species and populations. While in general not as heavily impacted by human activities as some endangered or threatened taxa, management strategies are required for game species to ensure sustainable harvesting. An assessment of gene flow and genetic variation using DNA markers can be useful in making recommendations for the management of sustainable harvests.

Moose are an example of a wildlife species that has been extensively studied due to its importance as a game animal (Timmerman and Buss 1998). Managers have utilised new technologies for management purposes, including DNA markers (Rodgers et al. 1998). Furthermore, the geographic range of moose extends into protected parks allowing the opportunity to examine differential local selection pressures. The relevance of a genetic characterisation of moose populations (Chapter 4) to the objectives of the thesis include:

1. Moose represent a game species that is subject to intermediate human disturbance through hunting and habitat modifications (Goddard 1970, Rempel et al. 1997) as well as natural impacts such as disease (Whitlaw and Lankester 1994). The proposed histories of a number of populations, i.e. isolation, small founding events, continuous distribution and protected park systems, provides an opportunity to assess DNA markers in reconstructing these histories.

2. Genetic analyses of moose comparing neutral and functional DNA markers with regard to population history allows an assessment of the concordance between neutral and adaptive genetic variation.

3. An assessment of moose population structure and genetic variation provides a valuable model for considering appropriate intra-specific conservation units and evaluating the roles of

isolation, adaptive history and adaptive potential when developing management strategies in the context of microevolutionary processes.

Relevance of Eastern North American Wolves and Coyotes

North America presently has three species in the genus *Canis*: the gray wolf (*Canis lupus*); the coyote (*C. latrans*); and the red wolf (*C. rufus*). The distribution of these three species has been significantly altered by human disturbances such as deforestation, farming, and hunting. Historically gray wolves (*C. lupus*) maintained a wide geographic distribution throughout North America but following European settlement the species has been extirpated from much of its original range (Goldman 1944) resulting in fragmented and isolated populations (Wayne et al. 1992). The historical range of coyotes (*C. latrans*) was dry arid regions in the southcentral and southwestern US, however, the decline in wolf numbers and the deforestation of the landscape to more agricultural use has resulted in the expansion of coyotes through much of North America (Moore and Parker 1992). Similar to the gray wolf, the red wolf (*C. lupus*) has seen a significant decline in numbers resulting in its endangered status and the subsequent recovery efforts by the USFWS through captive breeding and re-introduction programs (Goldman 1944, Nowak 1979, Nowak et al. 1995).

Wolves have been of great interest to taxonomists and the classification of North American wolves have undergone a number of changes in the past hundred years. Early morphological studies led to the recognition of 24 gray wolf subspecies in North America (Goldman 1944, Brewster and Fritts 1995). Recently, based on skull measurements, Nowak (1995) suggested five groupings of *C. lupus* subspecies. In addition to the gray wolf, the red wolf has received much attention from wolf taxonomists. Nowak (1979) has suggested that in contrast to the gray wolf, the red wolf is a North American evolved canid based on morphological characters. Wayne, using genetic markers, has hypothesized the red wolf is a hybrid resulting from the interbreeding of gray wolves and coyotes (*C. latrans*)(Wayne and Jenks 1991, Roy et al. 1994, Roy et al. 1996). This theory is based on the absence of mtDNA and microsatellite sequences from red wolf samples that were not of either gray wolf or coyote descent (Wayne and Jenks

1991, Roy et al. 1994, Roy et al. 1996). The origin of the red wolf, *C. rufus*, has continued to be a source of debate (Nowak and Federoff 1998, Wayne et al. 1998).

Ontario is an important example of the complexity of taxonomic classification of *Canis* species. Ontario represents an extensive history of wolf research and a series of studies have assessed the morphology of *Canis* and identified the variable nature of the wolves and coyotes (Kolenosky and Stanfield 1975, Schmitz and Kolenosky 1985). The morphology of the Ontario *Canis* complex includes a Boreal type wolf, an Algonquin type wolf, coyotes and intermediate forms of these three types. Both hybridisation and prey selection have been proposed to cause the different morphologies (Kolenosky and Stanfield 1975, Schmitz and Kolenosky 1985). As described above, anthropogenic factors have allowed the expansion of coyotes into the eastern wolf range leading to inter-specific mating (Lehman et al. 1991, Wayne and Lehman 1992).

The present classification of *Canis* types must be seen in the context of a highly disturbed ecosystem with severely impacted species. The loss or change in distributions and habitat and the existence of hybridisation significantly affect the attempts to reconstruct the history of wolves and coyotes in North America.

From a historical context, a smaller eastern North American wolf has been described since the late 1700s which precedes the arrival of coyotes in the eastern part of the continent (Goldman 1944). This eastern timber wolf (also called the eastern Canadian wolf) has also undergone a number of taxonomic reclassifications. Similar to the present status of the red wolf, the eastern timber wolf was originally recognised as a distinct species (*C. lycaon*) (Miller 1912). It has since been moved to its currently accepted gray wolf subspecies designation *C. l. lycaon* (Brewster and Fritts 1995). Based on morphology the historic range of the eastern timber wolf is thought to have been southeastern Ontario and southern Quebec to the north, extending south in the United States to an undetermined point near North Carolina (Brewster and Fritts 1995, Nowak 1995). Nowak (1979) predicts the eastern timber wolf (*C. l. lycaon*) and red wolf (*C. rufus*) existed in a southern refugium during the Pleistocene glaciation that subsequently led to its historical distribution following the retreat of the ice sheet. This southern refuge overlaps with the recent red wolf distribution predicted to have included the southeastern States including southeastern Kansas and

central Texas to Pennsylvania and Florida (Nowak 1995). Previous classification of *C. l. lycaon* by Hall and Kelson (1959) places this wolf in southern Manitoba and Minnesota.

Both the eastern timber wolf and the red wolf readily hybridise with coyotes (Lehman et al. 1991, Wayne and Jenks 1991, Wayne and Lehman 1992, Roy et al. 1994, Roy et al. 1996). Based on mtDNA analysis the presence of coyote genetic material in wolves was observed in northwestern Ontario, Algonquin Provincial Park, southern Quebec, and Minnesota (Lehman et al. 1991). Introgression of coyote DNA into red wolves has been shown using mtDNA analysis of animals from the red wolf captive-breeding program (Wayne and Jenks 1991). These results further suggest the potential for a common evolutionary relationship between the eastern timber wolf and red wolf since hybridisation appears to be limited to eastern North America despite the potential for coyote/gray wolf hybridisation in the west (Roy et al. 1994). The potential similarity between the eastern wolves offers an opportunity to evaluate the contradicting hypotheses on their origins and taxonomic status and attempt to reconstruct the history of "hybridising" wolves.

Similar debates have surrounded on the eastern coyote, particularly whether the introgression of wolf genetic material has caused the larger body size observed in the eastern range of *C. latrans* (Silver and Silver 1969, Mengel 1971, Lawrence and Bossert 1975, Schmitz and Kolenosky 1985, Nowak 1979, Lariviere and Crete 1993) or whether prey selection is the underlying cause of the increased size (Kolenosky and Stanfield 1975, Schmitz and Kolenosky 1985, Schmitz and Lavigne 1987). The original genetic data supported the lack of introgression of wolf genetic material into eastern coyotes, although limted samples were analysed from eastern regions (Lehman et al. 1991, Wayne and Lehman 1992, Roy et al. 1994). The ecological role of this canid in eastern North American as a deer predator, makes an accurate reconstruction of the eastern coyotes history important to predict the interactions with animals used in wolf recovery programs.

In this thesis DNA profiling was used to characterise eastern wolves and coyotes and to reconstruct the history of eastern North American *Canis*. Firstly we examined the hybridising wolves, the eastern timber wolf (*C. l. lycaon*) and the red wolf (*C. rufus*) to assess their relationship and to reconstruct their taxonomic history (Chapters 5 and 6). Secondly, we examined the genetics of the "Tweed" wolf, a

small canid south of Algonquin Provincial Park, and the eastern coyote to test for introgression of wolf genetic material (Chapter 7). Thirdly, we analysed a broad geographic distribution of Ontario *Canis* types to genetically characterise the wolf "types" described by Kolenosky and others (1975, 1985) throughout the province (Chapter 8). The relevance of these studies to the objectives of the thesis include:

1. Eastern wolves and coyotes represent a difficult biological situation for reconstruction of their historical background. This reconstruction requires consideration at the macro-evolutionary, species, level but also at the micro-evolutionary level due to extensive hybridisation. Future conservation plans relating to wolves are dependent on an accurate assessment of the history of *Canis* species, subspecies and populations.

2. Due to the heavily impacted nature of North American *Canis*, the importance of adaptive history and adaptive potential need to be assessed for future recovery and management plans. The analysis will focus on the impact of hybridisation and its adaptive significance to eastern wolves/coyotes and to identifying units of conservation.

Chapter 2

Genetic Structure of Lake Magadi Tilapia (*Oreochromis alcalicus grahami*) Populations

Genetic Structure of Lake Magadi Tilapia (*Oreochromis alcalicus grahami*) Populations

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ABSTRACT

Lake Magadi in Kenya is a soda lake inhabited by the tilapia *Oreochromis alcalicus grahami*, a sub-species of tilapia adapted within the last 5,000-10,000 years to conditions of high alkalinity, high pH (9.8-10.5) and high temperature (37-42°C). These adapations include the excretion of urea instead of ammonia, the use of the swim bladder as an accessory air-breathing organ and a modified gill structure. Lake Magadi is separated into lagoon systems by precipitated bicarbonate called 'trona'. Differences between the lagoon systems have resulted in subtle but distinct morphological adaptations in the fish inhabiting these areas. Genetic structuring between the two Magadi populations was assessed using DNA sequences of the control region of the mitochondrial DNA and multi-locus DNA fingerprinting. Samples of the sub-species *O. a. alcalicus* from Lake Natron in Tanzania, a body of water once continuous with Lake Magadi, were included in the analyses as an outgroup that has been isolated from *O. a. grahami* for approximately 10,000 years. The control region haplotype frequencies were significantly different among the three tilapia populations and DNA fingerprint similarity indices were significantly higher for intra-population comparisons of the two Magadi lagoon populations and the Lake Natron population than the inter-population similarity indices among these populations. A modified F_{st} measure indicated population sub-division and the phylogeographic partitioning of the VNTR fragments observed were unique to specific populations further indicating substantial genetic differentiation. The lagoon populations within Lake Magadi demonstrated the same degree of genetic differentiation as either of these populations did to the outgroup (the Lake Natron population). There appears to be limited gene flow between Lake Magadi

tilapia populations and this population structure has important implications for protecting locally adapted populations within this unique ecosystem.

INTRODUCTION

The species of the family Cichlidae in the African Rift Valley lakes demonstrate extraordinary rates of morphological, behavioural and physiological evolution (Strürmbauer and Meyer 1992, Meyer 1993). This study focuses on *Oreochromis alcalicus*, a tilapia endemic to two alkaline "soda" lakes of the Rift Valley. The sub-species *O. a. grahami* is found in Lake Magadi, Kenya and the sub-species *O. a. alcalicus* in Lake Natron, Tanzania. Once a continuous water body, Lake Magadi separated from Lake Natron approximately 10,000 years ago and has since developed a different geomorphology and water (Goetz and Hillaire-Marcel 1992). Lake Magadi water properties exhibit higher temperature, pH and alkalinity levels than Lake Natron. Magadi has a pH averaging 9.8-10.5 and total carbonate alkalinity over 300 mequiv.l^{-1}, and temperatures exceeding 40°C. Undoubtedly, Lake Magadi represents the most hostile environment that teleost fish have been found to inhabit. *Oreochromis alcalicus grahami* has developed unusual morphological and physiological adaptations to the high pH and alkalinity. These include the excretion of urea rather than ammonia as their sole nitrogenous waste, through expression of the ornithine-urea cycle (Randall et al. 1989, Wood et al. 1994). This condition of 100% ureotely is unique among teleost fish and renders the sub-species of special evolutionary importance. In addition, the Magadi tilapia exhibit an extremely thin blood-water diffusion barrier (Maina 1990, Laurent et al. 1995, Maina et al. 1996) and the use of the swim-bladder as a primitive air-breathing organ (Maina et al. 1995). Essentially nothing is known about the physiology of the Natron tilapia.

In Lake Magadi, floating precipitate of sodium bicarbonate ("trona"), underlain by anoxic lake water, divides the *O. a. grahami* habitat into three main areas: Fish Springs Lagoon, South East Lagoon and South West Lagoon. Almost all research on the physiology of *O. a. grahami* has been performed on the Fish Springs fish which have been shown to be unique relative to other teleosts in their extreme resistance to high environmental pH, carbonate alkalinity, and temperature (reviewed by Wood et al. 1994, Maina et al. 1996). One comparative morphological study identified differences in gill structure between the Fish Springs and South East Lagoon fish (Maina et al. 1996). The Fish Springs Lagoon temperature is

32-37°C, versus South East Lagoon temperature of between 37-45°C which may influence the availability of O_2 (Maina et al. 1996).

The present study applied molecular genetic analysis to determine if two of the populations within Lake Magadi are genetically divergent from each other, and/or those of Lake Natron, and whether they should be considered as separate conservation units. Lake Magadi typically undergoes at least biannual flooding events that cover the trona with rainwater thereby providing a potential route for the exchange of fish (Coe 1966). The objective of this study was to determine the degree of population sub-division that has occurred between the two lagoon systems in Lake Magadi. The genetic differentiation that has accumulated in the 10,000 years of isolation between Lake Magadi and Lake Natron was also determined as a reference point.

Selecting the appropriate DNA marker to examine the genetic structure of populations is dependent on the genetic variation detected by a particular marker. African cichlid species have evolved phenotypically at such a high rate that the mutation rates of certain 'neutral' markers, e.g. mitochondrial DNA, used in population studies does not demonstrate a corresponding increased rate of evolution (Stürmbauer and Meyer 1992, Meyer 1993). Although it is unlikely that new haplotype variants would have been generated at the mtDNA control region since Lakes Magadi and Natron separated, the maternal inheritance of this molecule causes it to be more susceptible to genetic drift (Moritz 1994). Given the severity of the environments where these fish exist, it is highly probable that these populations have undergone significant reductions in their effective population size at various times. This would further increase the rate of differentiation causing the divergence of haplotype frequencies following the generation of boundaries among populations.

We also applied multi-locus DNA fingerprinting that detects hypervariable minisatellite DNA or variable number of tandem repeats (VNTR) loci. Multi-locus DNA fingerprinting has the potential for examining the genetic structure of natural populations which have been genetically differentiated over time-scales which other DNA markers may not detect. Population structuring is a factor that will decrease the measure of genetic similarity of the two populations through genetic drift (Cohen 1990). If two

populations are isolated from each other the prediction is that the similarity indices will be higher for pair-wise comparisons within a population than comparisons of individuals between populations. A number of previous studies have applied DNA fingerprinting to identify population structuring in natural populations (Gilbert et al. 1990, Hoelzel and Dover 1991, Degnan 1993, Taylor and Bentzen 1993 1993, Rave 1995, Refseth et al. 1998).

MATERIALS AND METHODS

Sample Collection

The Fish Springs Lagoon (FSL) and South East Lagoon (SEL) collection sites are mapped on Fig. 1, adapted from Maina et al. (1996). The small size of the lagoons limited sampling to the specific region within the lagoon that the fish inhabited. Note that in Maina et al. (1996) the fish referred to as South West were in fact collected at the South East lagoon. At both sites, fish were sampled January 15 - 22 (1992), during a relatively dry period about 6 weeks after the "little rains" of December. A beach seine was pulled through each site to randomly collect fish. Heads were removed, and the bodies placed individually in lysis buffer (Guglich et al. 1993). The Fish Springs Lagoon samples (n=24) were collected from a population numbering in the thousands. South East Lagoon samples (n=16) were collected from a small population numbering less that 1000 fish in total. Lake Natron (n=20) samples were included in this study as a control population with a known period of isolation from Lake Magadi. Lake Natron samples of *O. a. alcalicus* were kindly provided by Dr. Jan Klein and were sampled according to Sültmann et al. (1995).

Mitochondrial DNA Control Region Amplification and Analysis

The mtDNA control region haplotypes of Lake Magadi and Lake Natron tilapia were identified using single stranded conformational polymorphism (SSCP) analysis and DNA sequencing of the haplotypes. DNA was amplified under the following reaction conditions: 1X PCR buffer (20 mM Tris-HCl pH 8.4, 50 mM KCl) with 2.0 mM $MgCl_2$ (GIBCOBRL), 0.5 U Taq polymerase (GIBCOBRL), 10 μM of primers 5'-TTCCACCTCTAACTCCAAAGCT-3' and 5'-CCTGAAGTAGGAACCAGATG-3

(Lee et al. 1995) and 25 ng of template DNA in a total volume of 20 µL. Amplification was performed under the following temperature regime: 95°C for 5 minutes, 55°C for 30 seconds and 72°C for 30 seconds. The amplified products were assessed by gel electrophoresis (Guglich et al. 1993).

Single stranded conformational polymorphism (SSCP) analysis was performed according to above reaction conditions with the following exceptions: a total reaction volume of 10 µL was used; radioactively labeled (γ ^{33}P-dATP (ICN)) primers were added in addition to the unlabeled primers. Amplified products were electrophoresed through a nondenaturing acrylamide Model S2 gel (GIBCOBRL) (5% acrylamide [59 acrylamide:1 bisacrylamide], 10% glycerol and 0.5 X TBE) for 16 hours at room temperature. Any unique conformational polymorphisms were sequenced from 1-5 individual fish to confirm the SSCP patterns.

The software package AMOVA (Analysis of Molecular Variance, version 1.55; Excoffier 1995) was used to test the hierarchical partitioning of genetic variability among the lagoon populations of Magadi and Lakes Magadi and Natron (Excoffier et al. 1993). Statistical significance was tested in AMOVA using 9999 permutated matrices. The following statistics were analysed: ϕ_{CT} which analysed the variation among lake systems and examined the correlation between random haplotypes within a group of populations against random pairs of haplotypes from the whole species; ϕ_{SC} which analysed the variation of populations within lake systems and examined the correlation between random haplotypes against random pairs of haplotypes drawn from the lake; ϕ_{ST}, analogous to F_{ST}, analysed variation within populations and examined the correlation of random haplotypes within populations against random pairs of haplotypes from the species. Pairwise ϕ_{ST} tests were performed to assess population structuring.

DNA Fingerprint Analysis

DNA fingerprints were generated using the minisatellite probes Jeffreys' 33.15 and *per* according to Guglich et al. (1993) using the restriction enzyme *AluI*. Within and between population similarity indices (Lynch 1991) were calculated from samples included on the same DNA fingerprint. Due to limitations in DNA quality and the need to score individuals from the same gel, a subset of samples was

used from each population in generating the DNA fingerprints. Differences in the within and between population similarity indices were assessed statistically using a Mantel test (1967) on intra/inter-population similarity indices using the computer package NTSYS (Rolhf 1990) and a modified F_{ST} measure (Wright 1978) (F'_{ST}) (Lynch 1991). The phylogenetic partitioning of VNTR alleles among the populations was determined by examining the number of common and unique VNTR alleles present in each population comparison. This phylogeographic approach has been applied to determining the genetic divergence between sub-species of the cheetah (Mennotti-Raymond and O'Brien 1993).

RESULTS & DISCUSSION

Mitochondrial DNA haplotype diversity within *O. alcalicus* populations ranged from 2-4 (Table 1) with a total of 6 (Fig. 2) partitioned among the Magadi and Natron populations examined. An analysis of the partitioning of the haplotype diversity (Table 2A) indicated 75% of the genetic variation was distributed within *O. alcalicus* populations. Only 8% of the variation was distributed between the Fish Springs Lagoon and Southeast Lagoon populations within Magadi and 17% of the observed variation was partitioned between the two lake systems. Genetic structuring was assessed by estimating pairwise ϕ_{ST} which indicated significant differences in all inter-population comparisons (Table 2B).

Haplotypes B and E were present in both Lakes Magadi and Natron while each lake had two specific haplotypes (Table 1). This pattern of haplotype distribution is consistent with two scenarios: a) the incomplete lineage sorting of haplotypes A and B and sorting of ancestral haplotypes (A, C, D and F) into specific lakes and populations; or b) haplotypes B and E represent haplotypes pre-dating the separation of Lakes Magadi and Natron with the B form representing the ancestral type to newly derived haplotypes specific to each lake following the geologic separation of the watershed system. The prediction for the lineage sorting of haplotypes pre-dating the sub-speciation of *O. a. alcalicus* and *grahami* is that divergent haplotypes from the historic populations should be partitioned among populations (Duvernell and Turner 1998). The similarity of lake-specific haplotypes (Fig. 3) supports lake-specific lineages that have diverged subsequent to isolation from the ancestral B haplotype. Although Lakes Magadi and Natron are

estimated to have separated 10,000 years ago it is possible that new haplotypes evolved subsequent to separation as the generation time of *O. alcalicus* has been estimated at approximately 30 days.

To determine the amount of genetic differentiation at VNTR loci among populations of *O. alcalicus*, the intra-population genetic similarity is compared to the inter-population genetic similarity. The prediction is that if animals from different geographic regions are structured into populations with limited or no gene flow, the S_{mean} value will be higher among individuals within the populations than among individuals between the populations. The S_{mean} values within the Magadi and Natron populations (Table 3A) were considerably higher than S_{mean} values observed in inter-population comparisons (Table 3B). The inter-population comparisons between the Lake Magadi lagoons demonstrated equivalent levels of genetic variability to the levels observed between the sub-species of *O. alcalicus* inhabiting separated lake systems. The Mantel test was used to compare the distribution of within population similarity indices to between population similarity indices. The three inter-population comparisons, South East Lagoon and Fish Springs Lagoon and each lagoon population to the Lake Natron population, demonstrated significance levels of $p = 0.0001$ for both minisatellite probes with approximate Mantel t-test statistic values in the range of $t = -0.5215$ to -6.038.

Genetic differentiation was also detected among the tilapia populations using a modified version of Wright's index of population sub-division (F'_{ST}) (Lynch 1991). An F'_{ST} value of 0.567 was calculated for all three populations when S_{mean} values for Jeffreys' 33.15 and *per* were combined. An F'_{ST} value of 0.585 was estimated for the two lagoon systems in Lake Magadi, again with the similarity values of the two probes pooled together. These values indicate that approximately 57% and 59% of the genetic variation observed is the result of inter-population variability. Substantial population differentiation is defined to exist with an F_{ST} value of 0.150, with a lower degree of population differentiation occurring below this value (Wright 1978). The F'_{ST} values in this study are considerably higher than the level described by Wright (1978) as defining substantially differentiated populations, i.e. approximately 15% inter-population variance. Wright's definition was on allozyme data and the hypervariable nature of the VNTR loci in the modified calculation of population sub-division may not reflect an equivalent estimate.

However, the equivalent F'_{ST} indices calculated in the outgroup comparison of known isolated populations, i.e. lake populations, and the Magadi lagoon population comparison supports substantial isolation of the fish inhabiting the different lagoon systems.

A total of 129 and 104 polymorphic VNTR fragments were scored with Jeffreys 33.15 and *per*, respectively, and were examined for their phylogenetic partitioning among the three populations. Of these fragments 11 (8.5%) were common to both Lake Natron and Lake Magadi tilapia with Jeffreys 33.15 and 14 (13.5%) with *per*. The remaining 91.5% and 86.5% of the VNTR fragments were unique to each sub-species. A similar comparison of the two lagoon systems within Lake Magadi indicated 14.9% and 10.0% of the 94 VNTR fragments detected by Jeffreys 33.15 and 140 detected by *per* were common to both populations with approximately 85% and 90%, respectively, of the VNTR fragments being specific to one of the two *O. a. grahami* populations. Therefore a large portion of the allelic variation at the VNTR loci appears to have accumulated by mutation or differentiated by drift since the isolation of Lake Magadi. Similar levels of geographic partitioning and genetic divergence of the VNTR alleles were detected between the lagoon populations of Lake Magadi. This assessment differs from the F'_{ST} statistic in that the presence or absence of a VNTR allele is considered in the phylogenetic partitioning while Wright's measure of population sub-division considers the frequencies of the VNTR alleles.

The genetic differentiation at the VNTR loci within Lake Magadi occurring on the same scale as the outgroup comparison suggests an extended period of isolation between Fish Springs Lagoon and South East Lagoon tilapia. These results, coupled with the majority of mtDNA control region variation partitioned within populations support that the two Magadi populations do not form a single breeding panmictic group. Coe (1966) concluded that "if it were not for the periodic gene flow allowed by flood connections, between otherwise isolated fish communities, no doubt sub-specific differences might well have arisen in the area." The structuring of mtDNA haplotypes and VNTR alleles is not consistent with this hypothesis and the morphological gill adaptations observed between two lagoon populations (Maina et al. 1996) strongly supports allopatric evolution in the absence of gene flow. Unfortunately, only one of the

populations investigated in the present study has been subject to physiological investigation (Fish Springs Lagoon), while two have received morphological study (Fish Spring Lagoon and South East Lagoons).

Genetic diversity within *O. alcalicus* populations was generally low for the mtDNA control region. The number of control region haplotypes within the populations is on the low end of the number of haplotypes observed within other cichlid species (Meyer et al. 1996, Agnese et al. 1997). The haplotype diversity is also comparable to Desert pupfish populations (genus: *Cyprindon*) which inhabit demographically isolated populations and demonstrate post-Pleistocene allopatric divergence with morphological, physiological and behavioural differences among populations (Duvernell and Turner 1998). The low haplotype diversity observed within Desert pupfish populations suggested large ancestral populations followed by historic population bottlenecks. Furthermore, the VNTR variation within the Magadi lagoon populations is comparable to levels observed in endangered species (Brock and White 1992, Patenaude et al. 1994, Schaeff et al. 1997) which demonstrate low genetic variability as a result of low effective population sizes during bottleneck events.

The low levels of genetic variability at nuclear and mitochondrial genetic markers within Lake Magadi likely reflects the population histories within the isolated lagoon systems. These levels of genetic variability are consistent with low historic effective population sizes (Ne) and would be predicted during severe shifts in the alkalinity, temperature and pH subsequent to the separation of Lakes Magadi and Natron. Major geochemistry and geomorphological changes in Magadi would result in strong selection pressures on the fish populations resulting in potentially repeated population bottlenecks. This seems consistent with the lower levels of genetic variation observed in the Southeast Lagoon, which has more severe environmental conditions, at least with respect to oxygen levels, than the Fish Springs Lagoon. Additional lagoon systems within Lake Magadi will be examined for the presence of tilapia. Genetic characterization of these fish is planned to help further assess the degree of isolation and attempt to reconstruct the evolutionary history of *O. a. grahami* within Lake Magadi.

The physiology of Fish Springs Lagoon fish appears unique in a number of aspects relative to other teleosts. In addition to extreme resistance to high environmental pH, carbonate alkalinity, and temperature (Reite et al., 1974), these characteristics include 100% ureotely through complete expression

of the ornithine-urea cycle enzymes in the liver (Randall et al., 1989; Wood et al, 1989, 1994). Associated with the latter is the greatest resistance to ammonia toxicity ever documented in a teleost fish (Walsh et al., 1993), a feature which makes the gene pool of interest for intensive aquaculture, where ammonia toxicity is a common problem. The great tolerance to fluctuations in environmental O_2 levels (Narahara et al., 1996), facilitated by an unusually high gill surface area and short blood-to-water diffusion distance (Maina, 1990; Laurent et al., 1995), is also relevant in this regard. Furthermore the Fish Springs Lagoon tilapia exhibit a remarkable ability for supplementary air-breathing through the swimbladder under hypoxic conditions (Maina et al., 1995). Other remarkable features include the highest temperature-specific blood pH's (Johansen et al., 1975; Wood et al., 1994) and the highest routine metabolic rates (Franklin et al., 1995; Narahara et al., 1996) ever recorded for poikilothermic teleost fish, and an unusual insensitivity of the blood O_2 dissociation curve to pH fluctuations in the physiological range (Lykkeboe et al., 1975; Narahara et al., 1996).

Inasmuch as conditions are even more severe at the South East Lagoons, it seems likely that the same suite of unusual physiological adaptations will be expressed in these tilapia, perhaps to an even greater degree. In this regard it is noteworthy that the gills of these fish are anatomically different from those of Fish Springs Lagoon specimens with greater numbers of gill filaments and respiratory lamellae, resulting in a 2.5 fold greater weight-specific O_2-diffusing capacity (Maina et al., 1996). Further, given the allopatric situation among the populations within Lake Magadi and the environmental differences between at least two microhabitats, i.e. the South East Lagoon and Fish Springs Lagoon, additional morphological and physiological differences may exist between these two populations and the yet uninvestigated South West lagoon fish.

Ideally, protected status should be extended to each Magadi population. Apart from the present evidence of genetic differentiation, we know nothing about the Lake Natron population (*O. a. alcalicus*), which may share many, some, or none of the adaptations of *O. a. grahami.* Nevertheless, the uniqueness of the species as a whole should be sufficient to warrant the protection of this population as well. Presently the protection of these populations is consistent with a classification of Management Units as described by

Moritz (1994) as both mtDNA and nuclear markers demonstrate significant structuring among the populations. Management Units (MU) are the components of 'Evolutionary Significant Units' (ESU): a biological population which is historically isolated and is distinguished by its presumed evolutionary significance (Ryder 1986, Waples 1991, Dizon et al. 1992). As further information is obtained on this species a classification of ESU may become appropriate or the taxonomic status *O. alcalicus* may warrant re-classification. A recent study of the Death Valley pupfish (Duvernell and Turner 1998) has addressed the need to apply a broader definition of an ESU by integrating molecular and phenotypic data as to the adaptive significance of a population.

In a larger context, this study demonstrates the importance of considering microhabitats within the lakes of the African Rift Valley. As these lakes can exhibit extreme environmental conditions, fish that have adapted to "life on the edge" would maintain highly specialized adaptations, i.e. locally adapted genomes, ultimately resulting in allopatry based on an inability to survive or reproduce in neighbouring habitats. Assumption of gene flow among populations based on the absence of obvious physical barriers may be premature when the physicochemical and environmental conditions among habitats can act as effective barriers. Protection efforts should maintain the genetic variability that contributes to the biodiversity of these taxa and the evolutionary potential these fish represent.

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Figure 1. Map of Lake Magadi, Kenya indicating the Southeast Lagoon, Southwest Lagoon and the Fish Springs Lagoon as well as the smaller Little Magadi to the north. Open water (alkaline lagoon) and trona are indicated on the map.

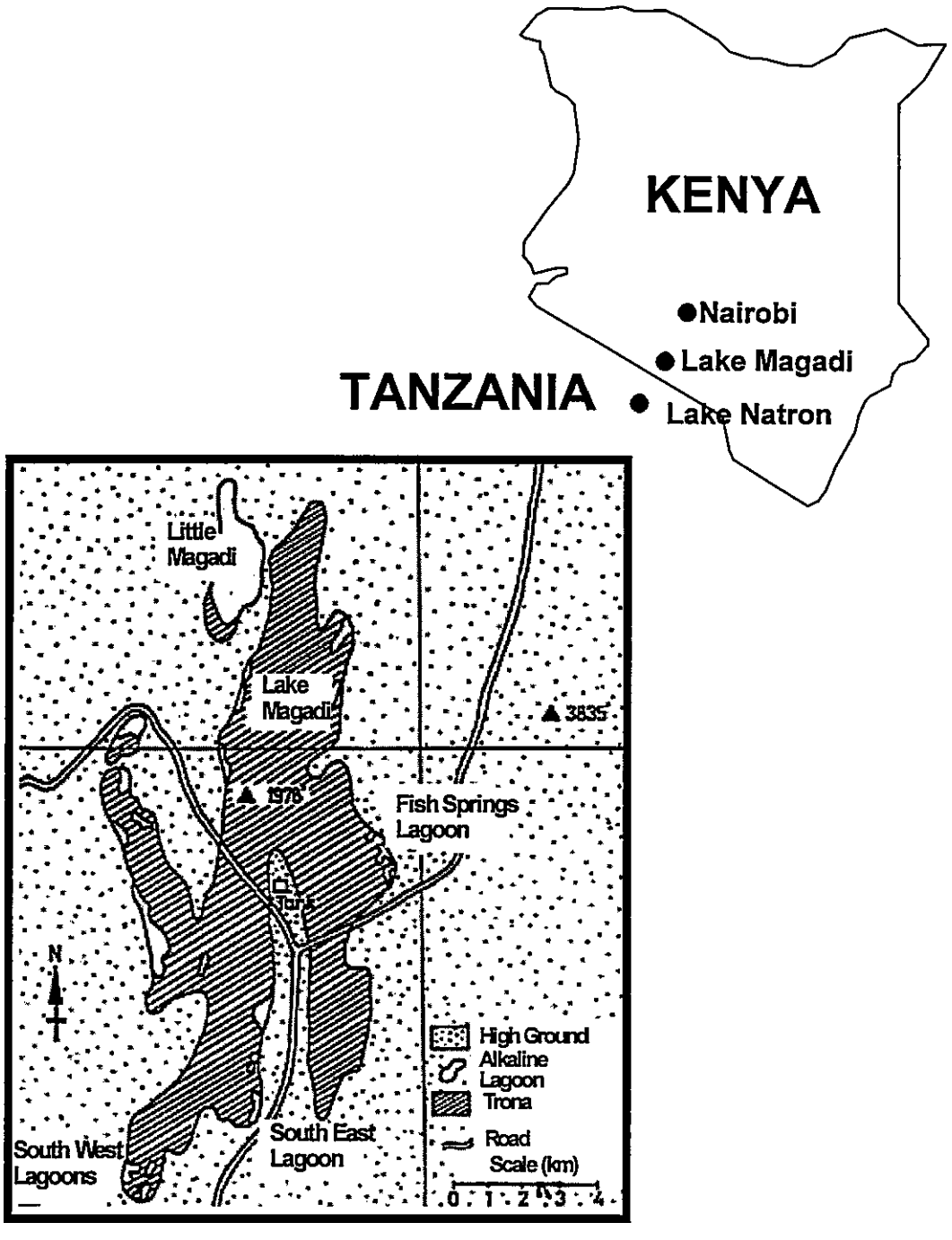
KENYA
Nairobi
Lake Magadi
Lake Natron
TANZANIA
Little Magadi
Lake Magadi
3835
1978
Fish Springs Lagoon
Tura
N
South West Lagoons
South East Lagoon
High Ground
Alkaline Lagoon
Trona
Road
Scale (km)
0 1 2 3 4

Figure 2. DNA sequences of five control region haplotypes from *O. alcalicus*. Dashes indicate identical nucleotides to haplotype A and nucleotide substitutions have been identified based on differences from haplotype A. Numbers in parentheses indicate the number of fish sequenced per haplotype.

```
                                                                                                                    50
Haplotype A(5)    GCTCTGCCTTCATGTAAAATGCAATGCATATATGTATTATCACCATTATT
Haplotype B(5)    ---------------------------------------------------
Haplotype C(1)    ---------------------------------------------------
Haplotype D(1)    ---------------------------------------------------
Haplotype E(1)    ---------------------------------------------------
Haplotype F(1)    ---------------------------------------------------
                                                                                                                   100
Haplotype A       TTATATCAAACATATCCTATATATAAATACATATATTTCTTAAAGAACAT
Haplotype B       ---------------------------------------------------
Haplotype C       ---------------------------------------------------
Haplotype D       ---------------------------------------------------
Haplotype E       ----------------------------------------------------C
Haplotype F       ------------------------G---T--------------------
                                                                                                                   150
Haplotype A       CCAATGTTCTCCCACATTTTTGTTATCAACATTTACATCTAAGGGATACA
Haplotype B       ------------------------------------A------------
Haplotype C       ---------------------------------------------------
Haplotype D       ----------------------------------A-A------------
Haplotype E       ------------------------------------A------------
Haplotype F       ----------------------------------A-A------------
                                                                                                                   200
Haplotype A       TAAACCAATAAATGAAATTTTCCAAAAATATTTCAAAACCACTGAACGAT
Haplotype B       ------------------C---------C--------------------
Haplotype C       ------------------------G---------------------
Haplotype D       ------------------C---------C--------------------
Haplotype E       ------------------C---------C--------------------
Haplotype F       ---------------------------------------------------
                                                                                                                   250
Haplotype A       AGTTTAAGACCGAACACAACTCTCATCCGTTAAGATATACCAAGTACCCA
Haplotype B       ---------------------------------------------------
Haplotype C       ---------------------------------------------------
Haplotype D       ---------------------------------------------------
Haplotype E       ---------------------------------------------------
Haplotype F       ---------------------------------------------------
                                                                                                                   300
Haplotype A       CCATCCTATACTTCCGAATTATTTAATGTAGTAAGAGCCCACCATCAGTT
Haplotype B       ---------------------------------------------------
Haplotype C       ---------------------------------------------------
Haplotype D       ---------------------------------------------------
Haplotype E       ---------------------------------------------------
Haplotype F       ---------------------------------------------------
                                                                                                                   350
Haplotype A       GATTCCTTAATGTTAACGGTTCTTGAAGGTCAAGGACAATTATTCGTGGG
Haplotype B       ---------------------------------------------------
Haplotype C       ---------------------------------------------------
Haplotype D       ---------------------------------------------------
Haplotype E       ------------C------------------------------------
Haplotype F       ---------------------------------------------------
```

Figure 3. Minimum-spanning tree of six control region haplotypes identified in *O. alcalicus*. Dashes indicate nucleotide substitution differences away from each haplotype. Haplotypes A and C were found only in Lake Magadi, haplotypes D and F were found only in the Lake Natron samples and haplotypes B and E were found in samples from both lakes.

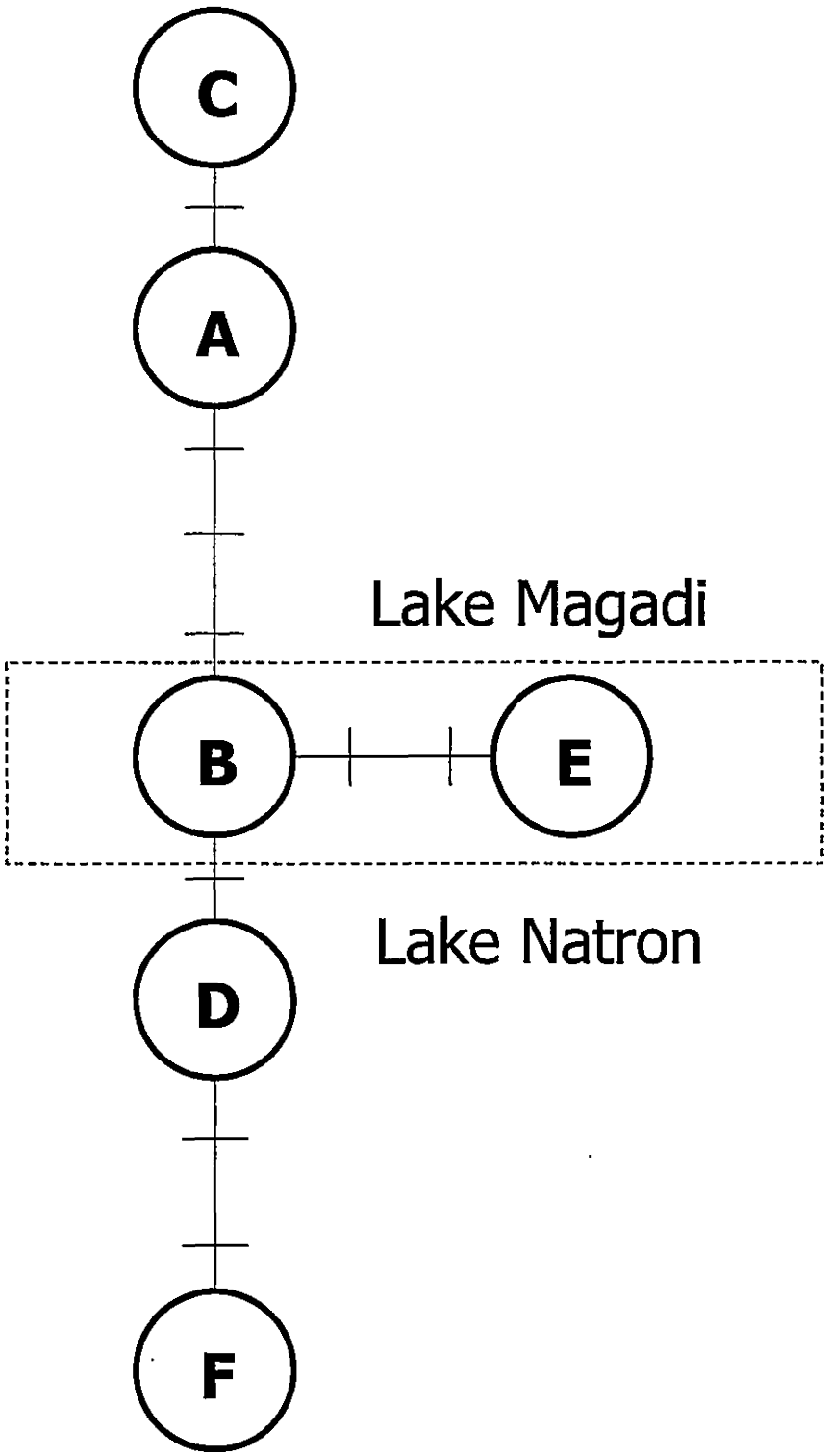
C
A
Lake Magadi
B
E
Lake Natron
D
F

Table 1. Frequencies of six control region haplotypes for *O. alcalicus* populations.

	POPULATION		
HAPLOTYPE	Fish Springs Lagoon, Lake Magadi	South East Lagoon, Lake Magadi	Lake Natron
A	**9**	**12**	**0**
B	**7**	**4**	**11**
C	**5**	**0**	**0**
D	**0**	**0**	**2**
E	**3**	**0**	**6**
F	**0**	**0**	**1**
Total	**24**	**16**	**20**

Table 2. Analysis of variance of *O. a. alcalicus* and *O. a. grahami* estimated by AMOVA (Excoffier 1995) with 9999 permutations of the data.

A. Partitioning of the genetic variation as measured by the variance, percentage (%Total) of the genetic variation of *O. alcalicus* and ϕ_{CT}, ϕ_{SC} and ϕ_{ST} measures with corresponding P-values are provided for tilapia in Lakes Magadi and Natron.

Variance Component	Variance	%Total	Measure	P-value
Among Lakes Magadi and Natron	0.140	16.97%	$\phi_{CT} = 0.170$	$p < 0.0001$
Among populations within Lake Magadi	0.069	8.34%	$\phi_{SC} = 0.100$	$P = 0.0549$
Within *O. alcalicus* populations	0.615	74.70%	$\phi_{ST} = 0.253$	$p < 0.0001$

B. Pairwise population comparisons of ϕ_{ST} from AMOVA tests (below diagonal) and the probability (P-value) of generating a random value greater than the observed value (above diagonal) as an indictor of population structuring.

Population	Southeast Lagoon	Fish Springs Lagoon	Lake Natron
Southeast Lagoon	-	0.0424	0.0000
Fish Springs Lagoon	0.1016	-	0.0071
Lake Natron	0.3979	0.1427	-

Table 3A and B. Levels of VNTR Genetic Variation Within and Between Populations of *O. alcalicus*. Values are provided for the number of individual fish, the number of pair-wise comparisons used in calculating the mean similarity indices and an estimate of the number of VNTR loci (S_{mean}) (± SE) (Lynch 1991).

Table 3A. Levels of Genetic Variability Within Populations of *O. alcalicus*.

Population Comparison	Jeffreys 33.15	*per*
South East Lagoon, (*O. a. grahami*)		
Number of Fish	8	8
Number of Pair-wise Comparisons	36	36
Number of VNTR Fragments	19.1 ± 0.8	18.9 ± 1.4
Number of VNTR Loci	11.2	10.4
S_{mean}	**0.515 ± 0.106**	**0.334 ± 0.097**
Fish Springs Lagoon, (*O. a. grahami*)		
Number of Fish	7	7
Number of Pair-wise Comparisons	28	28
Number of VNTR Fragments	19.3 ± 0.9	21.3 ± 1.0
Number of VNTR Loci	11.5	11.3
S_{mean}	**0.318 ± 0.095**	**0.229 ± 0.079**
Lake Natron *(O. a. alcalicus)*		
Number of Fish	8	8
Number of Pair-wise Comparisons	36	36
Number of VNTR Fragments	17.2 ± 2.1	16.7 ± 1.5
Number of VNTR Loci	8.8	8.5
S_{mean}	**0.180 ± 0.084**	**0.211 ± 0.091**

Table 3B. Levels of Genetic Variability Between Populations of *O. alcalicus*.

Population Comparison		Jeffreys 33.15	*per*
South East Lagoon & Fish Springs Lagoon,			
O. a. grahami			
Number of Fish			
	South East Lagoon	8	8
	Fish Springs Lagoon	7	7
Number of Pair-wise Comparisons		56	56
Number of VNTR Fragments		19.2 ± 0.7	20.1 ± 1.0
Number of VNTR Loci		10.0	10.2
S_{mean}		**0.132 ± 0.067**	**0.059 ± 0.048**
South East Lagoon & Lake Natron,			
O. a. grahami & O. a. alcalicus			
Number of Fish			
	South East Lagoon	6	6
	Lake Natron	6	6
Number of Pair-wise Comparisons		36	36
Number of VNTR Fragments		15.0 ± 1.2	17.0 ± 0.8
Number of VNTR Loci		7.8	8.8
S_{mean}		**0.080 ± 0.054**	**0.056 ± 0.048**
Fish Springs Lagoon & Lake Natron,			
O. a. grahami & O. a. alcalicus			
Number of Fish			
	Fish Spings Lagoon	6	6
	Lake Natron	6	6
Number of Pair-wise Comparisons		36	36
Number of VNTR Fragments		17.2 ± 2.1	16.7 ± 1.5
Number of VNTR Loci		8.8	8.5
S_{mean}		**0.084 ± 0.057**	**0.058 ± 0.049**

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Chapter 3

Discordance between genetic structure and morphological and physiological adaptation in Lake Magadi tilapia

Discordance between genetic structure and morphological and physiological adaptation in Lake Magadi tilapia

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ABSTRACT

The Magadi tilapia (*Alcolapia grahami*, formerly *Oreochromis alcalicus grahami*) is a remarkable example of teleost life in an extreme environment. Typical conditions include water pH 10, titration alkalinity > 300 mM, osmolality 525 mOsm, temperatures ranging from 32-42^{O}C, and O_2 levels fluctuating diurnally between extreme hyperoxia and anoxia. A number of relatively small populations exist in various thermal spring lagoons around the margin of the lake, in apparent isolation from one another, separated by kilometers of solid trona crust (floating Na_2CO_3) underlain by anoxic water. We examined the water conditions and collected cichlids for characterisation from six lagoon systems in Lake Magadi, Little Magadi, a small lake to the north, and Lake Natron, once connected to Lake Magadi in a large paleolake approximately 10,000 years ago. Despite the apparent isolation of different populations, annual floods may provide opportunities for exchange of fish across the surface of the trona, and subsequent gene flow. To assess the question of isolation within Lake Magadi we applied the variable control region of the mitochondrial DNA (mtDNA). Of a total of 7 mtDNA haplotypes, 3 were observed in all six lagoons, with the same two common sequences and a less common third sequence. Several of the Lake Magadi populations showed haplotype frequencies indicative of differentiation, while others showed very little. Remarkably, the observed similarities and differences were discordant with the geographical distribution of

lagoon systems along the shoreline. These findings were contrary to previous genetic structure analyses on two of the Magadi lagoon populations that indicated considerable differentiation. In addition, specialised morphological and physiological adaptations were observed among several of the lagoon systems supporting differentiation and locally adapted genomes. Different gut lengths were observed among several Magadi populations, likely due to dietary selection pressures. Distinct differences were observed in the osmolality among the lagoons with levels as high as 1400-1700 mOsm • kg^{-1} with corresponding differences in the natural levels of whole-body urea. These levels of osmotic pressure proved fatal to fish from less alkaline systems but remarkably were fatal to the fish that inhabited lagoons with this water chemistry. Upon more detailed inspection, specific adaptations to differential conditions in the lagoon habitat were identified that allowed survival of these cichlids. Additional evidence against gene flow amongst lagoons despite the sharing of common mtDNA haplotypes was the osmolality of the floodwaters following a heavy rain that showed lethal levels exceeding 1700 mOsm • kg^{-1}. In isolation, different mtDNA haplotypes are predicted to go to fixation in different populations due to rapid generation times and the small effective population sizes in a number of lagoons. We propose a model of balancing selection must be operating to maintain two common mtDNA sequences through a common selection pressure among lagoons that is based on microhabitats utilised by the tilapia.

INTRODUCTION

African cichlids (family Cichlidae, suborder Labroidei, order Perciformes) comprise the most speciose assemblages of closely related vertebrates. The African Rift Valley lakes of Lake Malawi, Lake Tanganyika and Lake Victoria harbour the majority of these cichlid species, with lake-specific flocks containing hundreds of endemic species. These species flocks have radiated within the past 5 million years (Meyer et al. 1990, Sturmbauer and Meyer 1992, Sturmbauer and Meyer 1993), but potentially as recently as 12,400 years (Meyer 1993, Johnson et al. 1996, Galis and Metz 1998). The cichlid species within each lake demonstrate specialisation into trophic groups for all available food resources (Fryers and Iles 1972, Ribbink 1991). In addition, a wide range of colour variation and behaviours distinguish cichlid species within and among species flocks (Fryers and Iles 1972, Trewavas 1983)

A recent examination of "soda" tilapia in Lakes Natron and Magadi (Seegers and Tichy 1999, Tichy and Seegers 1999, Seegers et al. 1999) identified a small species flock within these lakes. The several species in Lake Natron demonstrate differences in mouthparts typical of other cichlid species flocks in the Rift Valley lakes. Although cichlid species have been characterised in a number of Rift Valley alkaline "soda" lakes" (Trewavas 1983), the classification of species within these environments has focused on the standard morphological differences, e.g. jaw morphology and coloration.

The Lake Magadi tilapia in Kenya, *Alcolapia grahami* (Seegers and Tichy 1999), formerly *Oreochromis alcalicus grahami* (Fryers and Iles 1972, Trewavas 1983), has been well characterised with respect to the adaptations required to inhabit one of the most severe of the "soda lakes" within the African Rift Valley. Typical conditions within Lake Magadi include water of pH 9.8-10.5, titration alkalinity > 300 mM, osmolality 525 mOsm, temperatures as high as 42^{o}C, O_2 levels fluctuating diurnally between extreme hyperoxia and virtual anoxia, and intense levels of avian predation and UV radiation. Over the past 25 years, the physiological adaptations of the cichlids inhabiting this extreme environment have been studied in one lagoon population in detail, the Fish Springs Lagoon.

Of the many physiological adaptations to this severe environment, the most notable is the excretion of all N-waste as urea with no excretion of ammonia, presumably due to the difficulty of maintaining an outward P_{NH3} gradient in this highly buffered alkaline water (Randall et al., 1989; Wood et

al., 1989). This 100% ureotelism is unique amongst teleost fish and is achieved by expression of the Krebs ornithine-urea cycle throughout the white muscle mass (Lindley et al. 1999) as well as the liver (Randall et al. 1989, Walsh et al. 1993). Exceptionally high urea excretion rates are made possible by a facilitated diffusion type urea transporter which has been pharmacologically identified and cloned very recently (Walsh et al. 2000). Tolerance of high environmental alkalinity is also facilitated by the regulation of exceptionally high blood and tissue pH (Johansen et al. 1975, Wood et al. 1994). These cichlids have further evolved a unique pyloric bypass system to allow drinking of alkaline lakewater despite the presence of an acidic stomach (Narahara 2000). The metabolic rate is exceptionally high, reflecting the high environmental temperatures and activity levels of these remarkable fish (Franklin et al. 1995, Narahara et al. 1996). Respiratory adaptations include a very high blood O_2 affinity and a high Q10 of metabolic rate adaptive to the severe diurnal temperature and O_2 cycle (Narahara et al. 1996), together with a thin diffusion distance (Maina 1990, Laurent et al. 1995) and an exceptionally high diffusing capacity of the gills for O_2 (Maina et al. 1996). Furthermore, the Lake Magadi tilapia is most unusual amongst cichlids in that it is capable of supplementary airbreathing during hypoxic conditions (Narahara et al. 1996), facilitated by a well vascularized physoclistous swim bladder which seems to serve as a primitive lung (Maina et al. 1995). Unique ionoregulatory strategies and accompanying gill structural specializations have also been described (Eddy et al. 1981, Eddy and Maloiy 1984, Maloiy et al. 1984, Skadhauge et al. 1984, Wright et al. 1990, Maina 1991, Laurent et al. 1995).

Perhaps most remarkable is that these unusual morphological and physiological adaptations in *A. grahami* may have evolved within the last 7,000-10,000 years when the water chemistry of the lake began to change since the drying of Paleolake Orolonga resulted in separation of Lakes Natron and Magadi approximately 9,000 years ago (Butzer et al. 1972, Roberts et al. 1993). Seegers et al. (1999) provide a full discussion of the geological history of the lakes.

Within Lake Magadi, a number of small populations exist in various thermal spring lagoons around the margin of the lake, in apparent isolation from one another, separated by kilometers of solid trona crust (floating Na_2CO_3) underlain by anoxic water (Coe 1966). Despite the apparent isolation of different populations, annual flooding of the trona surface by torrential rainfall may provide opportunities

for the exchange of fish across the surface of the trona, resulting in subsequent gene flow. Early descriptions of these flooding events assumed homogenisation of lagoon populations based on dead fish on the dried trona following the rains (Coe 1966). This presumed genetic exchange was claimed to have prevented speciation and even sub-specific differences. However, subtle morphological differences between two of the lagoon populations, the well-studied Fish Springs Lagoon and the less studied South East Lagoon, in the size of the gills suggested differentiation of the two populations (Maina et al. 1996). Differentiation between these two populations was confirmed using multi-locus DNA fingerprinting and haplotype frequency differences at the mitochondrial DNA (mtDNA) control region (Wilson et al. 2000).

The overall goal of this study was to identify additional Magadi tilapia populations, determine the environmental conditions of the lagoon systems they inhabit, identify specific adaptations in *Alcolapia* populations, and identify adaptations specific to lagoon systems. Classic cichlid taxonomic descriptions of coloration and jaw morphology, while appropriate in other Rift Valley Lakes such as Malawi, Tanganyka and Victoria, may not accurately describe significant morphological, physiological and behavioural adaptations to more severe environmental conditions such as those in Lake Magadi and other Soda Lakes. For example, Seegers et al. (1999) recently separated *A. grahami* of Lake Magadi from the Natron species *A. alcalicus* based on different mitochondrial DNA (mtDNA) lineages and the allopatric nature of the two lakes, as the morphology of these two fish was very similar.

An expedition to Lake Magadi in 1997 provided us with the opportunity to identify and characterise cichlid populations from six lagoons around Lake Magadi, as well as two additional populations from neighbouring lakes, Little Magadi (only about 0.5 km to the north-west, but elevated by 20m above Lake Magadi) and Lake Natron, Tanzania (about 30 km to the south of Lake Magadi). The specific objectives of this study include: 1) Characterise the genetic relationship of *Alcolapia* populations with a focus on Magadi lagoon populations using the control region of the mitochondrial DNA. 2) Characterise the environmental conditions and chemistry of the water in which these populations live. 3) Characterise populations of the Lake Magadi basin, including Little Magadi, and Lake Natron using physiological, morphological and behavioural characters – a particular focus here was on whether the fish

were ureotelic and expressed the Krebs ornithine-urea cycle. 4) Examine the concordance between the genetic differences and specific phenotypic differences.

MATERIALS AND METHODS

Sample Collection

Samples of cichlids were collected in January and February, 1997 using seine or hand nets from six lagoon populations in Lake Magadi, Kenya (Fig. 1): the Fish Springs Lagoon; the Sports Club Lagoon; the South East Lagoon; the South West Lagoon; the Wilson Springs Lagoon; and the West Lagoon. Fish from Little Magadi (taken near shore from the northern area of open water) and Lake Natron, Tanzania (taken from a stream in the Shombole region running into the northern end the lake) were also collected. Samples were collected and transported alive to an outdoor field laboratory in the town of Magadi provided by the Magadi Soda company.

Genetic Analysis

The mtDNA control region haplotypes of Lake Magadi, Little Magadi and Lake Natron tilapia were identified using single-stranded conformational polymorphism (SSCP) analysis and DNA sequencing of the haplotypes according to Wilson et al. (2000). Samples were analysed from the Magadi lagoon populations: Fish Springs Lagoon (n=22), Sports Club Lagoon (n=46), Southeast Lagoon (n=20), the Southwest Lagoon (n=21), Wilson Springs (n=11), and the West Lagoon (n=17) (Fig. 1). Samples were also analysed from Little Magadi (n=21) and Lake Natron (n=27).

An analysis of molecular variance using the software package AMOVA, version 1.55 (Excoffier et al. 1992) was used to assess the partitioning of haplotypic genetic variation and population structure. The program Arlequin (Schneider et al. 1999) and was used to generate a minimum-spanning tree (Excoffier et al. 1992).

To assess the demographic history of the *Alcolapia* populations, we examined parameters associated with mismatch distributions of pair-wise sequence differences (Slatkin and Hudson 1991, Rogers and Harpending 1992, Rogers 1995). Population parameters were estimated using the program

Arlequin (Schneider et al. 1999). We applied a non-linear least-squared approach (Schneider and Excoffier 1999) to generate the test statistic SSD and estimate the population parameters based on the mismatch distributions. The parameter tau (τ) in mutational units was used to estimate the timing of past demographic events using the equation $\tau = 2ut$, where u is the mutation rate for the sequence and t is the number of generations since the expansion (Excoffier and Schneider 1999). The parameter theta (θ_1) was used to assess the effective female population size using the equation $\theta_1 = 2uN_1$, where N_1 is the effective population size following the population expansion.

Morphological Characterisation

Specific gross morphological differences (in mouth/head morphology, in coloration, and in gut length) were observed in a number of lake and lagoon populations from this and other studies. Due to limited time in the field the characteristics we observed were not compared among all populations.

Water Chemistry

The water chemistry and conditions of each Magadi lagoon system and Little Magadi and Lake Natron sites were characterised for pH, temperature, total CO_2 concentrations (reflecting the sum of carbonate and bicarbonate), sodium, chloride, and osmolality (mOsm $\bullet$ kg^{-1}). Additional measurements were taken along a transect near the Sports Club Lagoon site, following the flooding of Lake Magadi.

Water pH, temperature, total CO_2, chloride, and P_{O2} were measured on site using equipment and methods identical to those described by Wood et al. (1994). Alkalinity, expressed as HCO_3^- equivalents (i.e. $[HCO_3^-] + 2[CO_3^{=}]$) was calculated from pH and total CO_2 measurements using values for αCO_2 and pK^I and pK^{II} at the appropriate temperature, chlorinity, and ionic strength from Skirrow (1975). Samples were frozen and transported back to McMaster University for measurement of sodium (by atomic absorption spectroscopy = AAS, Varian 1275-AA), osmolality (by vapour pressure osmometry, Wescor 5100A), and some additional chloride determinations (by coulometric titration, Radiometer CMT10).

Physiological Characterisation

Fish were sampled from each of the identified field populations (with some exceptions) to determine the normal levels of whole body ions (Na^+, Cl^-), whole body urea-N, and hepatic enzyme activities. Immediately upon return of the live fish to the field laboratory in their natural water, 5 – 10 individuals of each population were sacrificed by cephalic concussion, blotted dry, then immediately freeze-clamped in liquid nitrogen for later determination of whole body urea-N, Na^+ and Cl^- concentrations. At the field laboratory, each frozen fish was weighed, ground to a fine powder under liquid nitrogen, then a weighed aliquot of the frozen powder was extracted in 9 volumes of 10% trichloracetic acid (TCA) at 4°C for 30-60 minutes. The extract was centrifuged for clarification, then assayed on site for urea-N by the colorimetric diacetyl monoxime method (Rahmatullah and Boyde 1980 - see below). The extracts were returned to McMaster University where they were appropriately diluted and assayed for Na^+ by AAS (as above) and Cl^- by the colorimetric assay of Zall et al. (1956). In all cases, standards were made up in the appropriate dilution of TCA.

A further 6 individuals from each population were similarly sacrificed and weighed. The liver was immediately excised, freeze-clamped in liquid nitrogen, weighed, then transported back to the University of Miami at liquid nitrogen temperature using a dry-shipper (Minnesota Valley Engineering). There, the samples were stored for less than two months at –80^{O}C prior to analysis of enzyme activities. Hepatosomatic index was calculated as the liver weight as a percentage of body weight. The activities of the following hepatic enzymes involved in the ornithine-urea cycle and/or contributing to other aspects of nitrogen metabolism were determined at 30.0 $\pm$ 0.2 ^{O}C according to Mommsen and Walsh (1989): glutamine synthetase (GSase), ornithine-citrulline transcarbamoylase (OTC), arginase (ARG), glutamate dehydrogenase (GDH), alanine aminotransferase (AlaAT), and aspartate aminotransferase (AspAt). GLNase (glutaminase) was determined according to Curthoys and Lowry (1973), and arginosuccinate synthetase/arginosuccinate lyase (AS/AL; as a coupled reaction), and carbamoyl phosphate synthetase III (CPSase III) metabolism were determined according to Anderson and Walsh (1995).

Urea production appears to be a function of metabolic rate (Wood et al., 1994). Therefore, to determine the extent of ureotelism in the various populations, measurements of urea-N ($M_{Urea\text{-}N}$) and ammonia-N ($M_{Amm\text{-}N}$) excretion rates and O_2 consumption rates (M_{O2}) were performed under conditions as close to those in the field as possible. Two approaches were used in parallel for some sites; for others, practical constraints dictated that only one or the other was used. Both employed the Tusker chamber system described by Wood et al. (1994) - 530 ml amber bottles fitted with sampling ports and aeration lines, each containing one fish. It was not feasible to aerate the individual Tusker chambers in the field. Therefore immediately after capture, one batch of fish from each site (N = 8-10) were placed immediately into individual Tusker chambers filled with water from Fish Springs Lagoon (see Table 1 for composition). This water had been pre-equilibrated with O_2 so as to allow the flux measurement to be started whilst the fish were being transported back to the field laboratory, a trip of 0.5 – 2.0 h (depending on the site) prior to the start of aeration. The other batch of fish from each site (N = 8-10) were transported back to the laboratory in a large bucket of their specific lagoon water, aerated with a battery driven pump. Immediately upon return to the laboratory, the Tusker chambers were filled with this water, individual fish were added, aeration commenced, and the flux measurements were started. In both cases, total flux periods for urea-N and ammonia-N were 3 – 5 h; in the middle of the period, aeration was suspended, and the chamber sealed for 40 min to allow the measurement of O_2 consumption. At the end of the experiment, exact time for the N-flux was noted, terminal water samples were taken for comparison to initial, and the fish were weighed. A chamber containing appropriate water but no fish was included in each run to serve as a blank.

Urea-N and ammonia-N concentrations in water were measured colorimetrically on site by the diacetyl monoxime method of Rahmatullah and Boyde (1980) and the salicylate-hypochlorite method of Verdouw et al. (1978) respectively. In each case it proved necessary to make up urea-N and ammonia-N standards in the appropriate lagoon water, and the assays were modified by the addition of an extra 1 ml of lagoon water to bring the volume to 3 ml so the samples could be read on a Spectronic 20 spectrophotometer (Bausch and Lomb). Nitrogen excretion rates ($M_{Urea\text{-}N}$, $M_{Amm\text{-}N}$) were calculated from the increases (blank-corrected) in water urea-N and ammonia-N concentrations over the entire flux period, factored by mass and time. Samples for water P_{O2} were analyzed using a Radiometer pHM 71 gas analyser

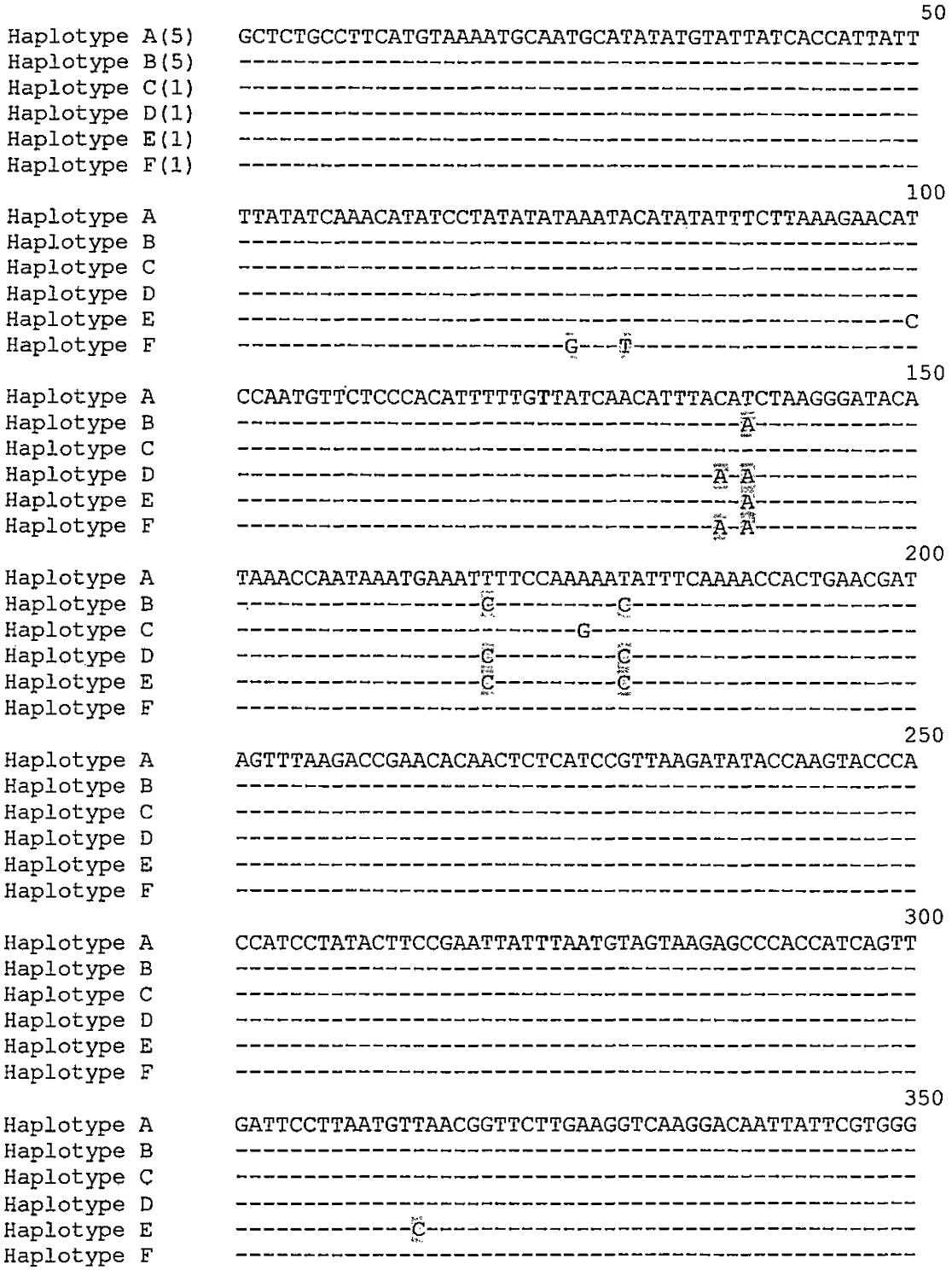
and Radiometer O_2 electrode at the experimental temperature. P_{O2} values were converted to O_2 concentrations using αO_2 values appropriate to the temperature and salinity from Boutilier et al. (1984). M_{O2} was calculated from the decrease (blank-corrected) in O_2 during the period of chamber closure, factored by mass and time.

In light of findings with respect to the chemistry at one site, Sports Club Lagoon, several tolerance tests were performed using water collected from the site (see Results). In addition, standardized tolerance tests were performed with each population (except Wilson Springs). The standardized test medium was designed to simulate a severe natural water chemistry and used Fish Spring Lagoon water (see Table 1 for composition) as a base. The test medium was made by adding sufficient NaCl to Fish Spring Lagoon water to raise measured Cl^- levels to 1000 mM and sufficient processed "trona powder" (largely $NaHCO_3$ and $(Na)_2CO_3$ plus other minor natural salts, kindly supplied by Magadi Soda PLC) to raise measured total CO_2 levels to 1000 mM respectively. The final Na^+ level was about 2800 mmol/l, osmolality was approximately 3300 mOsm, alkalinity was about 1980 mequiv/L, and pH was approximately 10.35. In all tolerance tests, the water was oxygenated prior to use and equilibrated to 32- 35 ^oC to ensure that only the osmotic pressure would be cause of toxicity. Freshly collected fish were returned to the field laboratory in their natural water as for the respirometry experiments. When possible, two tests were done: one test with fish collected the same day, i.e. allowed to settle for several hours, and a second test with fish acclimated to Fish Spings Lagoon water for at least 24 hours. Fish were added to a bucket containing 5L of the test medium; timed observations of mortality were continued until all fish were dead, allowing calculation of median lethal time (LT50).

In general the data have been expressed as means $\pm$ 1 SEM (N), where n = number of fish, and multiple comparisons have been performed by one way analysis of variance followed by the Bonferroni *post hoc* test for multiple comparisons to identify specific differences.

RESULTS

Genetic Analysis


50
Haplotype A(5) GCTCTGCCTTCATGTAAAATGCAATGCATATATGTATTATCACCATTATT
Haplotype B(5)
Haplotype C(1)
Haplotype D(1)
Haplotype E(1)
Haplotype F(1)
100
Haplotype A TTATATCAAACATATCCTATATATAAATACATATATTTCTTAAAGAACAT
Haplotype B
Haplotype C
Haplotype D
Haplotype E C
Haplotype F G T
150
Haplotype A CCAATGTTCTCCCACATTTTTGTTATCAACATTTACATCTAAGGGATACA
Haplotype B A
Haplotype C
Haplotype D A A
Haplotype E A
Haplotype F A A
200
Haplotype A TAAACCAATAAATGAAATTTTCCAAAAATATTTCAAAACCACTGAACGAT
Haplotype B C C
Haplotype C G
Haplotype D C C
Haplotype E C C
Haplotype F
250
Haplotype A AGTTTAAGACCGAACACAACTCTCATCCGTTAAGATATACCAAGTACCCA
Haplotype B
Haplotype C
Haplotype D
Haplotype E
Haplotype F
300
Haplotype A CCATCCTATACTTCCGAATTATTTAATGTAGTAAGAGCCCACCATCAGTT
Haplotype B
Haplotype C
Haplotype D
Haplotype E
Haplotype F
350
Haplotype A GATTCCTTAATGTTAACGGTTCTTGAAGGTCAAGGACAATTATTCGTGGG
Haplotype B
Haplotype C
Haplotype D
Haplotype E C
Haplotype F

Mitochondrial haplotypes that were previously identified in Magadi/Natron tilapia (Wilson et al. 2000) were observed within the 1997 sample collection with the identification of a number of additional haplotypes. One haplotype from the previous study was not observed in the 1997 samples, specifically haplotype E (Wilson et al. 2000) (Table 1). Haplotype B was present in all the lagoon populations in Lake Magadi and both Little Magadi and Lake Natron. Haplotypes A and C were present in all the Lake Magadi lagoon populations. Several lagoon- or lake-specific haplotypes were also identified.

The B sequence in all likelihood corresponds to the A1 mtDNA haplotype identified by Seegers et al. (1999) that was present in both Natron and Magadi lake systems. This haplotype was termed the "Orolonga haplotype" as it was interpreted to have existed within the Magadi/Natron paleolake and is the ancestral type for many of the existing haplotypes within the two lake systems at present. Also similar to Seegers et al. (1999) is the presence of a number of Magadi-specific sequences grouping with the haplotype A, likely the corresponding sequence for A17 (Fig. 2). Unlike the findings of Seegers et al. (1999) several apparently Magadi-specific haplotypes appear to have been derived directly from B (A1, Seegers et al. 1999) and not exclusively the A haplotype.

Within each Magadi lagoon population, haplotypes A and B were the most prevalent sequences with greater than 75% of the tilapia containing one of these two sequences in the majority of populations. Haplotype C was maintained at levels of 5-10% and as high as 30% in the South West Lagoon population. Haplotype B was prevalent in the two other lake systems, Little Magadi and Lake Natron. A number of rarer haplotypes were observed in almost all the populations we examined.

We applied an Analysis of Molecular Variance (AMOVA) (Excoffier et al. 1992) to the haplotype data to assess the partitioning of genetic diversity into the various lakes and populations within the lake systems and the degree of population structuring among the different populations. The first comparison examined the partitioning of haplotypic diversity among *Alcolapia* species from Lakes Natron and the Magadi basin, including the lagoon systems of Magadi proper and Little Magadi. The variance among lakes (11.13%) was slightly lower than the variance among the populations of the Magadi basin (16.79%) with the majority of the diversity (72.08%) being within populations. The ϕ_{ST} value of 0.279, analogous to

Wright's F_{ST} statistic, indicates structuring consistent with the inclusion of three isolated lake systems. The second comparison examined partitioning within the Magadi lake basin by comparing Lake Magadi lagoon populations to Little Magadi. The variance between the two lakes (35.20%) was higher than the comparison that included Lake Natron. The variance existing among populations within the lakes was 4.34% with 60.46% of the variance being partitioned within the tilapia populations. The ϕ_{ST} value of 0.395 was higher in the absence of Lake Natron when comparing the Magadi lakes. A ϕ_{ST} value of 0.055 was observed among the lagoon populations indicating minimal population structuring with 5.54% of the variance existing among lagoon populations and 94.46% existing within the lagoon populations.

Although the ϕ_{ST} value that included all the Magadi populations indicated low levels of structuring, we assessed the potential genetic exchange of fish among the specific Magadi lagoon populations by means of pair-wise comparisons of populations in an AMOVA analysis (Excoffier et al. 1992). An assessment of structuring using a ϕ_{ST} statistic indicated differential amounts of structuring among the lagoon populations of Lake Magadi (Table 2). Our interpretation of the ϕ_{ST} estimates was that a value of approximately 0.050 suggested low levels of differentiation with levels higher than 0.100 supporting substantial levels of structuring (Wright 1978). All comparisons of the Magadi lagoon populations to the different lakes, Little Magadi and Natron, indicated substantial structuring. Within Lake Magadi, populations such as the South West Lagoon indicated some level of structuring among all the lagoon populations within Magadi. A number of pair-wise comparisons indicated very low amounts of structuring between lagoon populations. The overall pattern of the potential movement of fish did not correspond well with geography, i.e. neighbouring lagoon populations, which would be predicted to have more fish exchanged during heavy flooding.

An SSD statistic (Schneider and Excoffier 1999) was used to infer the demographic history of populations based on the mismatch distribution. The SSD statistic significantly rejected population expansion for Fish Springs and West Lagoon and approached significant rejection for South East, South West and Wilson Springs (Table 8). Rejection of a population expansion in the above lagoons was further supported by overlap in the 90% and 95% confidence intervals of θ_0 and θ_1 in the SSD analysis using a

coalescence model (Schneider et al. 1999). A past population expansion could not be rejected for Sports Club based on the mismatch distribution parameter and was supported for Little Magadi and Natron at all three parameters. Analysing the mismatch distribution of all of the Magadi populations, assuming panmixia, indicated a rejection of population expansion consistent with the majority of lagoon populations analysed independently.

Estimates of the time for specific demographic events required an estimate of the substitution rate. A rate of 5.56×10^{-08} • site • year (Nagl et al. 2000) is the recent estimate substitution rate for cichlids, while 1.65×10^{-7} • site • year (Ward et al. 1991, Excoffier and Schneider 1999) has been used as the substitution rate for the mtDNA control region of various taxa including humans. We applied these rates to generate an estimate of the time of the historic demographic event (Table 3). The rate of 1.65×10^{-7} • site • year, assuming a generation time of 60 days for *Alcolapia* , provided estimates of the age of historic demographic events to approximately 10,000 years for all the Magadi lagoon populations and approximately 5,500 years ago for Little Magadi and Lake Natron. As the majority of Magadi lagoon populations had expansion signals that were rejected, the values of the timing of the demographic event may be biased. A generation time of 60 days is a conservative estimate based on estimates of 42 days interpreted from Coe (1966).

Estimates of female effective population size (θ_1) from the mismatch distribution analysis were generated and compared to field observations (Table 4). Field estimates of female effective population size assumed gender ratio and an effective population / census population ratio of 50:50, although the ratio of effective-to-census population size may be as low as 10% in many natural populations (Frankham 1995b). The population sizes in Little Magadi and Lake Natron were difficult to assess as they represented more lake-like systems. The three largest populations in Lake Magadi (Fish Springs, South West and West Lagoon) were in reasonable proximity with both expected N_F (female effective population size) and the field estimates numbering into 1000s of fish. However, the three smaller populations (Sports Club, Southeast and Wilson Springs) had 10-20 fold lower field estimates than values from the mitochondrial mismatch distributions.

Based on both estimates of the female effective population size (N_F), the mean time to fixation was estimated for a given mtDNA haplotype (Kimura and Ohta 1969, Parker and Kornfield 1997). Consistent with estimates of τ and the formation of Lake Magadi 9,000 years ago, the effective female population sizes were more consistent with field estimates in the large populations using a substitution rate approximately three times faster than recent published estimates for cichlids (Nagl et al. 2000) (Table 4). Estimated fixation times for mitochondrial DNA at female effective population sizes (N_F) based on the accelerated substitution rate ranged from 100 years, 600 generations, in Little Magadi to almost 3500 years in other populations. Fixation times based on field estimates ranged from 100 years in small lagoon populations to 1500 years in larger populations. A discrepancy among expected and field estimates of N_F, fixation was observed in the small lagoon populations of Sports Club lagoon, Southeast lagoon and Wilson Springs. Assuming that lagoon fish were exchanged constantly every 2-3 generations within Lake Magadi, the estimates of expected and field N_F values would have resulted in fixation to one haplotype potentially twice within the history of the lake based on field estimates and several times based on the mismatch distribution parameter N_1.

Morphological Characterisation

Specific morphological differences were observed in this study among the *Alcolapia* populations. Lake Natron fish had a very different coloration from all Lake Magadi populations. Lake Natron breeding males exhibited an olive-brown dorsal surface and intense golden yellow ventral surface with black-tipped dorsal and anal fins, identical to the photograph in Fig. 7B of Seegers and Tichy (1999) identified as "*Oreochromis alcalicus* male from Shombole Swamps", which is the same region from which the present Lake Natron cichlids were collected. Lake Natron females were duller, olive-grey on the dorsal surface and more brownish-orange on the ventral surface. We could not visually distinguish the six Lake Magadi populations based on external coloration. In all six, males exhibited a bluish upper surface, often quite brilliant in breeding individuals, with a prominent white lower lip and belly. This general appearance was very similar to the photograph in Fig. 26 of Seegers and Tichy (1999) identified as "*Oreochromis grahami*

male from South Western Lagoon, Lake Magadi". Females from the six Lake Magadi lagoon populations were much duller, with a greyish green upper surface and lips, and a dull white belly. Fish from Little Magadi were distinctly different in having a superior (i.e. upward slanted) mouth whereas all six Lake Magadi populations, as well as the Lake Natron fish, exhibited a simple terminal mouth. Coloration of all Little Magadi fish was comparable to that of females from the Lake Magadi populations with greyish-green upper surface and whitish belly. It is possible that no breeding males were captured.

There were very distinct differences in relative gut length amongst four populations that were examined in detail (Table 5). The mean gut length/body length ratio ranged from approximately 3.0 in Fish Springs Lagoon down to approximately 1.2 in the South East Lagoon population. Southwest Lagoon and Lake Natron fish exhibited intermediate ratios of approximately 1.8.

Water Conditions at Different Sites & Physiological Characterization

The six Magadi lagoon systems, Little Magadi and Lake Natron exhibited a relatively narrow range of temperature ($32^{o}C - 42^{o}C$) and pH (9.1 – 10.1) but marked differences in osmolality, ionic concentrations and alkalinity (Table 6). Using the well-studied Fish Spring Lagoon site as a reference, where the osmolality was about 50% that of full strength seawater, osmolalities varied from less than half this value at Wilson's Springs, to three-fold this value at Little Magadi. Amongst the different sites, Na^{+}, Cl^{-}, total CO_2, and alkalinity also varied in approximately the same order as osmolality, although there was obviously not a constant ratio of ions amongst the various sites. Most notable were alkalinities in excess of 1200 mequiv/l (in comparison to about 3 mequiv/l in full strength seawater) in both Little Magadi Lake and Sport Club Lagoon (in Lake Magadi), associated with very different Na^{+} and Cl^{-} concentrations but similar osmolalities at the two sites. It should be noted that the values reported in Table 6 for Sports Club Lagoon represent the maxima where fish were seen to occur; as outlined in detail subsequently, there was substantial chemical and physical heterogeneity within this environment.

Measurements of urea-N ($M_{Urea\text{-}N}$) and ammonia-N ($M_{Amm\text{-}N}$) excretion rates demonstrated that fish from all populations tested within Lake Magadi (only theWilson's Springs population was not evaluated), and also both Lake Natron and Little Magadi, were 100 % ureotelic (Fig. 3). $M_{Amm\text{-}N}$ was not

significantly different from zero after blank correction, but M_{Urea-N} varied almost 3-fold amongst different populations (Fig. 3). There was no obvious relationship to the chemical severity of the environment, as indicated, for example, by osmolality (Fig. 3). However, as these measurements were performed under field conditions, there were differences in ambient temperature, time-of-day, and likely feeding status amongst the different populations. Once M_{Urea-N} data were normalized for differences in M_{O2} according to Wood et al. (1994), virtually all of this variation disappeared, with N/O_2 ratios in the range of 0.18-0.24 (Fig. 4). Only the South East Lagoon fish exhibited significantly higher N/O_2 at 0.33. This difference can probably be explained by the very high levels of ammonia-N in the water (271 umol/l) due to the abundance of flamingo guano at this collection site; ammonia exposure is known to stimulate M_{Urea-N} in *Alcolapia grahami* (Wood et al., 1989). Water ammonia level was undetectable (< 2 umol/l) at all other sites.

For three populations (Little Magadi, South East Lagoon, and Lake Natron), measurements were started in the field in Fish Springs Lagoon water, and also performed in their natural waters using separate batches of fish at the laboratory. Again, while there were differences in absolute values of M_{Urea-N} between the two measurements, these disappeared after normalization to M_{O2}. Therefore Figures 3 and 4 illustrate data for tests performed in the natural waters of each population except for Fish Spring Lagoon (where the natural water proved toxic, as discussed below) and for South East Lagoon (the flamingo guano site, where the fish actually extracted ammonia from the natural water).

In accord with the M_{Urea-N} measurements, assay of hepatic enzyme activities demonstrated a full complement of all the enzymes of the Krebs ornithine-urea cycle (CPSase III, OTC, AS/AL, ARG) as well as the N-feeder enzymes (GSase, ALAat, and ASPat) and GDH in all populations examined (Table 8). These included Little Magadi, Lake Natron, and four Lake Magadi populations - Fish Springs Lagoon, Sports Club Lagoon, South East Lagoon, and South West Lagoon. GLNase was low and variable. There were some significant variations in specific enzyme levels amongst populations, but nothing that correlated consistently with either M_{Urea-N} (Figs 3, 4) or the chemistry of the respective environments.

With the exception of Little Magadi, whole body Na^+ and Cl^- concentrations were rather invariant amongst the different populations (Table 9), though the Wilson's Springs fish, which inhabit the most

dilute environment (Table 6), did have slightly lower whole body ions. Apart from this, whole body ion levels were not reflective of the concentrations in the water (Table 6), suggesting good homeostatic regulation. Little Magadi fish were a clear exception (Table 9), with whole body ions approximately 3-fold greater than those in the other populations, even though water Na^+ and Cl^- levels were comparable to levels at several other sites, and by no means the highest overall (Table 6). However, variations in whole body urea-N levels amongst populations were pronounced, and much more consistent with water chemistry. Thus urea-N was lowest in Wilson's Springs fish, where environmental osmolality was lowest, and highest in Little Magadi fish, where osmolality was highest (Table 9). Overall, there was a strong positive relationship (Fig. 5A) between whole body urea-N and environmental osmolality, suggesting that urea plays a small but important role as a regulated osmolyte.

To our surprise, we found that when fish from Sports Club Lagoon were collected and placed in what was ostensibly their own water (i.e. water collected from the site), they were all dead within 3h, so it was impossible to run the metabolism experiments reported in Figs. 3 and 4 in site water for this population (Fish Springs Lagoon water was used instead). Furthermore, when fish from Fish Springs Lagoon were placed in this water, they were all dead within 5 min, suggesting a marked difference in tolerance between the two populations. As outlined below, the explanation for the failure of Sports Club Lagoon fish to survive in "their own" water is the fact that they actually exploit a less severe microhabitat within their small lagoon. Nevertheless, these results stimulated us to evaluate the tolerance of different populations to challenge with severe environmental chemistry.

Upon analysis, the "toxic" water from Sports Club Lagoon had the following composition: Cl^- 1025 mmol/l, Na^+ 2400 mmol/l, total CO_2 1066 mmol/l, alkalinity 2130 mequiv/l, osmolality 3400 mosm/kg , pH 10.03. At the time, we were concerned that cyanobacterial toxins in this natural water might be the cause of toxicity. Therefore, we made up an artificial standardized challenge medium as outlined in the Methods which duplicated this chemistry fairly well: Cl^- 1000 mmol/l, Na^+ 2800 mmol/l, total CO_2 1000 mmol/l, alkalinity 1980 mequiv/l, osmolality 3300 mosm/kg , pH 10.35. Fish from all populations except West Lagoon were tested in separate, duplicate runs, and in all cases, 100% mortality occurred. Median lethal time (LT50) varied from a low of 2 min for Wilson's Springs to a high of

approximately 30 min for fish from Sports Club Lagoon and Little Magadi, with Fish Spring Lagoon fish intermediate at 11 min (Table 10). Among the different populations, LT50 was well correlated with the osmolality of their natural environment (c.f. Table 6) and with whole body urea concentration (Fig. 5B), reinforcing the importance of urea as an osmolyte.

We then re-examined the water chemistry and fish distribution in the Sports Club Lagoon, and found considerable heterogeneity in both. This whole lagoon in Jan –Feb 1997 was only about 165 m along the shore and 30 m wide, and fish appeared to be restricted to the inshore 10 m. The depth here was only 2.5 to 5.0 cm. We identified two sources of spring fed water: hot, very alkaline springs at the bottom of the lagoon (one sampled : 45.3^{O} C, Cl^- 1283 mmol/l, Na^+ 4500 mmol/l, total CO_2 1329 mmol/l, alkalinity 2618 mequiv/l, osmolality above 6000 mosm/kg , pH 9.91.) and a single much fresher water spring at the shoreline (37.0 ^{O}C, Cl^- 20 mmol/l, Na^+ 102 mmol/l, total CO_2 48 mmol/l, alkalinity 69 mequiv/l, osmolality 153 mosm/kg , pH 9.80) that provided cooler less alkaline water along the lagoon surface. Evaporative cooling at the surface plus photosynthesis by freeliving cyanobacteria in the surface waters was also occurring. Distinct temperature and O_2 gradients with depth were observed – for example at one site 40.6°C, P_{O2} 290 torr (~ twice air saturation) at the surface and 44.5°C, P_{O2} 8 torr (almost anoxic) at the 4.0 cm bottom. More importantly, there were great differences in Cl^- and total CO_2 concentrations with depth. For example, at one site the following values were read at the surface (Cl^- 71 mmol/l, total CO_2 123 mmol/l) whereas from 1.0 to 2.5 cm at the bottom, the values were more than 10-fold higher (Cl^- approximately 1350 mmol/l, total CO_2 approximately 1400 mmol/l.). At a second site, the differences were even more extreme. The "toxic water" we had collected had unavoidably consisted of mainly this deeper water.

To investigate the question of tolerance further, we ran a test with the Sports Club Lagoon fish to determine whether it was the NaCl or the $NaHCO_3$ + $(Na)_2CO_3$ (i.e. alkalinity) components, or both, which were toxic. Each component was tested separately on 8 fish using Fish Spring Lagoon water dosed with NaCl to raise measured Cl^- to 1000 mmol/l, or dosed with trona powder to raise measured total CO_2 to

1000 mmol/l. Both components separately caused 100% mortality within 45 min, though the LT50 was slightly shorter in the trona test.

Upon closer examination, it was clear that the Sports Club fish were living "on the edge" by exploiting a non-toxic surface layer no more than 1.0 cm deep, and congregating close to the shore edge at the source of the freshwater spring system for the majority of time we observed their behaviour. Samples from this area provided the "typical" water chemistry reported for the Sports Club Lagoon in Table 6. When the observers made sudden movements, the fish would briefly enter into the deeper "toxic" water, likely an anti-predator response, although this behaviour diminished when a series of movements were made. Thus the input of "fresher" water into this system appears to be crucial for the survival of this population.

A final laboratory challenge experiment examined the surface behaviour in the Sports Club Lagoon fish and whether this behaviour existed in another population from Lake Magadi, the Fish Springs Lagoon fish. In a bucket, we duplicated the stratified environment of Sports Club Lagoon by first adding a slightly concentrated version of the standardized toxic challenge medium, and then slowly pumping onto it a layer of diluted Fish Spring Lagoon water. The stratification was confirmed with Cl^- readings from the surface layer (380 mmol/l) and depths of 2.5 cm (1400 mmol/l) and 5.0 cm (1400 mmol/l). The Sports Club cichlids (n=6) swam slowly at the surface, one fish died at 30 minutes, another at 60 minutes and no additional fish died by 4 hours when the experiment was terminated. In a similar test with Fish Springs Lagoon fish, the cichlids did not swim at the surface and destroyed the stratification within minutes of starting the experiment. All died, with an LT50 of 18 min. In this test, the initial Cl^- concentrations were approximately 80 mmol/l (surface), 900 mmol/l (2.5 cm), and 900 mmol/l (5.0 cm), respectively. Although differences existed in chemistry between the two tests, it was a conservative error as the Sports Club fish were tested with more severe conditions.

Finally, a sudden rainstorm on the night of Jan. 16-17, 1997 provided an opportunity to evaluate conditions on the flooded surface of the trona, a potential route for intermittent genetic interchange amongst populations. The transect of Table 5 was performed in a direction perpendicular to shore in the region of Sports Club Lagoon at about 16:00, approximately 12h after the end of the storm. At this time the

depth of the floodwater on the trona was 2.0 to 6.0 cm, and the elevated levels of total CO_2, Na^+, Cl^-, alkalinity, osmolality, and pH indicated much dissolution of the trona into the rainwater. . Based on the results of the standardized challenge tests, the measured water chemistry alone of the trona floodwater would likely have proved lethal (e.g. osmolality approaching 3000 $mosm.kg^{-1}$, total CO_2 approaching 1000 $mol.l^{-1}$, alkalinity approaching 2000 $mequiv.l^{-1}$; Table 5), and this would have been exacerbated by temperatures significantly above the upper lethal limit of 42.5°C and a mean P_{O2} below the lethal threshold of 16 torr (c.f. Narahara et al, 1996). Considering that the next nearest lagoon was more than 3 km away the likelihood of fish surviving a journey through this extended zone of toxicity seems highly unlikely.

DISCUSSION

Genetic Structure

In this study, several of the population comparisons indicated some population differentiation based on haplotype frequencies, while other comparisons showed very little differentiation indicative of gene flow among certain lagoons, although the observed similarities and differentiation were discordant with the geographic distribution of lagoon systems along the shoreline (Fig. 1, Table 2). These findings contradict previous genetic structure analyses with mtDNA haplotypes and more importantly multilocus DNA fingerprinting analysing nuclear minisatellite loci that indicated significant population structuring between two of the lagoon populations (Wilson et al. 2000). The discrepancy between the mtDNA results of the two studies may be the result of the lower sample size of one of the lagoon populations within the initial study. Despite this difference, the previous comparison indicated similar predominant A and B haplotype distribution within the two Magadi lagoon populations (Wilson et al. 2000).

The lack of significant differentiation among the lagoon populations in this study was further complicated by lagoon-specific specialised adaptations (summarised in Table 11) that support locally adapted genomes existing in an allopatric situation within the different habitats. The common A and B haplotype distribution among all six Magadi lagoon populations was particularly surprising given that in allopatry we would predict that the lagoon populations should maintain different haplotypes through lineage sorting (Duvernall and Turner 1998). There are two alternative hypotheses to explain the common

pattern of mtDNA haplotypes among all the lagoon systems. Firstly, gene flow is prevalent among the lagoon systems during the annual rains and flooding events. Secondly, a common selection pressure is acting on the mitochondrial DNA maintaining the haplotypes within isolated lagoon systems.

Extensive gene flow among the Magadi lagoon populations during annual flooding events could maintain the common haplotypes through the large metapopulation of tilapia that are subdivided into lagoon populations for the majority of the year and homogenised every 3-6 generations. This has been proposed by Coe (1966) for the Magadi tilapia, however, Coe's (1969) conclusion for Lake Natron during heavy rains was that deoxygenation of the water due to the flush of blue-green algae resulted in severe mortality and restricted fish to their springs and creeks. Tichy and Seegers (1999) have proposed that barriers to gene flow existed in Lake Natron as a result of deoxygenation and the lethal increase in concentrations of salt and brine, eventually resulting in the re-formation of the soda crust within 48 hours. This explanation is consistent with our findings of extreme osmotic pressure following the flooding of Lake Magadi. At present it is unknown whether Lake Natron tilapia have evolved a primitive air-breathing lung from the swim bladder (Maina et al. 1995) and whether deoxygenation is a significant barrier to movements during flooding. For the Magadi situation it is more likely that the high osmotic pressure of the floodwaters, lethal to all the tilapia we examined under experimental conditions (Table 7), acts as a barrier to dispersal and gene flow among lagoon populations. The floodwater temperature is also likely an effective barrier to gene flow as a range of 39-43°C surrounds the lethal limit for Magadi tilapia.

Fine-scale population structuring has been identified in other cichlid species inhabiting the more lacustrine environment of Lake Malawi. Intra-specific population differentiation in four rock-dwelling cichlid species was observed at distances of 700-1400m (van Oppen et al. 1997), although the biological significance of the genetic divergence estimated in this study have been questioned (Hedrick 1999). Fine-scale structuring was observed using haplotype frequencies among replicate sample collections of *mbuna* cichlids from Malawi (Moran and Kornfield 1995). Proposed barriers to gene flow in *Labeotropheus fuelleborne* include long stretches (>2 km) of sandy bottoms and deep water (Arnegard et al. 1999). The Rift Valley lakes of Malawi, Tanganyka and Victoria are presently stable water bodies while Lake Magadi

enjoys a lake-like environment of toxic water for 2-3 days, one to two times a year (Coe 1966), a system predicted to be less conducive to gene flow than other lake systems.

Alternative explanations for gene flow include movement by predatory birds or inter-connections among the spring systems allowing an alternate and less severe route to other lagoons. The first explanation would not likely result in the levels of gene flow required to maintain the common haplotypes in all the Lake Magadi lagoons and at present there is no evidence to support the second alternative explanation. Even if fish are translocated via these alternate routes, low levels of movement may not result in gene flow due a failure to reproduce, consistent with morphological, physiological and behavioural differences that we have observed (Table 11 – see below).

Morphological Evidence of Lagoon-specific Adaptation

The very different upwarding facing mouth in the Little Magadi fish suggests a different feeding habit, perhaps associated with feeding at the surface in this open-lake habitat. Here the bottom is covered with mud, and there appear to be no exposed rocks to support periphytic cyanobacterial growth. Nevertheless, the gut still appeared to be full of blue-green algae, suggesting that these fish ate floating or free-living rather than crustose forms. Feeding has only been studied in the Fish Springs and South West Lagoon populations where the terminal mouth is used for grazing periphytic blue-green algae off rocks (Coe 1966, Narahara et al. 1996, Narahara, 2000). It may be that the blue-green algae species differ amongst sites because of differences in water chemistry, and that the differences in relative gut length (Table 5) reflect differences in algal digestibility. In this regard, the very short gut length of the Southeast Lagoon population is notable. Here the bottom is covered with flamingo guano, and inspection of gut contents suggests that while some cyanobacteria are eaten, guano is also an important component of the diet. This "pre-digested" food source may only require a short intestinal length for processing.

Additional evidence of lagoon-specific adaptations are the different gill structure and size, with a 2.5 fold greater weight-specific O_2-diffusing capacity in the Southeast lagoon compared to the Fish Springs lagoon (Maina et al. 1996).

Assuming that gene flow occurs among the lagoons, Magadi tilapia would have to demonstrate phenotypic plasticity to adapt to the differential lagoon systems and express the morphological adaptations we observed. In general, there is not strong evidence for phenotypic plasticity among the cichlid species flocks for morphological characters. Adaptations in jaw morphology, the character most often studied in cichlids, have been shown to evolve independently (Ruber et al. 1999) and rapidly as a result of independent bones in the flexible jaw structure (Galis and Metz 1998). However, although this flexibility is a mechanism for rapid divergence, ultimately reproductive isolation, whether allopatric, sympatic or microallopatric (Meyer 1993), reinforces speciation within cichlid species flocks. The morphological adaptations we observed among several of the lagoon populations strongly supports selection pressures acting on nuclear genes within each lagoon system, thereby differentiating into locally adapted genomes.

Water Conditions at Different Sites & Physiological Characterisation

There was substantial variation in water conditions amongst the eight different sites studied (Table 6), presumably reflective of the characteristics of the inflowing hotsprings. These measurements were all made at one time of the year (January-February) which intervenes between the "little rains" of December and the "big rains" of March-May. It would be of great interest to know how much variability occurs on a seasonal basis within individual sites, especially during the summer period of greatest aridity (c.f. Coe 1966). Consistent features amongst all sites however were the presence of high pH's, alkalinities, and Na^+ and Cl^- levels. Osmolalities were also high, but generally much lower than might be predicted by adding up all the measured electrolytes. This is probably explained by substantial ion pairing in solution at these high concentrations.

Fish Springs Lagoon is the only site where water chemistry has been studied previously, and the claim has often been made the fish here live in one of the most hostile aquatic environments on earth (e.g. Johansen et al. 1975, Wood et al. 1994, Narahara et al. 1996). Clearly this claim must be re-evaluated in light of the current findings that Sports Club Lagoon and Lake Little Magadi have 3-5 fold higher alkalinities and osmolalities yet still support thriving cichlid populations. The exploitation of a less severe surface microhabitat may provide part of the explanation for the tolerance of the Sports Club Lagoon fish,

but the same cannot apply in Little Magadi, which is an open lake. Little Magadi clearly deserves further chemical study (as well as biological study, see below), because the water ion ratios are very different from those at other sites, with much lower Na^+ and Cl^- relative to alkalinity and osmolality, suggesting the presence of substantial concentrations of other strong electrolytes.

An important physiological finding of this study is that the condition of 100% ureotelism, previously identified only in the Fish Springs population (Randall et al. 1989, Wood et al. 1989), was expressed in all of the *Alcolapia* populations (Figs. 3, 4) which were examined (7 of 8, Wilson's Springs fish not tested)). This conclusion was backed up by the finding that all the enzymes of the Krebs ornithine-urea cycle were expressed in the livers of all 6 of the populations which were examined (Table 8; Wilson's Springs and Western Lagoon fish were not assayed). At the time these collections were taken, we had not yet discovered that these enzymes are expressed to an even greater extent in the white muscle, such that this large tissue, rather than the small liver, is probably the major site of urea production, at least in the Fish Springs Lagoon fish (Lindley et al. 1999). Therefore white muscle was not collected from the other populations, and the meaning of the variations in liver enzyme activities and HSI amongst different populations (Table 8) is difficult to evaluate. The key point is that the complete cycle is present.

While it was originally believed that the genes for the Krebs ornithine-urea cycle were silent or deleted in most teleost fish, their full expression has now been found in the embryonic forms of several ammoniotelic teleosts, followed by switching off sometime after hatch (Wright et al. 1995, Chadwick and Wright 1999). Furthermore the gulf and oyster toadfishes, *Opsanus beta* and *Opsanus tau* (Mommsen and Walsh 1989, Walsh 1997, Wang and Walsh 2000) and some catfishes of the Indian subcontinent (Saha and Ratha 1998) are also known to express the ornithine-urea cycle as adults. These fish are normally ammoniotelic, but can convert facultatively to ureotelism in response to environmental stressors. Therefore this characteristic probably represents retention of an embryonic characteristic in adult life. However the present *Alcolapia* species are the only teleost species which are 100% ureotelic as adults, and additional tests suggest that this is obligate ureotelism (unpublished results). As discussed previously in detail elsewhere (Randall et al. 1989, Wood et al. 1989, Wood et al. 1994, Walsh et al. 1993), this

ureotelism is highly adaptive to life in an alkaline environment because of the problems in excreting ammonia into high pH water.

When expressed as the N/O_2 ratio, urea-N production was uniform across populations (Fig. 4), in accord with earlier work on just the Fish Spring Lagoon population (Wood et al., 1994). This indicates that urea-N production is normally a function of metabolic rate. The one exception, a high N/O_2 ratio in the South East Lagoon fish (Fig. 4) was explained by the fact that these fish were living in a high ammonia environment due to bacterial ammonia production from the guano-laden benthos. In our tests, these fish actually extracted sufficient ammonia-N from the water to explain the elevated urea-N production (unpublished results). Earlier, this potential for ammonia-N detoxification by elevated urea-N production was demonstrated in laboratory tests with Fish Spring Lagoon specimens (Wood et al. 1989), but its significance was problematical as elevated environmental ammonia levels have never been found at this site. The Southeast Lagoon findings demonstrate that this phenomenon has real ecological significance, and is an additional adaptive benefit of ureotelism.

The present findings suggest that urea production may play a third adaptive role, that of contributing to osmoregulation. Whole body urea levels correlated extremely well with environmental osmolality in the different populations, as well as with LT50 in the standardized challenge tests (Fig. 5). To date, osmoregulatory ability has been studied only in the Fish Springs Lagoon fish which maintain an internal osmolality of about 350 mosm/kg (Wright et al. 1990, and unpublished results). The measured whole body urea levels in these fish (~ 11mmol/kg; Fig. 5), assuming a body water compartment of 65-70%, might account for about 5 % of internal osmotic pressure. While this is relatively small, its contribution may be significant as we have found that whole body urea concentrations (in Fish Spring Lagoon specimens) can be rapidly adjusted in the face of sudden upwards or downwards osmotic challenge (unpublished results).

The same may not be true of differences in whole body Na^+ and Cl^- concentrations that were fairly uniform in most populations, but three-fold higher in Little Magadi fish (Table 9). This appears to be unique to the Little Magadi poulation, and was not correlated with particularly high environmental Na^+ and Cl^- levels at this site although it is obviously adaptive to the very high osmolality at this site (Table 6).

Such differences have never been seen in physiological challenge tests with Fish Spring Lagoon fish (unpublished results).

The same is true of the marked differences amongst populations in tolerance to a standardized severe environmental challenge test (Table 10, Fig. 5B), and in the fascinating behavioural adaptations of the Sports Club Lagoon population to exploit a livable micro-environment at the surface overlaying toxic water below. Nevertheless, the results of these toxicity tests, together with the severe chemistry measured along the transect of floodwater over the trona (Table 7), suggests for even the most resistant populations, the toxic floodwater will serve as an impenetrable barrier to movement and gene flow among the lagoon systems.

In drawing conclusions about selection pressures on physiological characteristics, phenotypic plasticity (in physiological terms "acclimation") must be considered as an alternate explanation to genetically based adaptation. Firstly, the 100% ureotelism among all the *Alcolapia* populations is obviously an important evolutionary adaptation to the environmental conditions of these two Soda lakes but does not address the issue of lagoon-specific adaptation. Secondly, ammonia-N detoxification through elevated urea-N production, while ecologically important, can be induced in Fish Springs fish despite the absence of high ammonia concentrations in this lagoon supporting a physiological response based mechanism. Thirdly, although the utilisation of urea for osmoregulation is a physiological response (Fig. 5), differences in whole-body urea concentrations at similar water conditions (unpublished data) suggest that there is a genetic basis for range and response differences of urea retention in different populations inhabiting different environmental severity. This may be supported by the elevated difference in the HSI values of the Sports Club lagoon fish compared to the other *Alcolapia* populations (Table 8). Also differences among groups of lagoons with whole-body urea concentrations, i.e. the higher levels in Sports Club, Southwest and Little Magadi fish from environments with the highest osmotic pressure, suggests a genetic basis for the physiological range observed with urea retention.

Urea retention is estimated to account for 5% of the osmoregulation required by tilapia in Magadi and Natron environments. Therefore it is likely additional genetically based adaptations, e.g. behavioural adaptations in Sports Club Lagoon tilapia, and presently undetermined adaptations have evolved to allow

survival within extreme environmental osmotic pressure. This is supported by the mortality observed in the experimental challenges (Table 10). While adult fish should not survive migration into the Sports Club lagoon environment, the possibility does exist that larval fish transferred by mouth-brooding adults may rapidly adapt to the specific environmental conditions. The cichlid *Asterochromis alluadi* demonstrates flexibility in jaw morphology depending on the diet in the early stages, mollusc vs. insect, although this is dependent on the amount of calcium intake from a mollusc diet such that the feeding behaviour re-enforces the jaw adaptation (Barel et al. 1991). Overall the evidence supports phenotypic limitations on survival among some lagoon systems potentially supporting genetic divergence but at the very least resulting in reproductive isolation among some lagoons.

Maintenance of Mitochondrial DNA Haplotypes

The severe floodwater conditions and genetic, morphological and to a lesser degree physiological evidence supports isolation with differentiation as a result of selection pressures among isolated lagoons. These findings reject the hypothesis of gene flow, and are consistent with the maintenance of mitochondrial DNA haplotypes as a result of a common selection pressure among the Magadi lagoon systems. The well-documented geological history of the Lake Magadi basin allows certain predictions of historic population events and the likelihood of maintaining mtDNA haplotypes.

Lake Orolonga was proposed to be present during a period of evolutionary stasis with one predominant tilapia form similar in appearance to *A. alcalicus* and *A. grahami* (Tichy and Seegers 1999). This period of stasis was followed by rapid morphological and "neutral" haplotype radiation (Seegers et al. 1999). Seegers et al. (1999) suggest that the *Alcolapia* species flocks may demonstrate elevated rates of mutation, potentially as a result of high environmental stress. We found that the estimates of past demographic events, based on the parameter τ, were consistent with the separation of Lake Orolonga 9000-10,000 years ago when using a substitution rate three times higher than presently published estimates for African cichlids (Nagl et al. 2000) and accounting for the rapid generation time of *Alcolapia grahami* (Coe 1966) (Table 3). Given that the majority of haplotypes are specific to one or the other of the soda lakes, it

seems reasonable that the generation of the haplotypes in both systems occurred following the separation of the paleolake, a point also made be Seegers et al. (1999). Calibrating the substitution rate with the geological evidence and accounting for the rapid generation in the tilapia provides the most biologically realistic estimate for this system.

Selection pressures would likely have intensified as the lake levels lowered, dividing the paleolake, and shifting the water source to the thermal hot springs and less on lake water from a more humid environment at the end of the Pleistocene. Selection would likely have further intensified in Lake Magadi 7,000 years ago when the salinity increased and separation into lagoon systems began. Intense selection pressure would have potentially resulted in population bottlenecks both in the larger Lake Magadi basin followed by the isolation of lagoon environments. Evidence for population bottlenecks was observed in two of the Magadi populations that were previously analysed, the Fish Springs and Southeast Lagoons (Wilson et al. 2000). This was in contrast with Lake Natron tilapia that showed reasonable low levels of band-sharing and therefore higher levels of genetic variation not suggestive of past population bottlenecks. SSD statistics (Schneider and Excoffier 1999) did not support a historic population expansion in the majority of lagoon populations, consistent with potential bottlenecks. Population expansion was not excluded for the Sports Club Lagoon tilapia, a surprising result as this lagoon has the most severe environment and maintains one of the lowest effective population sizes (see below).

Levels of genetic variation observed at nuclear loci with the DNA fingerprints (Wilson et al. 2000) also reflect small effective population sizes in combination with historic population bottlenecks. This is more likely for the Southeast Lagoon as the field estimates indicate a relatively small number of fish compared to the Fish Springs Lagoon with fish in the 1000s (Table 4). Independent population bottlenecks specific to lagoon systems and/or small effective population sizes are predicted to result in lineage sorting of mtDNA haplotypes (Duvernall and Turner 1998) resulting in differential haplotype distributions among isolated lagoons. The ubiquitous nature of the common A and B haplotypes and to a lesser extent the C haplotype suggests the maintenance of these haplotypes despite potential isolation, historic bottlenecks and small effective population sizes

Estimates of female effective population size (N_F) based on nucleotide diversity and a mismatch distribution analyses appeared consistent with field estimates for the larger lagoon populations (Fish Springs, Southwest and West Lagoon) in that the numbers were in the 5,000-10,000 range (Table 4). Discrepancies were apparent for estimates of the smaller lagoons, where highly conservative field estimates were no higher than 250 female breeding fish and estimates from mtDNA control region sequences were 20-30x higher. Considering the rate that mtDNA haplotypes are predicted to go to fixation based on the rapid generation time, all the lagoons should have gone to fixation for one mtDNA a number of times within the history of Lake Magadi. Although this may argue for extensive gene flow and a large effective population size, it is important to note that N_F estimates based on τ and assuming a panmictic Magadi population, revealed values three times lower than the combined field estimates. Furthermore, even assuming panmixia, fixation of a mtDNA haplotype should have occurred multiple times within Magadi since the formation of the lake based on mismatch distribution estimates of N_F (Table 10).

The Sports Club Lagoon system is perhaps the best supporting evidence for the maintenance of common haplotypes in isolation. Unless the hypothesis of rapid larval adaptation is supported, it is highly improbable that migration into this severe environment will occur from other sources given the results of the LT50 experiments and the observation of the behavioural adaptation to the less severe surface water from an alternative spring. As a result, this population has likely maintained a small effective population size for a long period of time. With no influx of mtDNA haplotypes to maintain the observed distribution, this population should have gone to fixation to one of the observed haplotypes many times within its history.

Balancing Selection Acting on Mitochondrial DNA

In the absence of gene flow, the maintenance of two common haplotypes in small populations may be explained by balancing selection where there is selective advantage to more than one mtDNA variant within heterogeneous microhabitats of the Magadi lagoon systems. Evidence for environmental heterogeneity of microhabitats was observed in the water chemistry and fish distribution in the Sports Club

Lagoon. We identified both a source of hot alkaline water and a source of fresher water over the surface that provided a cooler less alkaline environment. The ubiquitous maintenance of haplotypes A and B suggests that any environmental heterogeneity that maintains these haplotypes through balancing selection is common among all the Magadi lagoons.

We propose a model of balancing selection acting on a gene or genes on the mitochondrial DNA that is reflected in the control region haplotype distribution. The "Orolonga" haplotype (B, this study and A1 according to Seegers et al. (1999)) was predicted to represent the period of stasis prior to the separation of the paleolake and changes in the environmental conditions. This seems reasonable as it appears as the progenitor sequence for all the observed sequences in the two lakes. Assuming an accelerated substitution rate and generation time consistent with *Alcolapia*, the other common haplotype (A or A17 (Seegers et al. 1999)) evolved in the range consistent with the separation of Orolonga. Dry periods corresponding to low water levels would increase the exposure of Lake Magadi fish to water from thermal springs, predicted to be higher in temperature, salinity, alkalinity and therefore osmotic pressure. We predict the mtDNA corresponding to haplotype A evolved in response to high osmotic pressure, although other possibilities exist including adaptations to anoxia, hyperoxia and high temperature. The ubiquitous nature of haplotype B suggests that this sequence may be maintained for other properties common to Lakes Magadi, Natron and Little Magadi. For the purpose of this model we consider additional haplotypes to be associated with either the A or the B mtDNA lineages, although, additional selection pressure may be acting on functional sequence differences on those mtDNA molecules, e.g. haplotype C.

Similar stable frequencies of two common haplotypes and rare endemic types were observed in natural populations of *Drosophila subobscura* (Garcia-Martinez 1998, Castro et al. 1999). Balancing selection was proposed as one of the explanations for the mtDNA haplotype distribution but negative Tajima's D scores did not support this, although this was not rejected based on the use of only a few segregating sites (Castro et al. 1999). Experiments on flies from natural populations of *D. subobscura* within caged conditions provided strong evidence for selection acting on the mtDNA as fixation to one haplotype occurred within 20 generations when flies were bred in captivity (Garcia-Martinez 1998).

Discordance between allozyme allele frequencies and both morphology and haplotype frequencies in spiders of the Genus *Metepeira* supported balancing selection acting on allozyme loci (Piel and Nutt 2000). The original interpretation of the allozyme data supported extensive gene flow while morphological and behavioural differences and mtDNA haplotype distributions suggested differentiation to the species level. Although opposite to our findings with respect to the loci, i.e. Magadi mtDNA demonstrate low differentiation while morphological, physiological and behavioural characters and nuclear loci show significant differentiation. Evidence of selection acting on the mtDNA COI gene has been observed in deep-water vent clams (Genus *Vesicomydae*) (Peek et al. 2000) based on higher number of non-synonymous to synonymous substitutions in more severe environmental conditions, conditions not dissimilar to the Soda Lakes. We are currently examining mtDNA genes including COI to determine if significant sequence difference may reflect differential selection pressures.

Taxonomic Status

Although we did not characterise *Alcolapia* specimens to the full extent as in Seegers et al. (1999), Little Magadi and Lake Natron did demonstrate distinct morphological and genetic differences. In light of the isolation among the lake systems and the differential morphology it is reasonable to assign Little Magadi species status within the *Alcolapia* genus. At present it would be premature to consider any of the lagoon populations for distinct species classification until isolation among the lagoons was confirmed and an equivalent assessment of characters (morphological, physiological and behavioural) could be described for each population. The proposed model offers strong potential that the *Alcolapia* species flock may be larger than originally assessed in Seegers et al. (1999). Furthermore, the use of physiological characters in cichlid taxonomic classifications is a potentially new and important criterion for which to identify these speciose taxa.

Summary

In summary, we identified discordant patterns between genotypic and phenotypic characters within the Lake Magadi tilapia and further identified evidence against gene flow despite the similar

mtDNA haplotype patterns. An alternative explanation for the discordant patterns we observed is balancing selection acting on a gene or gene(s) on different mtDNA, represented by control region haplotypes, as an adaptation to heterogenous micro-environments within each Magadi lagoon system. These results demonstrate the need to consider the interpretation of genetic structure in the biological context of the system under investigation (Hedrick 1999). We are also presently examining other "neutral" DNA markers, specifically microsatellites, with the assumption they will provide an accurate assessment of genetic structure and potential isolation among the Magadi lagoons.

The findings in this study raise a number of important questions focusing on rates of adaptation, sequence substitution rates and selection. It appears that cichlids from Soda Lakes can demonstrate rates of evolution in physiological adaptations comparable to morphological characters such as jaw morphology and coloration (Trewavas 1983, Seegers et al. 1999, Tichy and Seegers 1999). Also, generation time, while important in estimating various population parameters, may be extremely important in the evolution of *Alcolapia* tilapia to the high pH of the Soda lakes. Larval phases of other teleosts express the ornithine-urea cycle required for the excretion of urea (Wright et al. 1995). Adult Magadi and Natron tilapia may maintain a life cycle more consistent with a larval phase maintaining this pathway, and potentially other important adaptations, within this environment. Furthermore, evidence of accelerated substitution rates and selection of mtDNA genes in stressful environments clearly warrants further study. We did not have sufficient time to characterise all the Magadi and Natron tilapia for all the phenotypic characters we described and the list of adaptations is unlikely to be exhausted. Future expeditions will further characterise these fish and address many of the questions raised in this study. The Lake Magadi and Lake Natron *Alcolapia* tilapia clearly represent important biological systems to study evolutionary processes.

Table 1. Frequencies of mitochondrial DNA control region haplotypes from *Alcolapia* populations.

MtDNA	Fish Springs Lagoon	Sports Club Lagoon	South East Lagoon	South West Lagoon	Wilson Springs	West Lagoon	Little Magadi	Lake Natron
A	10 (0.455)	30 (0.652)	11 (0.550)	5 (0.238)	6 (0.545)	8 (0.471)	0	0
B	10 (0.455)	7 (0.152)	6 (0.300)	8 (0.381)	3 (0.273)	7 (0.412)	20 (0.952)	17 (0.630)
C	2 (0.090)	2 (0.043)	2 (0.1050	8 (0.381)	1 (0.090)	1 (0.059)	0	0
D	0	0	0	0	0	0	0	7 (0.259)
E	0	0	0	0	0	0	0	0
F	0	0	0	0	0	0	0	1 (0.037)
G	0	0	0	0	0	1 (0.059)	0	0
H	0	0	0	0	1 (0.090)	0	0	0
I	0	6 (0.130)	1 (0.050)	0	0	0	0	0
J	0	1 (0.022)	0	0	0	0	0	0
K	0	0	0	0	0	0	1 (0.095)	0
L	0	0	0	0	0	0	0	1 (0.037)
M	0	0	0	0	0	0	0	1 (0.037)
Total	22	46	20	21	11	17	21	27

Table 2. Analysis of variance of *Alcolapia* populations estimated by AMOVA (Excoffier et al. 1992) on pair-wise comparisons of ϕ_{ST} as an indicator of population structure.

	FSL	*SCL*	SEL	SWL	WS	WL	LM	LN
FSL	-							
SCL	0.0906	-						
SEL	-0.0192	0.0015	-					
SWL	0.0559	0.2111	0.0844	-				
WS	-0.0295	-0.0089	-0.0654	0.0701	-			
WL	-0.0491	0.0624	-0.0347	0.0643	-0.0482	-		
LM	0.3769	0.5561	0.5001	0.3872	0.5620	0.4193	-	
LN	0.1893	0.3941	0.2756	0.1883	0.2719	0.1990	0.1728	-

Table 3. Mismatch distribution analysis of expansion times (τ) and SSD statistics of population expansion and estimates of historic demographic events in *Alcolapia* populations.

Population	τ^{*}	SSD**	t (years)† μ (5.56×10^{-8} • site • year)	t (years)†† μ (3.36×10^{-7} • site • year)	t (years)††† μ (9.90×10^{-7} • site • year)
Fish Springs	**5.348**	**0.020**	**173,636**	**28,752**	**9,759**
Sports Club	**5.526**	**0.470**	**179,415**	**29,902**	**10,083**
South East	**5.445**	**0.070**	**176,785**	**29,464**	**9,936**
South West	**5.855**	**0.060**	**190,097**	**31,682**	**10,684**
Wilson Springs	**5.510**	**0.070**	**178,896**	**29,816**	**10,054**
West Lagoon	**5.250**	**0.010**	**170,454**	**28,409**	**9,580**
Lake Magadi	**5.344**	**0.440**	**173,182**	**28,731**	**9,752**
Litte Magadi	**3.000**	**0.180**	**97,402**	**16,233**	**5,474**
Lake Natron	**3.008**	**0.300**	**97,662**	**16,172**	**5,489**

* Expansion time (t) in years obtained from the expansion time in units of mutation rate ($\tau = 2ut$) assuming a generation time of 1 year.

** Expansion time expressed in units of mutation rate ($\tau = 2ut$) where u is the mutation rate and t is the number of generation since the expansion or demographic event.

**P values given for SSD statistic (Schneider and Excoffier 1999) estimated by simulated demographic parameters and compared to observed parameters. The P value is the proportion of expected distributions larger than the observed distribution.

† Expanion time (t) in years obtained from the expanion time in units of mutation rate ($\tau = 2ut$) assuming a mutation rate of 5.56×10^{-8} • site • year and a generation time of 1 year.

†† Expansion time (t) in years obtained from the expansion time in units of mutation rate ($\tau = 2ut$) assuming a mutation rate of 5.56×10^{-8} • site • year and a generation time of 60 days.

††† Expansion time (t) in years obtained from the expansion time in units of mutation rate ($\tau = 2ut$) assuming a mutation rate of 1.65×10^{-7} • site • year and a generation time of 60 days.

Table 4. Estimates of female effective population size (N_F) using the mismatch distribution parameter θ_1 and field estimates. Estimated times to fixation of a single mitochondrial haplotype based on N_F were calculated.

Population	θ_1	Expected N_F* ($\mu = 3.36 \times 10^{-07}$)	Expected N_F* ($\mu = 9.90 \times 10^{-07}$)	Estimated Mean Time to Fixation	Field Estimate N_F	Estimated Mean Time to Fixation
Fish Springs	**3.091**	**16,618**	**5640**	**2000 yrs**	**>5,000**	**1500 yrs**
Sports Club	**5.526**	**21,709**	**10,083**	**3500 yrs**	**250**	**100 yrs**
South East	**5.445**	**21,274**	**9,936**	**3500 yrs**	**250**	**100 yrs**
South West	**3.821**	**20,543**	**6972**	**2400 yrs**	**>5,000**	**1500 yrs**
Wilson Springs	**3.330**	**17,903**	**6076**	**2000 yrs**	**250**	**100 yrs**
Lake Magadi	**2.355**	**12,554**	**4261**	**1500 yrs**	**≈16,000**	**5000 yrs**
West Lagoon	**3.457**	**18,586**	**6308**	**2000 yrs**	**>5,000**	**1500 yrs**
Little Magadi	**0.111**	**597**	**200**	**100 yrs**	**†**	**†**
Lake Natron	**1.573**	**8457**	**2870**	**1000 yrs**	**†**	**†**

* Estimated based on mismatch distributions where $\theta_1 = 2N_1\mu$, where θ_1 is the parameter for population size, N_1 is the present effective population size and μ is the mutation rate per haplotype. The mutation rate per haplotype is estimated using the substitution rate per site by the number of base pairs being analysed. Substitution rates of 5.56×10^{-8} and 1.67×10^{-7} were used based on cichlid (Nagl et al. 2000) and human control region estimates (Ward et al. 1991), respectively, and to account for the rapid generation time among Magadi tilapia, i.e. approximately 60 days (Coe et al. 1967).

† Lake conditions made field estimates difficult.

Table 5. Mean Gut Length (GL) / Total Length (TL) values in mm for ± SEM for fish <58mm from Fish Springs Lagoon, South East Lagoon. South West Lagoons and Lake Natron.

Population	Sample Size (N)	Mean GL/TL	SEM
Fish Springs	**56**	**2.956**	**0.103a**
South East Lagoon	**12**	**1.240**	**0.147c**
South West Lagoons	**6**	**1.742**	**0.087c**
Lake Natron	**5**	**1.858**	**0.218c**

Means sharing the same letter are not significantly different from one another

Table 6. Values of water chemistry (for areas containing fish) measured from multiple replicates (typically 3-10).

Population	pH	ΣCO_2 (mM/L)	$[Cl^-]$ (mM/L)	$[Na^+]$ (mM/L)	Osmotic Pressure (mOsm • kg)	Alkalinity (mEq/L)
Fish Springs	**9.86**	**216**	**113**	**355**	**581**	**378**
Sports Club	**10.05**	**835**	**693**	**978**	**1465**	**1625**
South East	**9.55**	**245**	**203**	**326**	**617**	**402**
South West	**9.65**	**226**	**190**	**454**	**884**	**380**
Wilson Springs	**9.60**	**120**	**46**	**183**	**278**	**184**
West Lagoon	**9.13**	**254**	**156**	**390**	**695**	**350**
Little Magadi	**9.32**	**834**	**192**	**372**	**1689**	**1251**
Lake Natron	**9.91**	**189**	**60**	**193**	**442**	**323**

Table 7. Values of water chemistry and temperature along a transect outwards across the surface of the trona from Sports Club Lagoon, measured on Jan. 17, 1997 approximately 12h after a torrential rainfall.

Transect Distance	Temperature (°C)	pH	ΣCO_2 (mM/L)	$[Cl^-]$ (mM/L)	$[Na^+]$ (mM/L)	Osmotic Pressure (mOsm • kg)	Alkalinity (mEq/L)
0 m	37.0	9.97	360	507	1145	1627	688
30 m	39.5	9.91	569	667	1310	1858	1099
50 m	41.5	9.88	731	865	1531	2119	1421
100 m	44.6	10.01	1007	632	1724	2316	1956
150 m	43.8	10.05	597	622	1897	2634	1167
200 m	42.0	9.94	638	655	2414	3058	1239
300 m	40.1	9.93	469	520	2166	2967	900

Note: P_{O2} averaged 11 torr (range = 2.5 – 22.5 torr) at the 7 sites

Table 8. Activities (umols product/g wet weight of liver/min) of hepatic enzymes of the Krebs ornithine-urea cycle, associated enzymes of N-metabolism, and hepatosomatic index (HSI, % of body weight) in fish from different populations. Abbreviations include: glutamine synthetase (GSase), ornithine-citrulline transcarbamoylase (OTC), arginase (ARG), glutamate dehydrogenase (GDH), alanine aminotransferase (AlaAT), and aspartate aminotransferase (AspAt). GLNase (glutaminase) arginosuccinate synthetase/arginosuccinate lyase (AS/AL; as a coupled reaction), and carbamoyl phosphate synthetase III (CPSase III. Means ± 1 SEM (N).

Enzyme	Fish Springs (6)	Sports Club (3)	South East (6)	South West (6)	Little Magadi (6)	Lake Natron (6)
GSase	7.260a ± 1.220	5.040a.b ± 0.760	6.320a ± 0.430	7.760a ± 0.620	4.260b ± 0.500	9.080a,c ± 1.340
CPSase III	0.044a ± 0.005	0.029a ± 0.012	0.024a ± 0.008	0.050a ± 0.003	0.039a ± 0.013	0.054a ± 0.009
OTC	3.44a ± 0.17	4.93b ± 0.17	4.19a.b ± 0.60	4.90b ± 0.31	3.32a 0.32	3.44a ± 0.30
AS/AL	0.018a ± 0.005	0.014a ± 0.001	0.021a ± 0.007	0.019a ± 0.008	0.240a ± 0.010	0.008a ± 0.004
ARG	36.51a ± 1.87	25.15a,b ± 2.72	19.86b ± 1.29	25.57a,b ± 1.71	34.16a,b ± 4.54	26.46a,b ± 2.10
GLNase	0.430a ± 0.190	ND	0.49a ± 0.16	0.82a ± 0.16	0.32a ± 0.17	0.78a ± 0.25
GDH	19.49a ± 1.22	15.83a ± 0.76	20.30a ± 1.51	20.70a ± 1.09	19.89a ± 1.57	19.49a ± 1.51
ALAat	19.35a,b ± 1.50	15.69b ±0.54	18.67a,b ± 1.33	25.04a ± 1.02	20.44a.b ± 1.81	17.05b ± 1.60
ASPat	79.43a ± 3.15	55.89b ± 2.51	81.46a ± 4.13	96.48c ± 2.81	86.47a,c 4.61	91.48a,c ± 4.97
HSI	1.09a ± 0.07	3.11a,b ± 0.65	1.91b ± 0.09	2.30b ± 0.16	1.64a,b ± 0.18	1.83b ± 0.10

Means sharing the same letter are not significantly different from one another

Table 9. Whole body urea-N, chloride, and sodium concentrations in fish from the different populations, sampled in their native waters. Means ± SEM.

Population	Sample Size (N)	Whole-body Urea Concentrations (umol -N•kg^{-1})	[Cl$^-$] (mM. •kg^{-1})	[Na$^+$] (mM. •kg^{-1})
Fish Springs	**9**	**22,397a,b ±1,562**	**73.5a ±5.7**	**124.9a.b ±13.2**
Sports Club	**15**	**39,291c ±3,102**	**85.3a ±8.5**	**140.7a,b ±35.2**
South East	**10**	**25,633b ±2,120**	**67.8a ±3.8**	**91.6b ±6.8**
South West	**8**	**35,339c ±3,508**	**67.3a ±1.9**	**121.7a ±6.0**
Wilson Springs	**10**	**10,889d ±6,28**	**57.4b ±2.1**	**94.7b ±6.1**
Little Magadi	**10**	**42,017c ±2,918**	**167.7c ±10.0**	**381.1c ±32.2**
Lake Natron	**10**	**17,126a ±2,119**	**82.1a ±5.0**	**102.7a,b ±12.4**

Means sharing the same letter are not significantly different from one another

Table 10. Median lethal times (± SEM) for fish from the different populations in a standardized challenge test using two trials when possible. The trials included fish tested within one day of their arrival to the laboratory and fish acclimated to Fish Springs water for at least one day and (see text for details).

Population	Sample Size (N) of Fish Exposed Directly from Field Conditions	Sample Size (N) of Exposed Acclimated Fish	LT50 (minutes)
Fish Springs	**10**	**10**	**11.0 ± 1.0**
Sports Club	**10**	**10**	**30.0 ± 6.0**
South East	**10**	**7**	**10.8 ± 1.3**
South West	**12**	**10**	**16.3 ± 7.8**
Wilson Springs	**8**	**-**	**2.0 ± 0.0**
Little Magadi	**11**	**10**	**29.5 ± 2.5**
Lake Natron	**11**	**-**	**8.0 ± 0.0**

Table 11. Summary of genetic, adaptive or distinct characteristics among *Alcolapia* populations from Lake Magadi lagoon populations, Lake Natron and Little Magadi.

Population	Character	Reference
Fish Springs Lagoon	DNA fingerprinting indicates significant differentiation with South East Lagoon. Differentiation equivalent to comparison to Lake Natron fish.	Wilson et al. 2000
Fish Springs Lagoon	Relatively large gut length	This study
South East Lagoon	DNA fingerprinting indicates significant differentiation with Fish Springs Lagoon. Differentiation equivalent to comparison to Lake Natron fish.	Wilson et al. 2000
South East Lagoon	Increased gill size and modified structure.	Maina et al. 1996
South East Lagoon	Relatively small gut size.	This study
Sports Club Lagoon	Increased tolerance to high osmotic pressure.	This study
Sports Club Lagoon	Relatively high natural whole-body urea concentrations.	This study
Sports Club Lagoon	Relatively high HSI values.	This study
Sports Club Lagoon	Behavioural adaption to fresh surface water.	This study
Little Magadi	Mouth morphology.	This study
Little Magadi	Increased tolerance to high osmotic pressure.	This study
Little Magadi	Relatively high natural whole-body urea concentrations and NaCl levels.	This study
Lake Natron	Mouth morphology and coloration.	This study and Seegers et al. 1999

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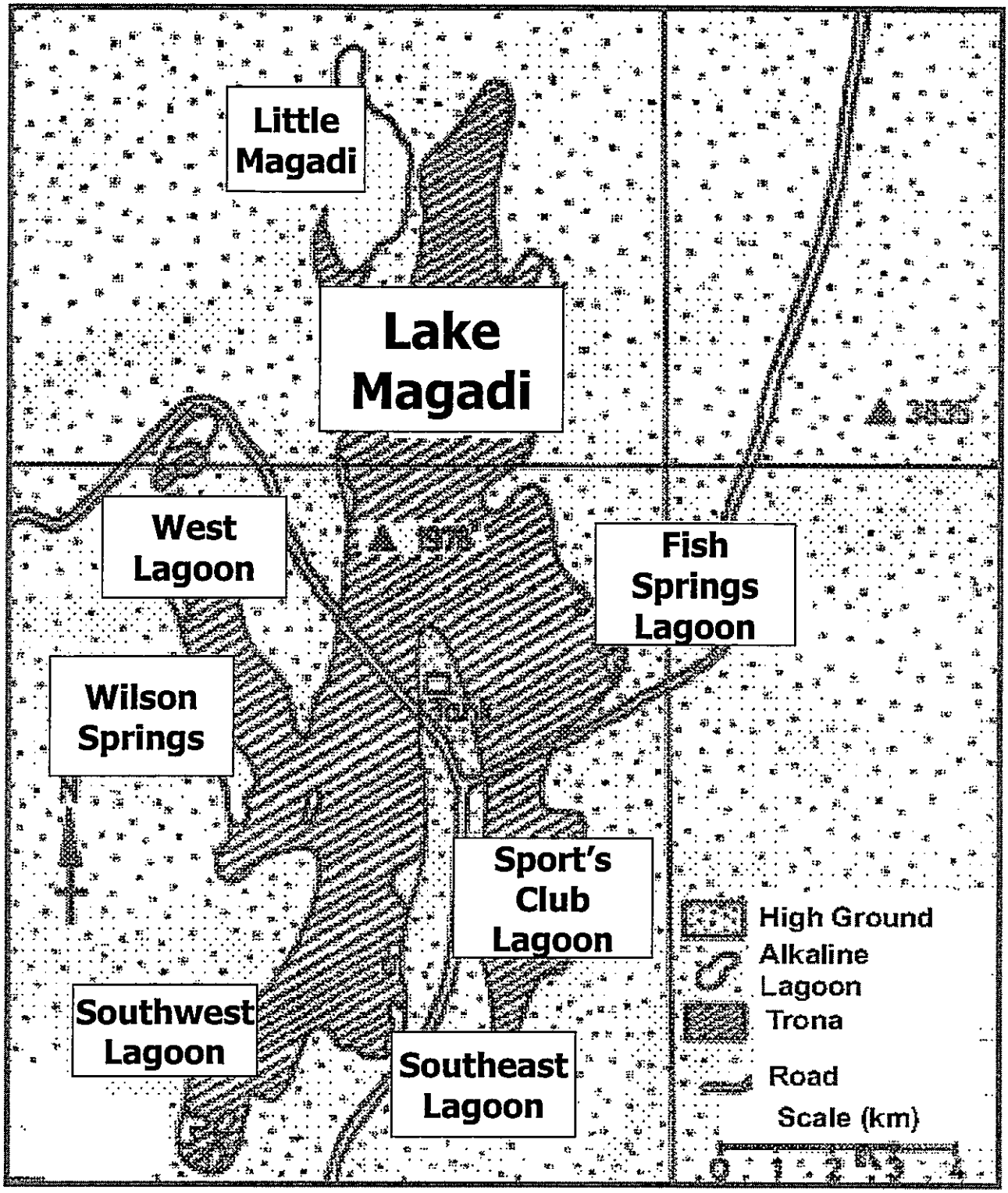
Figure 1. Map of Lake Magadi, Kenya indicating the Fish Springs Lagoon, Sports Club Lagoon, the South East Lagoon, the South West Lagoon, Wilson Springs, the West Lagoon and Little Magadii. Open water (alkaline lagoon) and trona are indicated on the map (adapted from Maina et al. 1996).


Little Magadi
Lake Magadi
West Lagoon
Fish Springs Lagoon
Wilson Springs
Sport's Club Lagoon
Southwest Lagoon
Southeast Lagoon
High Ground
Alkaline Lagoon
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Road
Scale (km)
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Figure 2. Minimum-spanning tree (Excoffier et al. 1992) of *Alcolapia* mitochondrial DNA control region sequences.

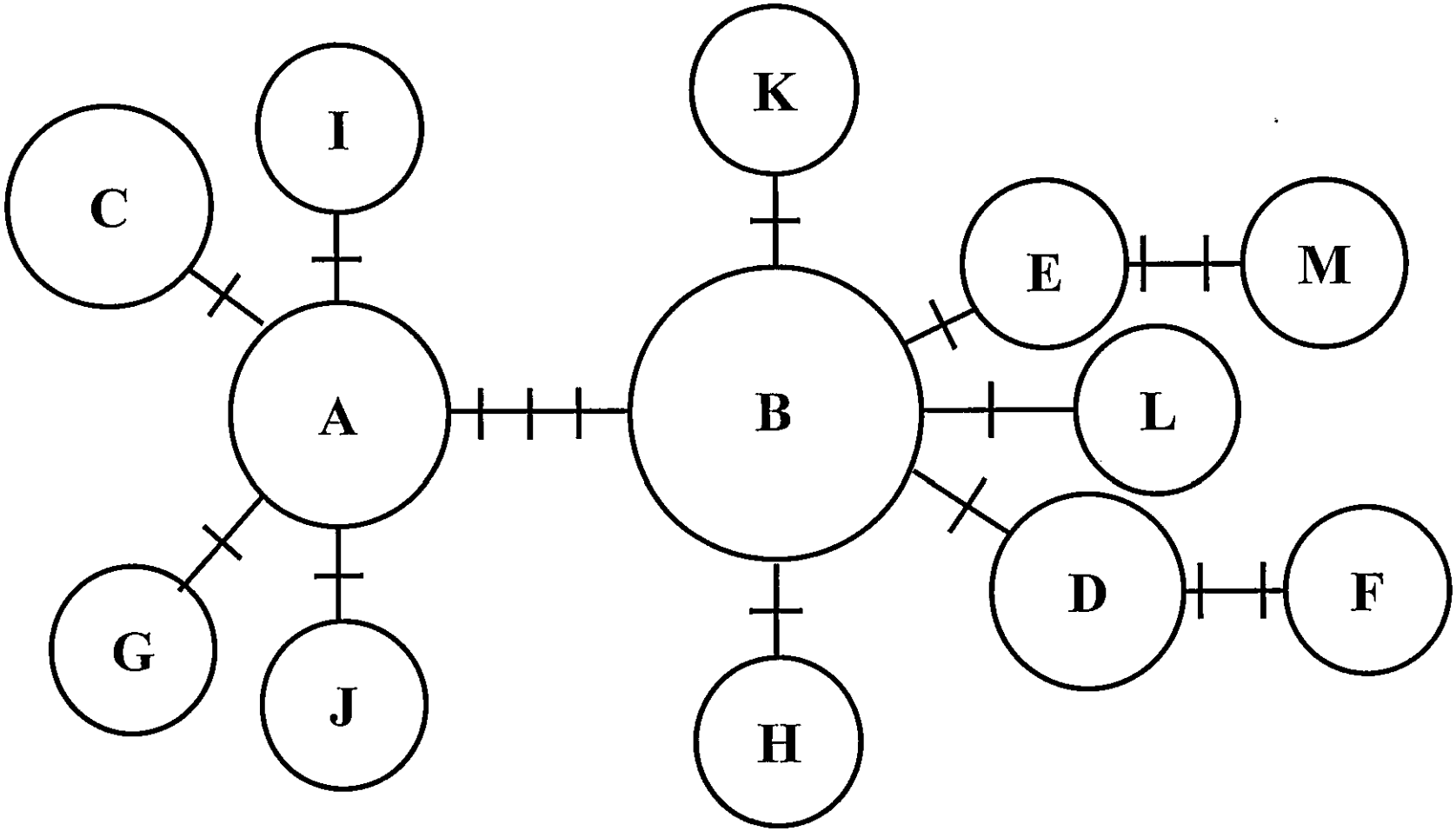
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Figure 3. Mean rates of urea-N excretion ($M_{Urea\text{-}N}$) and ammonia-N ($M_{Amm\text{-}N}$) in different *Alcolapia* populations (LN = Lake Natron; FSL = Fish Springs Lagoon; SEL = South East Lagoon; WL = Western Lagoon; SWL = South West Lagoon; SCL = Sports Club Lagoon; LM = Little Magadi). The corresponding osmolalities of the environments are shown. Means + 1 SEM (N = 8-10). Means sharing the same letter are not significantly different.

N-Excretion

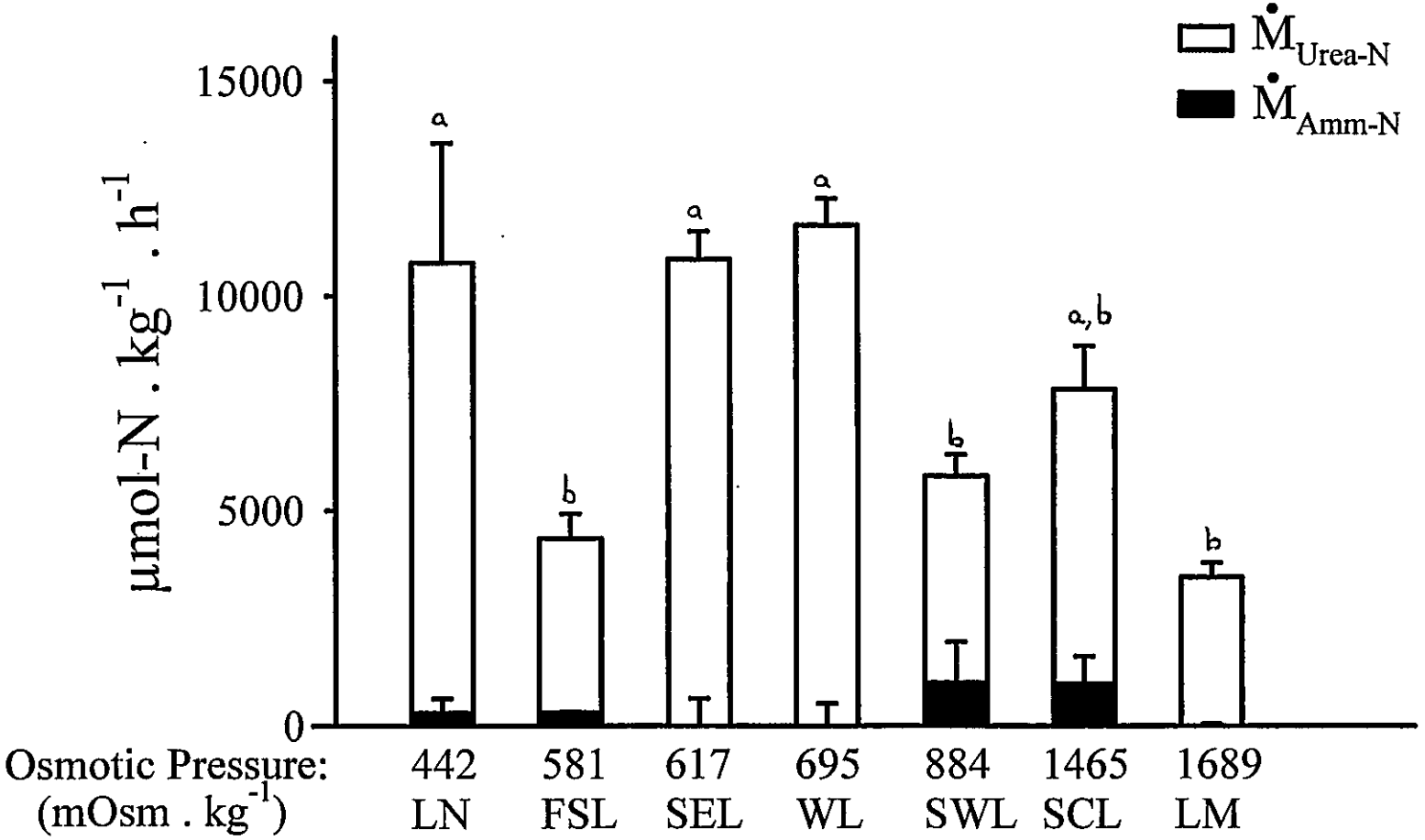

$\dot{M}_{Urea-N}$
$\dot{M}_{Amm-N}$
15000
10000
5000
0
µmol-N . kg^{-1} . h^{-1}
a
b
a
a
b
a, b
b
Osmotic Pressure:
(mOsm . kg^{-1})
442 LN
581 FSL
617 SEL
695 WL
884 SWL
1465 SCL
1689 LM

Figure 4. Mean N/O_2 ratios ($[M_{Urea\text{-}N}]/[M_{O2}]$) in different *Alcolapia* populations. Abbreviations, N numbers, and other information as in legend of Fig. 3.

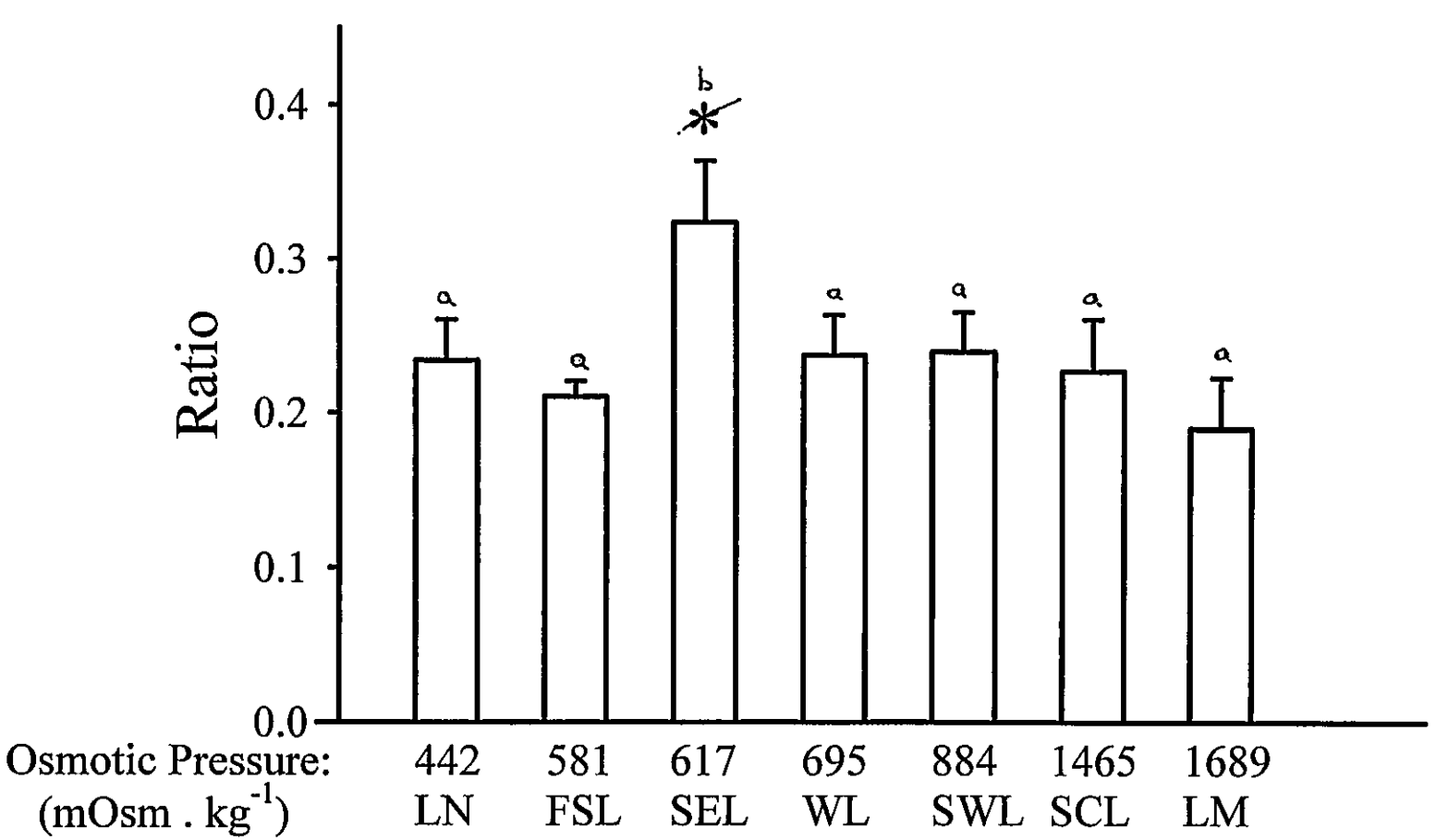

N/O2
Ratio
0.4
0.3
0.2
0.1
0.0
b
a
a
a
a
a
a
Osmotic Pressure:
(mOsm . kg-1)
442 LN
581 FSL
617 SEL
695 WL
884 SWL
1465 SCL
1689 LM

Figure 5. Relationships between (A) whole body urea concentration and environmental osmolality, and (B) median lethal time (LT50) and whole body urea concentration in standardized challenge tests for the different populations. Means ± SEM. Abbreviations as in legend of Fig. 3, and N numbers as in Table 3 (for A) and Table 4 (for B).

Figure 5A

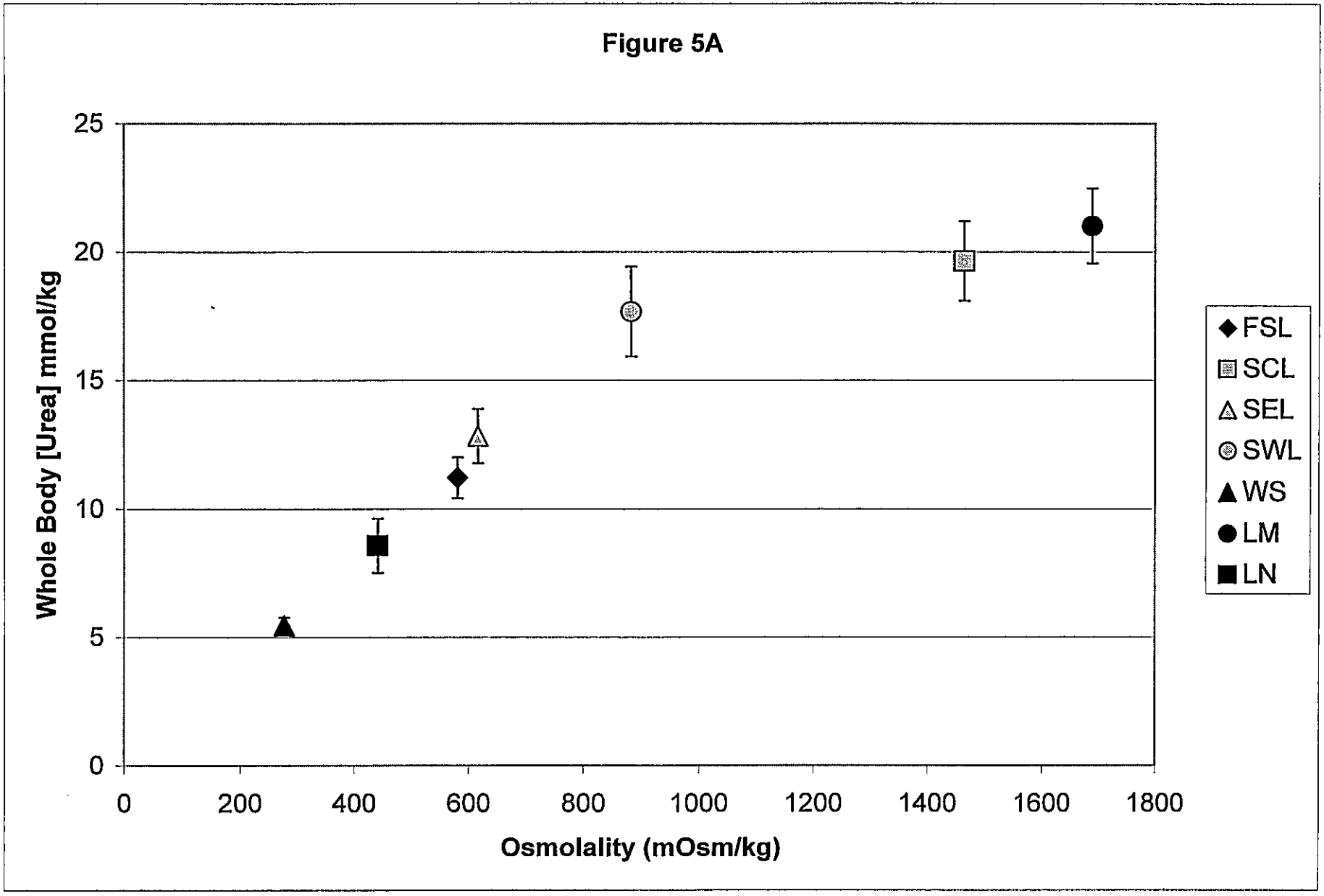
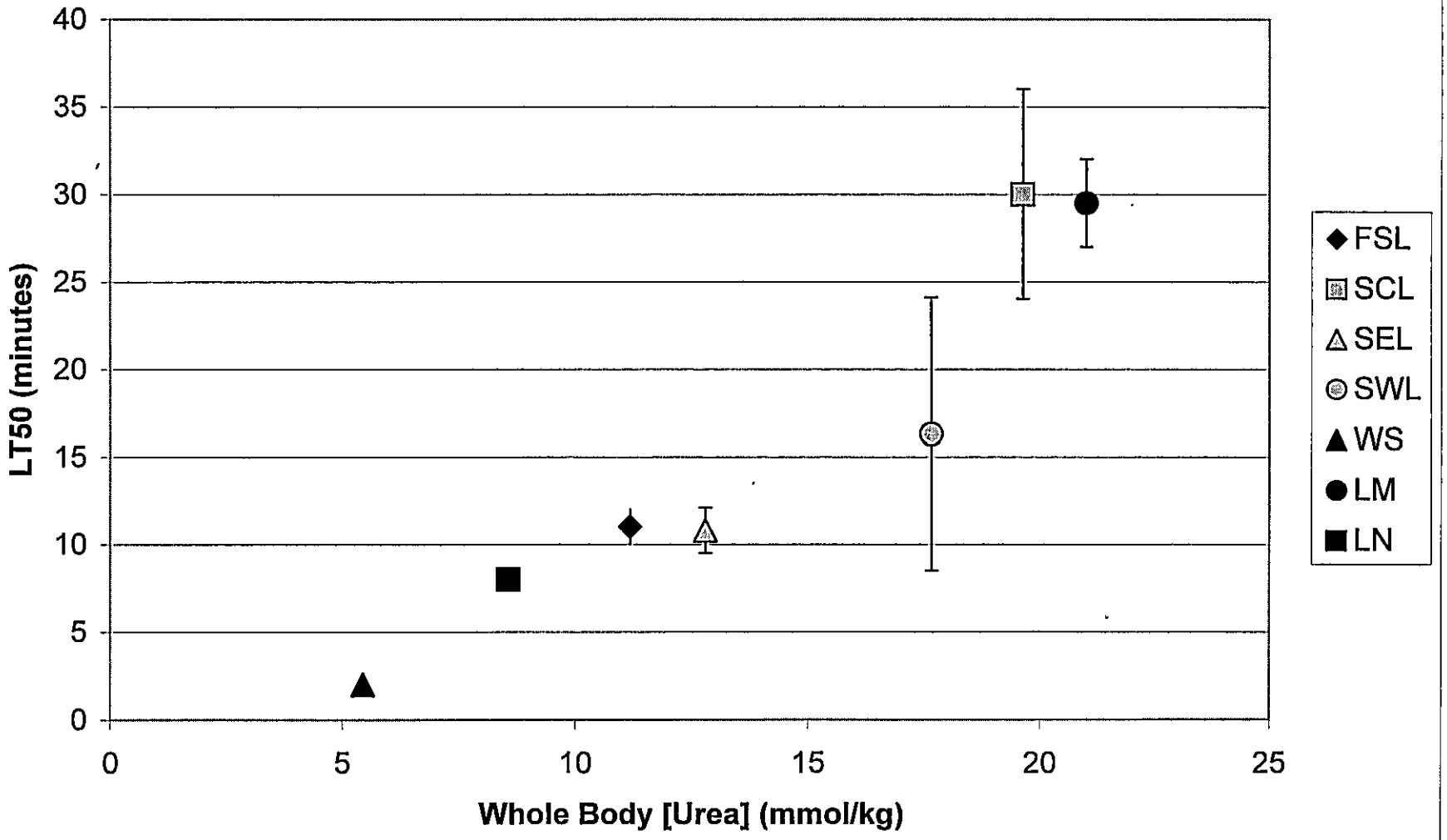

Whole Body [Urea] mmol/kg
25
20
15
10
5
0
0
200
400
600
800
1000
1200
1400
1600
1800
Osmolality (mOsm/kg)
FSL
SCL
SEL
SWL
WS
LM
LN

Figure 5B


LT50 (minutes)
40
35
30
25
20
15
10
5
0
0
5
10
15
20
25
Whole Body [Urea] (mmol/kg)
FSL
SCL
SEL
SWL
WS
LM
LN

Chapter 4

Genetic variation and population structure of moose (*Alces alces*) at neutral and functional DNA loci

Genetic variation and population structure of moose (*Alces alces*) at neutral and functional DNA loci

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ABSTACT

Genetic variation and population structure was examined for moose from nine geographic regions: Riding Mountain, Isle Royale and Pukaskwa National Parks; northwestern, Nipigon, northeastern and central Ontario; New Brunswick and Newfoundland. These regions were selected to represent different population histories including those exhibiting isolation and/or small historic founding events versus contiguous ranges. Furthermore the National Parks were identified as maintaining potentially different local selection pressures due to the absence of hunting and the presence or absence of the parastite *Parelaphostrongylus tenuis*. Genetic variation was estimated using neutral DNA markers, assessed by multi-locus DNA fingerprinting and five microsatellite loci, and the functional antigen binding region (ARS) (exon 2) of the Major Histocompatibility Complex (*Mhc*) gene *DRB*. There was discordance in the genetic variation observed at the neutral loci compared to the *Mhc DRB* locus in a number of populations. Ontario populations demonstrated higher levels of variability at the neutral loci and relatively low levels at

the *DRB* locus. Conversely, Isle Royale has the lowest genetic variability consistent with a historic small founding event at the neutral DNA markers and relatively high variability at the *MHC* gene. Riding Mountain and Pukaskwa National Parks demonstrated relatively high levels of genetic variation at both types of loci and Newfoundland showed consistently low levels of variation at both neutral and functional loci. Relatively high levels of genetic variation at the *DRB* locus were observed in protected park populations with the absence of white-tailed deer or the parasite *P. tenuis* and an absence of hunting. An assessment of population structure supported Riding Mountain National Park as an isolated moose population as were the island populations of Isle Royale and Newfoundland. Gene flow was observed among the neighboring geographic regions within Ontario including Pukaskwa National Park with evidence of isolation-by-distance among more distant regions within Ontario. The discordant patterns between mini/microsatellite and *DRB* loci shows that neutral DNA markers may not accurately reflect adaptive variation present at functional loci. However, assessing these discordant patterns in the context of identifying both gene flow and local selection pressures offers the potential for reconstructing the metapopulation structure of wildlife species.

INTRODUCTION

Maintaining genetic variation is an important factor in protecting the evolutionary potential and promoting the long-term persistence of populations (Leberg 1992, Frankham 1995, Lacy 1997). The movement of animals and subsequent gene flow within a metapopulation structure can maintain genetic variation among local sub-populations (Harrison and Hastings 1996). Low levels of genetic variation within sub-populations of a larger metapopulation has been identified as a causal factor in increasing the probability of extinction within local populations in greater prairie chickens (Westemeier et al. 1998) and the Glanville frittiliary butterfly (Saccheri et al. 1998). Neutral DNA markers have been used extensively in studying genetic variation and population structure in natural populations (Avise 1994, Ferris and Palumbi 1996, Carvalho 1998, Estoup and Angers 1998).

The application of neutral DNA markers in assessing population structure and levels of genetic variation assumes that these loci reflect adaptive variation within populations (King and Burke 1999). However, local selection pressures within a metapopulation framework affecting the levels of genetic variation in sub-populations at adaptively important loci may not be readily detected using neutral DNA marker systems (Hedrick 1999) and conversely, functional loci may not accurately reflect gene flow among different geographic regions with different selection pressures. Despite this concern, few studies have directly compared neutral genetic variation to adaptive variation at the intra-specific level (Carvalho 1998, Crandall et al. 2000).

In this study, we present results on genetic variation and population structure in natural moose (*Alces alces*) populations using neutral and functional DNA markers. Firstly, neutral DNA markers such as minisatellite loci and microsatellites loci have identified levels of genetic variation as representative of over-all levels of genome variation (Lacy 1997). Minisatellite loci, as detected with multi-locus DNA fingerprinting, has had limited application in assessing population differentiation (Schenk et al. 1998, Wilson et al. 2000) due to technical limitations (Estoup and Angers 1998), while microsatellites have proven more effective at assessing the population structure in natural wildlife populations such as bears (Paetkau et al. 1994), wolves (Roy et al. 1994), African water buffalo (Simonsen et al. 1998) and moose (Broders et al. 1999).

Secondly, the Major Histocompatibility Complex (*Mhc*) is the most common DNA marker system for assessing genetic variation at a functional gene locus (Hedrick 1994, Murray et al. 1995, Murray and White 1998, Wenink et al. 1998, Murray et al. 1999). *Mhc* proteins bind pathogen-derived foreign peptides to T-cells to initiate an immune response. As a result of its role in disease resistance, *Mhc* genes can be highly polymorphic and genetic variation is proposed to be adaptive to the large numbers of pathogens for which natural populations are exposed (O'Brien and Evermann 1988). One mechanism proposed to maintain high levels of variation at *Mhc* genes is balancing selection (Hedrick and Thomson 1983, Hughes and Nei 1988, Yuhki and O'Brien 1990, Hughes et al. 1994) through overdominance or negative frequency-dependence. Overdominance or heterozygote advantage increases the range of pathogens recognized by the *Mhc* proteins while negative frequency-dependent selection is based on host-parastite interactions causing an increase of low-frequency alleles followed by a shift in the composition of the parasite population (reviewed in Potts and Wakeland 1993, Hedrick 1994, Hughes and Yeager 1998). The importance of *Mhc* genes in a conservation context has been identified for captive breeding (Hughes 1991) and isolated populations (Yuhki and O'Brien 1990) indicating low variation at these loci may result in increased disease susceptibility and potential local extinction.

Moose (*Alces alces*) are the largest land mammals in the circumpolar boreal forests of Canada and Eurasia (Telfer 1984). Despite the importance of moose as a game species within most Canadian provinces and a number of US states (Cumming 1974, Ritchey 1974, Timmerman and Buss 1998) there is uncertainty about the movement patterns among moose populations. Moose movements have been classified as either dispersal or migration: dispersal being defined as the movement of moose from the natal range to the area where it will potentially breed and migration defined as the seasonal movement of moose between mating and non-mating ranges (Shields 1983, Shields 1987, Hundertmark 1998). Previous studies of moose populations have indicated differential amounts of moose migration (LeResche 1974, Tefler 1994). Limited dispersal has been described for moose (Hundertmark 1998) suggesting the amount of dispersal may be inadequate to effectively influence neighbouring populations and the recovery of moose populations in heavily hunted regions in Ontario was attributed to reproduction and not to the immigration of moose from adjacent areas (Goddard 1970). Moose populations have also been proposed

to be composed of both migratory and non-migratory animals (LeResche 1974), although, exclusively "island" populations of non-migratory moose have been described in the prairie provinces of Canada (Karns 1998).

Previous genetic studies on moose have left the question of local movement patterns and gene flow unresolved. A genetic survey of North American moose (Cronin 1992) could not distinguish among proposed *A. alces* sub-species with restriction fragment length polymorphisms (RFLP) of the mitochondrial DNA (mtDNA) suggesting that the high potential for dispersal in moose has caused a genetic homogenization among moose populations. However, only one mtDNA haplotype was detected in this survey, likely the result of the resolution of RFLP analysis in detecting variation. Population structure was identified among Canadian moose representing populations from different provinces (Broders et al. 1999) using microsatellite loci, although this geographic scale could not address levels of migration and dispersal in the range consistent with moose biology (LeResche 1974, Hundertmark 1998). Other evidence in European moose populations suggests limited home ranges and site fidelity to the natal home range (Cederlund et al. 1987) and population structuring in Scandinavian moose was detected using protein polymorphisms over geographic distances of approximately 50 km (Ryman et al. 1980, Chesser et al. 1982).

In addition to the amount of gene flow through dispersal and migration, local selection pressures such as disease and hunting further impact levels of genetic variation within wildlife populations. The impact of disease on *Mhc* variation in a moose populations may be of particular importance in Ontario given the distribution of white-tailed deer (*Odocoileus virginianus*) and incidence of *Parelaphostronglylus tenuis* throughout the province (Whitlaw and Lankester 1994). Furthermore, computer simulations of different hunting regimes on moose and white-tailed deer populations modeled a loss of genetic variability and a decrease in the ratio of effective population size to census population size over time (Ryman et al. 1981). The amount of inbreeding under the most severe hunting regime, i.e. random hunting of any age and sex, was modeled to be the equivalent of full-sibling matings after 50 years.

The potential impact of disease and hunting on the genetic variation of game species makes protected areas such as Provincial, State and National Parks potentially important reservoirs for

maintaining genetic diversity within a metapopulation structure. However, the role of parks as a source of genetic variation will be effective in a larger metapopulation only if the genes associated within these protected areas can be exchanged among adjacent populations through animal movements and gene flow. An assessment of Canadian National and Provincial Parks showed parks surrounded by human altered landscapes reduced immigration of mammals into park systems (Gurd and Nudds 1999) demonstrating the potential for isolation. As a result it is important to determine the level of "genetic connectivity" between protected and non-protected areas and the amount of genetic variation within park populations in comparison to neighboring geographic regions.

We have performed genetic analyses on moose populations using minisatellite loci detected using multi-locus DNA fingerprinting, microsatellite loci, and the functional antigen-recognition binding region (ARS), exon 2, of the *Mhc* gene *DRB* from moose collected from islands populations, continuous geographic areas and three National Parks. The moose populations in this study have different histories ranging from continuous geographic ranges compared to island and isolated populations originating from small founder events. Furthermore, these regions have potentially differential local selection pressures based on the presence or absence of hunting and the parasite *P. tenuis* corresponding to park and non-park environments. The objective of this study is to assess the genetic variation and population structure at neutral and functional genetic loci in moose within the context of differential historic factors, ecological factors and human impacts, specifically the degree of isolation, presence or absence of *P. tenuis* and hunting, respectively.

MATERIALS & METHODS

Sample Collection

Tissue samples were collected from moose representing nine geographic regions (Table 1). Riding Mountain National Park, Manitoba represents a potentially isolated population based on the difference in habitat within the park in comparison to the highly developed agricultural areas outside the park boundaries (Karns 1998). Various regions within Ontario were sampled and categorized on a provincial scale. Moose were analysed from northwestern Ontario, representing Red Lake and Sioux

heterozygotes through balancing selection (Hedrick and Thomson 1983) (see below). The heterozygote deficiency in both the neutral microsatellite loci and the functional *Mhc* gene supports a Wahlund effect. Riding Mountain and Isle Royale populations may maintain local subpopulations within these "island" populations. The higher densities in these populations, estimated at between 1.0-4.0/km^2 (Tefler 1984), may have resulted in the formation of local congregates of moose within the boundaries of the isolated populations, consistent with the apparent Wahlund Effect. As a result, the presence of local structured populations may have actually under-estimated expected heterozygosity at both the microsatellite and *DRB* loci. A consistent deficiency in heterozygotes was observed in African buffalo (*Synercus caffer*) from National Parks at the *DRB* locus (Wenink et al. 1998) and microsatellite loci (Simonsen et al. 1999).

Isle Royale and Newfoundland demonstrated the lowest genetic variability at the neutral DNA markers consistent with the small founding events approximately 100 years ago. Two discrepancies were observed when comparing the two neutral markers. Firstly, Nipigon moose demonstrated a relatively low APD value by comparison to microsatellite H_E, likely the result of using moose from a localized region around Thunder Bay, Ontario. DNA fingerprint profiles may detect higher genetic relatedness on smaller geographical scales than microsatellites due to the resolution of the two markers with higher mutation rates at minisatellite loci (Estoup and Angers 1998, Hansson et al. 2000). Unfortunately, technical limitations associated with DNA fingerprinting make large numbers of samples difficult to compare, i.e. intra-gel comparisons (Guglich et al 1993), thereby limiting the scale that can be effectively monitored with this technique. These limitations have resulted in the decline of studies using DNA fingerprinting in favour of microsatellite loci (Estoup and Angers 1998).

Secondly, although consistently lower than moose from other regions, the H_E of microsatellite loci for Isle Royale moose was considerably higher than its comparable APD value. This discrepancy is likely the result of H_E being less sensitive to population bottlenecks / small founding events and APD reflecting allelic loss and not heterozygosity. Levels of heterozygosity can be maintained following a population bottleneck despite the loss of alleles (Spencer et al. 2000) or increase immediately following a bottleneck event (Cornuet and Luikart 1996). Bottlenecks can equalize allele frequencies as rare alleles are lost through the population reduction causing and increase in heterozygosity (Leberg 1992, Spencer et al.

2000). Natural and experimental studies have shown that allelic diversity is significantly affected by population bottlenecks and more accurately reflects the loss of genetic variation (Leberg 1992, Luikart and Cornuet 1998, Spencer et al. 2000)

This relationship between allele loss and bottlenecks was supported with the observed loss of genetic variation at APD for minisatellite loci and H_E and allelic diversity for microsatellite loci between source and island founded populations (Table 3). The DNA fingerprint data (APD) showed identical loss to microsatellite allelic diversity in both comparisons of source population to island founding events. Again, DNA fingerprinting analyses examines allele sharing (Guglich et al. 1993) and will therefore reflect the loss of rarer alleles through a bottleneck.

At present, heterozygosity is one of the most common measures of assessing genetic variation. The recent study examining experimentally bottlenecked populations (Spencer et al. 2000) and other studies (Leberg 1992, Cornuet et al. 1996, Luikart et al. 1998, Luikart and Cornuet 1998, Luikart et al. 1999) have demonstrated the importance of allelic diversity in considering genetic diversity for conservation biology. Allelic diversity of *Mhc* loci in particular has been recommended for consideration in captive breeding programs (Hughes 1991) and natural populations (Yuhki and O'Brien 1990). Our data suggests that the assessment of neutral genetic variation may not accurately reflect adpative loci such as *Mhc*.

We observed several discordant results in this study when comparing neutral and functional loci. Moose in Isle Royale, Riding Mountain and Pukaskwa show relatively high levels of genetic variation at the *DRB* locus compared to the *Mhc* diversity in the other populations of Ontario that maintain gene flow and relatively high levels of genetic variation at the neutral DNA loci. The *DRB* variation observed in Isle Royale moose is contrary to the variation observed at the mini- and microsatellite loci that are more consistent with the history of the island population, specifically a small founding event. Newfoundland has a similar history to Isle Royale with its small founding population and shows consistently low levels of genetic variation at the micro- and minisatellite loci and monomorphism at the *DRB* locus.

A number of studies have shown concordance between *Mhc* and micro- or minisatellite variation including big horn sheep (Boyce et al. 1997), pocket gophers (Sanjayan et al. 1996) and European beavers

(Ellegren et al. 1993). However, a survey of three marine mammal species (Slade 1992) indicated lower *Mhc* diversity compared to terrestrial mammals with no equivalent reduction at allozyme loci in the one seal and two whale species examined. This finding was attributed to reduced pathogen exposure in a marine environment. A similar explanation was proposed for the overall low *Mhc* variation observed in Swedish moose (Ellegren et al. 1996) suggesting that the solitary lifestyle of moose may reduce the lateral transmission of pathogens. The marine mammal and moose studies concluded that the reduced genetic variation at *Mhc* was not indicative of low genome-wide variation and supported reduced balancing selection pressures.

Balancing selection is predicted to result in high allelic diversity and an even distribution of allele frequencies at *Mhc* loci that are maintained through heterozygote advantage (Hedrick and Thomson 1983). However, empirical evidence in non-humans of balancing selection in natural populations is sparse. A study of island populations of the bush rat (*Rattus fuscipes greyii*) reported a loss of allelic diversity at neutral and *Mhc* loci but an excess of heterozygosity at the *Mhc* gene *RT1.Ba* in 3 of 14 island populations suggestive of balancing selection through overdominance (Seddon and Baverstock 1999). Paterson and Pemberton (1997) have shown direct evidence of selection acting on specific alleles rather than heterozygosity, predicted under balancing selection, in a natural ungulate population as alleles at the *DRB* locus in Soay sheep (*Ovis aries*) were significantly associated with juvenile survival and resistance to intestinal nematodes. However, the heterozygosity at the *DRB* locus in Soay sheep of all age classes in the population revealed an even allele frequency distribution supporting balancing selection (Paterson 1998). Both the bush rat and Soya sheep studies (Seddon and Baverstock 1999, Paterson and Pemberton 1997, Paterson 1998) observed greater non-synonymous to synonymous substitutions at *DRB*, further supporting balancing selection through the favoring of novel *Mhc* alleles.

Although the *DRB*-a allele was prevalent (Appendix I), we observed higher *DRB* allelic diversity and more even allele frequency distributions within National Parks. Although a deficiency in H_E was detected, discordant levels of genetic variation at the *DRB* locus compared to the neutral micro/minisatellite DNA markers within Isle Royale (high and low, respectively) and mainland Ontario

populations (low and high, respectively) supports differential selection pressures acting on the *Mhc* locus. High allelic diversity and even distributions of allele frequencies at *DRB* were also observed in African buffalo National Park populations despite historic population bottlenecks caused by disease (Wenink et al. 1998). The analyses of genetic distances and tree topologies revealed different spatial patterns of variation between the microsatellite and DRB loci with respect to Pukaskwa (Fig. 3). Similar results were interpreted as limited evidence for seletion on the *Mhc* (Boyce et al. 1997). Furthermore, the *DRB* sequences of this study and Mikko and Andersen (1995) have greater non-synonymous to synonymous substitutions in the ARS region supporting long-term balancing selection. The differential patterns are suggestive of balancing selection within the park systems and pathogen-driven selection, i.e. selection acting on specific alleles in non-park regions. The levels of gene flow we observed do not support genetic drift as a factor resulting in a loss of alleles in the continuous Ontario region. We considered two potential factors that differ between the parks and neighbouring regions we examined: the absence of white-tailed deer infected with the parasite *P. tenuis* within National Parks; and the absence of hunting within the park systems.

Firstly, the impact of white-tailed deer infected with the menengial worm *P. tenuis* may be influencing the *Mhc* locus independent of the neutral loci resulting in a shift to predominant allele frequency within non-park regions inhabited by deer. Pukaskwa National Park has contact with moose from the northern and central regions of Ontario, yet the other regions within the province show a more pronounced trend towards the *DRB*-a allele. White-tailed deer and *P. tenuis* are absent within Pukaskwa (Whitlaw and Lankester 1994) which maintains an additional allele not observed exterior to the park. The dominant *DRB*-a distribution in other regions of Ontario, despite the gene flow detected with the microsatellite loci, is consistent with the presence of *P. tenuis*. Also supporting the potential impact of *P. tenuis* on *DRB* allele distributions are the alleles observed in British Columbia moose (Mikko and Andersen 1996). These moose have maintained the four North American *DRB* alleles and do not demonstrate the predominant *DRB*-a allele frequency observed in the more eastern range of moose we examined in this study consistent with the absence of *P. tenuis* in western Canada.

In the absence of *P. tenuis*, balancing selection may be the primary factor influencing *Mhc* variation and this may be further enhanced by high moose densities in two of the park systems – Isle Royale and Riding Mountain. The higher densities of moose in these populations may be atypical of the solitary life history of moose in other regions (Ellegren et al. 1996) resulting in increased balancing selection due to increased contact and pathogen exchange. High densities in Isle Royale and Algonquin Provincial Park, with the highest moose per km^2 in Ontario (Whitlaw and Lankester 1994), have shown high incidences of mortality from the winter tick (D. Strickland, personal communication). Pukaskwa National Park maintains a stable population of approximately 0.200 moose per km^2 (Whitlaw and Lankester 1994) compared to 1.0-4.0 moose per km^2 in Isle Royale and Riding Mountain.

Secondly, although hunting has not directly been associated with a loss of diversity at *Mhc* loci, there is the potential for such an association similar to pathogen-driven selection. An examination of the impacts of selective hunting, i.e. preference for large body size and high quality antler racks, on red deer indicated a loss in the frequency of alleles associated with such traits (Hartl et al. 1991). *Mhc* alleles have been shown to have an association with growth in cattle (Grignola et al. 1995) and this gene complex is linked to a large number of growth-related genes (Lewin et al. 1992). Hunting pressure on "larger" moose outside of protected areas may influence a loss of allelic diversity observed at the *Mhc* genes that would not be readily detected at micro- or minisatellite loci.

Our data is consistent with the suggestion that the presence or absence of white-tailed deer with *P. tenuis*, differential hunting pressure, singly or in combination, are potential factors influencing *Mhc* allelic diversity in moose populations. At present, it would be premature to connect specific *DRB* alleles to any disease resistance against *P. tenuis* or fitness characteristics, however, *Mhc* alleles are inherited in haplotypic combinations (Murray et al. 1999) and alleles at other genes in linkage may be influencing the observed shift in the *DRB* alleles. Furthermore, a complete assessment of the population structure of isolated parks would be required to fully assess heterozygote excess due to balancing selection versus a deficiency based on a Wahlund effect. More direct comparisons would be required to examine the role, if any, of hunting pressure on moose populations and its impact of levels of genetic variation on *Mhc* or other functional loci.

Population Structure and Metapopulations

An assessment of connectivity and gene flow among different geographic areas identified both non-migratory and migratory moose populations. Riding Mountain National Park was shown to be isolated from its nearest geographic neighbor, northwestern Ontario, consistent with a non-migratory prairie "island" population (Karns 1998). Isle Royale was confirmed as being isolated from its corresponding mainland populations, i.e. northwestern Ontario / Nipigon. Moose movement and gene flow were prevalent throughout Ontario with gene flow, extending along the periphery of Lake Superior (Fig. 2). Differentiation between northwestern / Nipigon and northeastern Ontario likely reflects isolation-by-distance (Table 4). Genetic distances between populations can reflect the effects of geographical separation (Simonsen et al. 1998) and the Neighbour-joining tree of Nei's unbiased genetic distance (Nei 1978) supports isolation-by-distance within the continuous Ontario range (Table 4, Fig. 3A).

The monomorphic *DRB* locus in Newfoundland moose, in the absence of deer and *P. tenuis* on the island, indicates that historic population events such as founder events can significantly influence levels of genetic variation at functional DNA markers. Similar monomorphism at *DR*B was observed in a number of the island populations of bush rat (Seddon and Baverstock 1999). The population turnover that may result from over-hunting (Goddard 1970) may accelerate genetic drift in isolation due to decreased effective population size thereby reducing genetic variation (Harrison and Hastings 1996). However, our findings suggest the recovery of moose numbers in continuous geographic ranges such as Ontario are supplemented by immigration, contrary to the proposed limited impact of neighbouring populations (Goddard 1970, Hundertmark 1998). The recovery of moose populations through local movements, immigration or re-colonization, is consistent with a metapopulation of potential "source" and "sink" sub-populations (Harrison and Hastings 1996).

National and Provincial Parks represent important areas for maintaining genetic variability and further represent potential "source" populations within a connected metapopulation structure. The one notable example from this study is Pukaskwa National Park, which appears to represent an important reservoir of genetic material within the northern Ontario moose metapopulation. Different levels of

genetic variability at *DRB* within the park (Fig. 3B) were observed despite connectivity to other regions in Ontario (Table 3, Fig. 3A). The maintenance of an additional allele at this one *Mhc* locus within this region provides the potential transfer of allelic diversity into other regions of Ontario through the existing levels of gene flow if local selection pressures are altered. Contrary to Pukaskwa, Riding Mountain and Isle Royale National Parks, while maintaining potentially important allelic diversity, are in apparent isolation from neighboring regions limiting their contribution as natural "source" populations.

Recent studies have examined the genetic structure of populations within a metapopulation context by examining genetic variation combined with local extinction and re-colonization (Saccheri et al. 1998, Westemeier et al. 1998). Also, local selection pressures affecting morphological traits despite gene flow have been observed in conifers (Karhu et al. 1996) and positive-selection has been identified acting on the transferrin gene in salmon populations (Ford et al. 2000). Our data demonstrate the potential of combining DNA profiles from neutral loci such as microsatellite to functional loci such as moose *Mhc* to completely elucidate metapopulation structure. Although variable neutral loci such as microsatellites can be useful in assessing population structure, they may have limited use as a proxy for genome-wide variation particularly in detecting local selection pressures influencing genetic variation within local "source" and "sink" populations.

Conclusions

There are obvious limitations in the range of genetic variability that can exist in moose populations based on the number of alleles present in North American animals. As a result, large differences in allelic diversity and heterozygosity are constrained and any interpretation on the role of specific selection pressures, disease and hunting, influencing *Mhc* allele frequencies in moose populations requires large sample sizes from a number of sites with different selection pressures. However, the low overall-levels of genetic variation in moose in otherwise viable populations can provide a useful model for understanding genetic variation for comparison to threatened and endangered populations.

This study has identified differences between neutral genetic variation and variation at an adaptively significant locus. Neutral and functional loci in combination may provide a very different

interpretation of genetic variation and population structure than if one marker system was used exclusively. A comprehensive comparison of both neutral and functional genetic loci will provide a more accurate composite of population structure and genetic variation within a metapopulation framework. The management strategies of wildlife species should examine larger metapopulations as the primary conservation unit. Many studies focus on isolated populations as the primary criterion for identifying conservation or management units (Moritz 1994). Isolation may be a reasonable criterion for recommending protection but identifying adaptive variation provides a sound basis for effective management and is a proactive approach to preventing isolation among the differential selection pressures within a larger metapopulation. Priority should be placed on identifying locally adaptive genomes using morphological, physiological or genetic characters; high allelic diversity; and population structure, migration and gene flow.

For moose specifically, increasing the number of park systems and surrounding areas, as well as increasing the number of microsatellite and *Mhc* loci will allow a more accurate reconstruction of the metapopulation structure and potential local selection pressures acting on moose from different regions. Based on our present findings, we recommend managing moose as a metapopulation containing protected park systems with no hunting and no white-tailed deer, or more importantly, *P. tenuis*, that maintains connectivity through corridors to support migration and gene flow to neighboring regions.

Table 1. Sampling sites of moose (*Alces alces*) included within geographical regions. The total sample size (N) used in profiling multilocus DNA fingerprinting, microsatellite loci and/or the *DRB* locus and proposed histories for each region are provided.

Geographical Region	Sites	N	Proposed Histories
Manitoba	Riding Mountain National Park	23	Island prairie population (Karns 1998)
Northwestern Ontario	Red Lake, Sioux Lookout	27	Continuous Ontario population (Banfield 1974)
Nipigon, Ontario	Lake Nipigon, Geraldton, Thunder Bay	39	Continuous Ontario population (Banfield 1974)
Isle Royale, Michigan	Isle Royale National Park	17	Founded in the early 1900s, increasing in size to about 200 moose in 1915, population expansion in late 1920s to 5000 animals followed by a population crash in early 1940s. Levels remained at approximately 1000 moose upon introduction of wolves to the island in the late 1940s (Mech 1966)
Pukaskwa, Ontario	Pukaskwa National Park	37	Continuous Ontario population (Banfield 1974)
Central Ontario	North Bay, Sault Ste. Marie, Sudbury	32	Continuous Ontario population (Banfield 1974)
Northeastern Ontario	Kirkland Lake, Cochrane, Moosonee	36	Continuous Ontario population (Banfield 1974)
New Brunswick	North shore	19	Unknown
Newfoundland	Province-wide	29	Moose introduced in 1870 from Nova Scotia and four moose from New Brunswick in the early 1900s (Pimlott 1953, Broders 1999).

Table 2. Average percent difference (APD) from multi-locus DNA fingerprints (DNAfp), number of alleles, expected heterozygosity (H_E) and inbreeding coefficient (F_{IS}) for five microsatellite loci (Micro) and the *DRB* locus and the for nine moose (*A. alces*) populations. Sample sizes (actual or mean) analysed per type of locus are included.

Population	DNAfp		Micro				*DRB*			
	N_t	APD	N_{tt}	$Alleles_t$	H_E	F_{IS}	N_t	$Alleles_{tt}$	H_E	F_{IS}
Riding Mountain National Park, Manitoba	10	0.593	23	4.4	0.534	0.120	23	4.0	0.529	0.171
Northwestern Ontario	18	0.573	24	4.4	0.595	0.039	27	3.0	0.419	0.047
Nipigon, Ontario	9	0.375	38	4.2	0.610	0.098	25	2.0	0.365	0.143
Isle Royale, Michigan	9	0.238	15	2.8	0.482	0.176	17	3.0	0.607	0.329
Pukaskwa National Park, Ontario	-	-	33	4.2	0.532	-0.037	37	3.0	0.551	0.082
Central Ontario	10	0.561	31	4.2	0.520	0.057	15	2.0	0.358	-0.273
Northeastern Ontario	19	0.487	36	4.0	0.534	0.075	30	2.0	0.433	0.016
New Brunswick	18	0.429	18	2.8	0.537	-0.132	19	3.0	0.381	0.060
Newfoundland	17	0.337	19	2.2	0.378	0.017	29	1.0	0.000	-

† Mean number of alleles or sample size.
††Total numer of alleles or sample size.

Table 3. Loss of genetic variation between proposed source populations and the island populations of Isle Royale and Newfoundland.

Founding Event	**DNA Fingerprinting: APD**	**Microsatellite: Expected Heterozygosity (H_E)**	***Microsatellite:* Allelic Diversity**
Nipigon-to-Isle Royale	**36.0% Decrease**	**21.0% Decrease**	**36.4% Decrease**
New Brunswick-to-Newfoundland	**21.4% Decrease**	**30.0% Decrease**	**21.4% Decrease**

Table 4. R_{ST} (top diagonal) and F_{ST} (bottom diagonal) for each pairwise comparison of nine moose populations. Moose populations include: Riding Mountain National Park (RMNP); northwestern Ontario (NWON); Nipigon (NIP); Isle Royale (IROY); Pukaskwa National Park (PNP); Central Ontario (CEON); northeastern Ontario (NEON); New Brunswick (NB); and Newfoundland (NFLD).

Population	RMNP	NWON	NIP	IROY	PNP	CEON	NEON	NB	NFLD
RMNP	-	0.1042	0.0579	0.0424	0.0601	0.0944	0.1072	0.1138	0.2011
NWON	0.1033	-	0.0011	0.0389	0.0343	0.0648	0.0579	0.0792	0.1300
NIP	0.0918	0.0239	-	0.0439	0.0045	0.0350	0.0294	0.0664	0.1652
IROY	0.1366	0.1036	0.1154	-	0.0673	0.1416	0.1150	0.0913	0.1095
PNP	0.0602	0.0237	0.0430	0.0927	-	0.0282	0.0124	0.1041	0.1553
CEON	0.0872	0.0688	0.0871	0.1674	0.0255	-	0.0266	0.1898	0.2523
NEON	0.0829	0.0645	0.0790	0.1647	0.0265	0.0343	-	0.1386	0.2110
NB	0.1228	0.1252	0.1132	0.1869	0.1083	0.1465	0.0737	-	0.1727
NFLD	0.3013	0.1614	0.1897	0.2371	0.1758	0.2028	0.1321	0.1370	-

Table 5. Nei's unbiased genetic distance for the *DRB* locus (top diagonal) and five microsatellite loci (bottom diagonal). Moose populations include: Riding Mountain National Park (RMNP); northwestern Ontario (NWON); Nipigon (NIP); Isle Royale (IROY); Pukaskwa National Park (PNP); Central Ontario (CEON); northeastern Ontario (NEON); New Brunswick (NB); and Newfoundland (NFLD).

Population	RMNP	NWON	NIP	IROY	PNP	CEON	NEON	NB	NFLD
RMNP	-	0.0212	0.0257	0.0458	0.0248	0.0370	0.0455	0.0142	0.0454
NWON	0.2085	-	0.0013	0.0552	0.0015	0.0256	0.0075	0.0070	0.0538
Nipigon	0.1980	0.0680	-	0.0736	0.0001	0.0265	0.0082	0.0046	0.0475
IROY	0.2043	0.1740	0.1947	-	0.0742	0.0935	0.0758	0.0815	0.1362
PNP	0.1068	0.0520	0.0764	0.1380	-	0.0280	0.0096	0.0040	0.0443
CEON	0.1528	0.1258	0.1560	0.2638	0.0491	-	0.0168	0.0283	0.1194
NEON	0.1401	0.1263	0.1586	0.2497	0.0534	0.0068	-	0.0218	0.0973
NB	0.2404	0.2610	0.2495	0.3291	0.1925	0.2661	0.1435	-	0.0305
NFLD	0.5449	0.4201	0.5574	0.4715	0.3998	0.4540	0.2620	0.2142	-

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Figure 1. Graph showing average percent difference (APD) for multilocus DNA fingerprint profiles and expected heterozygosity (H_E) for five microsatellite loci and the *Mhc DRB* locus for moose from nine regions.

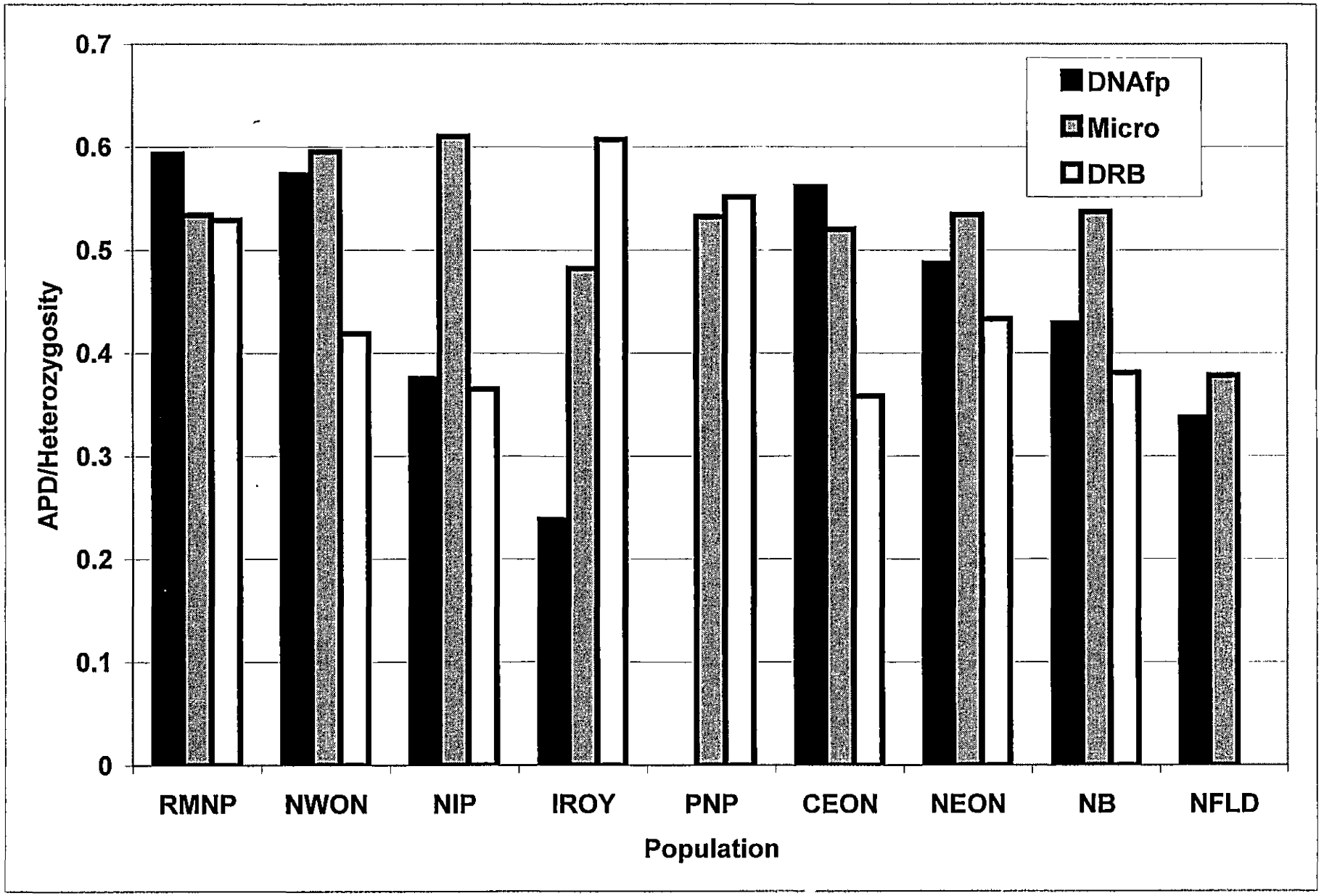

0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
APD/Heterozygosity
DNAfp
Micro
DRB
RMNP
NWON
NIP
IROY
PNP
CEON
NEON
NB
NFLD
Population

Figure 2. A map indicating the population structure of moose based on R_{ST}/F_{ST} estimates for three National Parks, Ontario, New Brunswick and Newfoundland. Arrows represent animal movement and gene flow among regions based on values of both R_{ST} and F_{ST} below 0.050. A population within a square box represents an isolated "island" population.

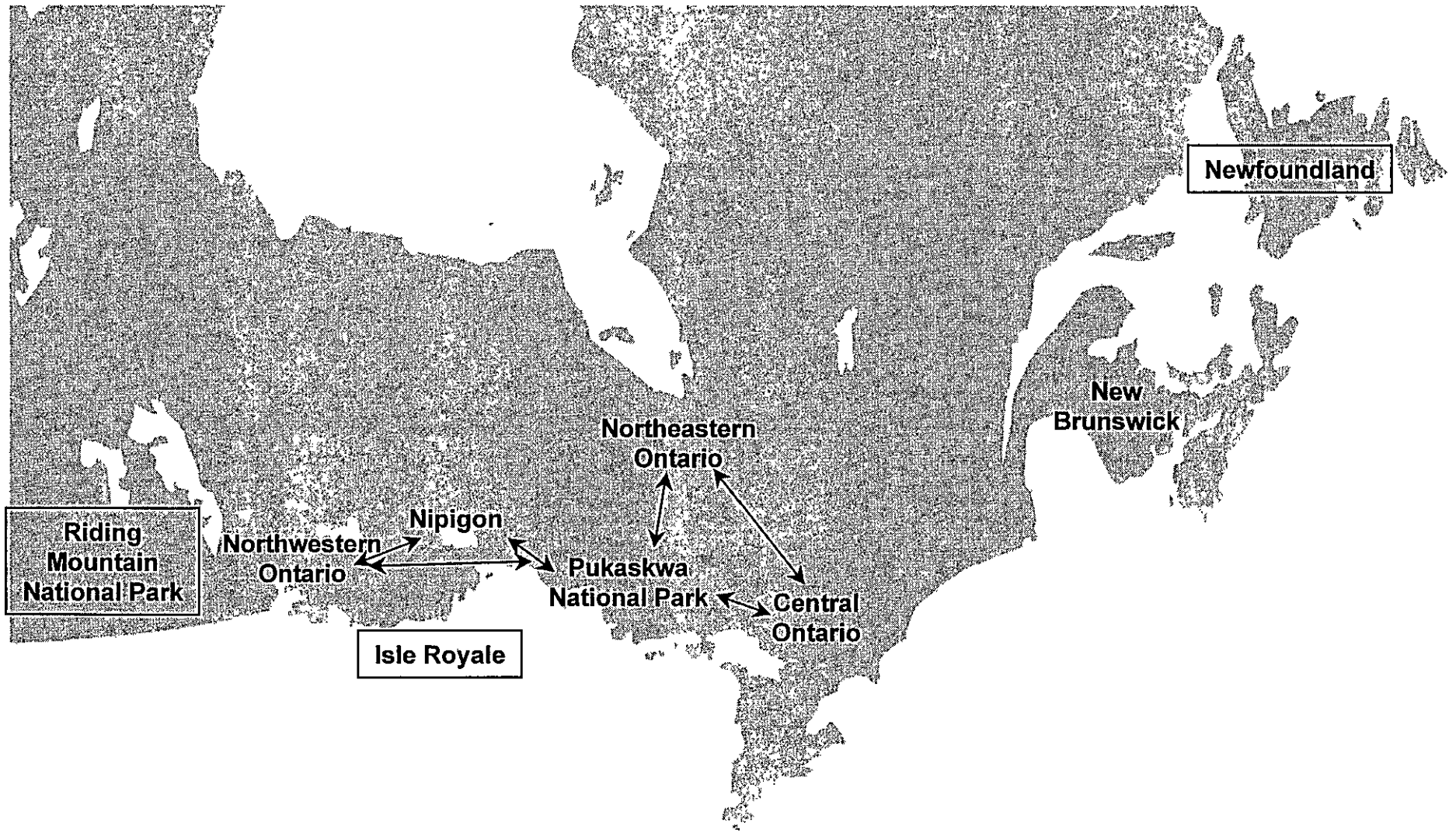
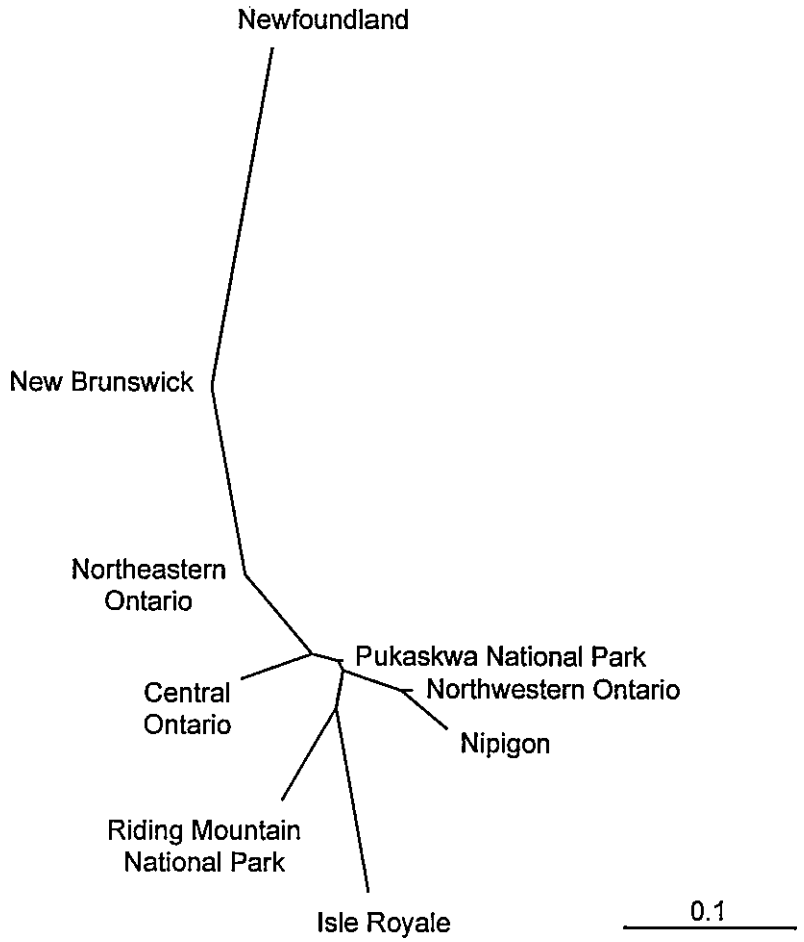

Newfoundland
New
Brunswick
Northeastern
Ontario
Riding
Mountain
National Park
Nipigon
Northwestern
Ontario
Pukaskwa
National Park
Central
Ontario
Isle Royale

Figure 3. Neighbour-joining trees using Nei's unbiased genetic distance on allele frequencies from five microsatellite loci **(A)** and the exon-2 region of the *MHC* gene *DRB* **(B)**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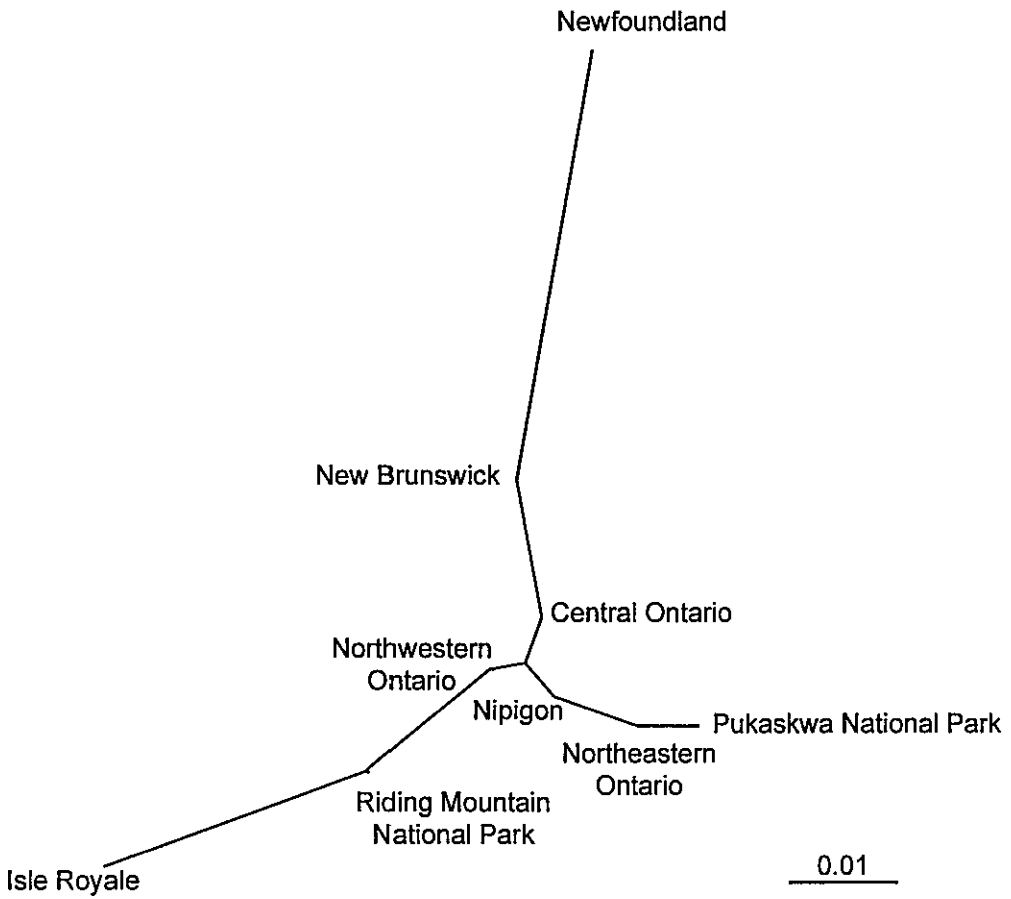
New Brunswick
Northeastern Ontario
Pukaskwa National Park
Northwestern Ontario
Central Ontario
Nipigon
Riding Mountain National Park
Isle Royale
0.1


Newfoundland
New Brunswick
Central Ontario
Northwestern
Ontario
Nipigon
Pukaskwa National Park
Northeastern
Ontario
Riding Mountain
National Park
Isle Royale
0.01

APPENDIX I. Moose Allele Frequencies for five microsatellite loci (Map2C, Cervid14, IGF-1, BM1225 and BM4513) and the *DRB* locus. Alleles are provided in base pair sizes for the microsatellite loci.

Locus/ Allele	RMNP	NWON	NIP	IROY	PNP	CEON	NEON	NB	NFLD
MAP2C									
113	0.000	0.042	0.083	0.000	0.015	0.000	0.015	0.000	0.000
111	0.115	0.063	0.000	0.000	0.000	0.000	0.000	0.531	0.625
109	0.577	0.604	0.514	0.867	0.676	0.545	0.424	0.250	0.000
107	0.192	0.167	0.306	0.067	0.191	0.121	0.348	0.219	0.375
105	0.115	0.125	0.097	0.067	0.118	0.333	0.212	0.000	0.000
CEV14									
221	0.000	0.000	0.038	0.000	0.000	0.016	0.013	0.000	0.000
219	0.694	0.660	0.700	0.367	0.809	0.891	0.795	0.650	0.694
217	0.167	0.080	0.000	0.000	0.015	0.031	0.064	0.000	0.167
215	0.028	0.120	0.200	0.633	0.147	0.047	0.115	0.350	0.028
213	0.000	0.140	0.063	0.000	0.029	0.016	0.013	0.000	0.000
207	0.111	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.111
IGF-1									
109	0.786	0.333	0.500	0.500	0.485	0.438	0.500	0.700	0.159
107	0.214	0.667	0.500	0.500	0.515	0.563	0.500	0.300	0.841
BM1225									
250	0.053	0.022	0.100	0.000	0.045	0.172	0.132	0.184	0.000
248	0.132	0.174	0.000	0.000	0.015	0.063	0.000	0.000	0.000
246	0.000	0.022	0.050	0.167	0.015	0.016	0.000	0.000	0.000
238	0.026	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
236	0.105	0.413	0.412	0.233	0.348	0.172	0.118	0.237	0.000
232	0.026	0.022	0.262	0.000	0.000	0.000	0.000	0.000	0.000
230	0.658	0.348	0.175	0.600	0.576	0.578	0.750	0.579	1.000
BM4513									
142	0.079	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
140	0.079	0.000	0.053	0.000	0.145	0.400	0.092	0.000	0.000
138	0.079	0.083	0.053	0.000	0.129	0.033	0.197	0.452	0.452
136	0.421	0.167	0.276	0.433	0.226	0.200	0.066	0.024	0.024
134	0.289	0.354	0.329	0.367	0.210	0.100	0.197	0.214	0.214
132	0.053	0.104	0.013	0.033	0.032	0.017	0.066	0.000	0.000
130	0.000	0.000	0.013	0.000	0.000	0.000	0.000	0.000	0.000
122	0.000	0.292	0.263	0.167	0.258	0.250	0.382	0.310	0.310
DRB									
DRB-a	0.656	0.722	0.760	0.529	0.595	0.767	0.683	0.763	1.000
DRB-b	0.100	0.037	0.000	0.206	0.000	0.000	0.000	0.000	0.000
DRB-c	0.144	0.241	0.240	0.265	0.283	0.233	0.317	0.184	0.000
DRB-d	0.100	0.000	0.000	0.000	0.122	0.000	0.000	0.053	0.000

DNA profiles of the eastern Canadian wolf and the red wolf provide evidence for a common evolutionary history independent of the gray wolf.

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ABSTRACT

The origin and taxonomy of the red wolf (*Canis rufus)* has been the subject of considerable debate and it has been suggested that it is a recently formed taxon as a result of hybridisation between the coyote and gray wolf. Like the red wolf, the eastern Canadian wolf has been characterised as a small "deer-eating" wolf that hybridises with coyotes. While studying the population of eastern Canadian wolves in Algonquin Provincial Park we recognised similarities to the red wolf based on DNA profiles at eight microsatellite loci. We examined whether this relationship was due to similar levels of introgressed coyote genetic material by comparing the microsatellite alleles with other North American populations of wolves and coyotes. These analyses indicated that it was not coyote genetic material that led to the close genetic affinity of red wolves and eastern Canadian wolves. We then examined the control region of the mitochondrial DNA and confirmed the presence of coyote sequences in both. However, we also found sequences in both that were 150,000-300,000 years divergent from sequences found in coyotes. None of the red wolves or eastern Canadian wolf samples from the 1960s contained gray wolf (*C. lupus*) mtDNA sequences. The data are not consistent with the hypothesis that the eastern Canadian wolf is a sub-species of gray wolf, as it is presently designated. We suggest both the red wolf and eastern Canadian wolf evolved in North America sharing a common lineage with the coyote until 150,000-300,000 years ago. We propose that it retain its original species designation of *C. lycaon.*

INTRODUCTION

The origin of the red wolf, *Canis rufus*, has been the subject of considerable debate and controversy. Nowak (1979; 1995) proposed that the species evolved in North America from a wolf-like canid representing a transitional form between a coyote-like ancestor and the gray wolf (*C. lupus*) that evolved in Eurasia. Contrary to this hypothesis Wayne and Jenks (1991) and Roy et al. (1994, 1996) has suggested that *C. rufus* is not a valid species but the result of recent extensive hybridisation between *C. lupus* and coyotes (*C. latrans*) in the south central U.S. The taxonomic designation of *C. rufus* together with all North American canids has been fluid in this century ranging from less distinct than a sub-species, eg. *C. lupus* var. *rufus*, to its present species status (Brewster and Fritts 1995). There is general agreement that the red wolf hybridizes with the coyote.

The eastern Canadian wolf, *C. l. lycaon*, like the red wolf, has been the subject of several taxonomic treatments that have moved it from species status, *C. lycaon*, to its presently accepted status as a gray wolf sub-species (Brewster and Fritts 1995). Since the late 1700s, eastern North American wolves were described as among the smallest on the continent (Goldman 1944), long before any documented arrival of coyotes (*C. latrans*) in the 1900s. As with the red wolf, there is general agreement *C. l. lycaon* readily hybridizes with coyotes and studies of mitochondrial DNA have shown hybridisation between wolf populations east of Minnesota and coyotes (Lehman et al. 1991, Wayne and Lehman 1992).

Wolf and coyote populations have been further compared using microsatellite loci (Roy et al. 1994); all coyote populations are closely related, whereas gray wolf populations representing different sub-species of *C. lupus* are more divergent. "Hybridizing wolf" populations in Minnesota and southern Quebec were genetically most similar to each other and then to captive red wolves. The original interpretation of these relationships was that "hybridizing wolves" of southern Quebec and Minnesota and the red wolf contained similar amounts of coyote genetic material (Roy et al. 1994).

The cause of wolf/coyote hybridisation has been attributed to the destruction of forested habitat and the increased expansion of coyotes in the last 90 years (Wayne and Lehman 1992). While these are clearly important factors, the introgression of coyote mtDNA and nuclear DNA into wolf populations appears limited to the eastern portion of North America. The hybrid zone that has been identified based on

mtDNA and microsatellite DNA markers has not been assessed with respect to the sub-species of *C. lupus* that is involved (Lehman et al. 1991, Wayne and Lehman 1992, Roy et al. 1994, Nowak 1995). The proposed sub-species distribution of Nowak (1995) shows that the boundary of the hybrid zone corresponds closely to the historical distribution of the eastern Canadian wolf, *C. l. lycaon.* The absence of any introgression of coyote DNA into western wolf populations sympatric with coyotes, such as those in Alberta (Roy et al. 1994, Pilgrim et al. 1998) and Alaska (Thurber and Peterson 1991, Roy et al. 1994) suggests that only the eastern wolves, *C. l. lycaon* and *C. rufus*, readily hybridize with coyotes.

While studying a population of the eastern Canadian wolf, *C. l. lycaon*, from Algonquin Provincial Park we found a surprisingly close relationship with the red wolf based on allele frequencies at microsatellite loci. Although both wolves are known to hybridize with coyotes, we perfomed several analyses to determine if it was introgressed coyote genetic material that led to their close affinity. We further examined mitochondrial control region sequences from captive red wolves, from coyote samples and from wolf teeth samples collected in Algonquin Park and elsewhere in Ontario during the 1960s. These represent wolves that had contact with coyotes for a period of less than 30 years. They are the best available natural sample set of eastern Canadian wolves to detect representative eastern Canadian wolf mtDNA. In this paper we test two alternative hypotheses that the red wolf/eastern Canadian wolf are hybrids of coyotes and gray wolves or that these wolves both derived independently of gray wolves in North America.

MATERIALS & METHODS

Samples and DNA Extraction

Eastern Canadian wolves, representing the putative gray wolf sub-species *C. l. lycaon*, were sampled from Algonquin Provincial Park and surrounding area from 1960-1965 (n=19) and 1985-1996 (n=49). *Canis rufus* samples from the captive red wolf breeding program (n=60) were also analysed. Texas coyotes (n=24) were used to represent *C. latrans*. Gray wolves, *C. lupus*, were sampled from the Northwest Territories (n=67). DNA was extracted by methods described in Guglich et al. (1994) from

frozen organ samples (liver, heart, kidney, or muscle) or from whole blood obtained by venipuncture of individuals that were live trapped and released. DNA from the captive red wolf program, Texas coyotes and historic teeth collected in Ontario during the 1960s was extracted following a modified Qiagen (Qiagen) extraction protocol using the lysis buffer described in Guglich et al. (1994).

Microsatellite Analysis

Ten microsatellite loci (Roy et al. 1994; 1996; Ostrander et al. 1993) were amplified using 4.6 pmol γ^{33}P T4 polynucleotide kinase (Boehringer-Mannheim) end labeled primer ATP in a total reaction volume of 10µl per tube using 25ng of genomic DNA, 200 µM dNTPs, 1x amplification buffer, 2.0 mM $MgCl_2$, unlabelled primer (0.2 mM), 1.0 µg of Bovine Serum Albumin (BSA) (BRL) and 0.5 units of *Taq* polymerase (BRL). Products were amplified under the following conditions: 94°C for 5 min., 55-65°C for 30 sec., 72°C for 15 sec. 1 cycle; 94°C for 15 sec., 55°C for 30 sec., 72°C for 15 sec. 30 cycles; 94°C for 15 sec., 55°C for 30 sec., 72°C for 2 min. 1 cycle. Products were then mixed with an equal volume of formamide loading buffer and were heated at 95°C for 5 minutes before loading onto a 6% sequencing gel containing 50% (w/v) urea. A control sequencing reaction of phage M13 DNA was run adjacent to the samples to produce size markers for the microsatellite alleles.

Control region Sequencing and Sequence Analysis

The following primers were used to amplify the control region of the mitochondrial DNA.

Primer 1 5'-GAA GCT CTT GCT CCA CCA ATC-3' (Pilgrim et al. 1998)

Primer 2 5'-GGG CCC GGA GCG AGA AGA GGG AC-3'

The control region was amplified in a total reaction volume of 20µl per tube using 25ng of genomic DNA, 200 µM dNTPs, 1x amplification buffer, 2.0 mM $MgCl_2$, primers 1 and 2 (0.2 mM) and 0.5 units of *Taq* polymerase (BRL). Products were amplified under the following conditions: 94°C for 5 min., 55°C for 30 sec., 72°C for 30 sec. 1 cycle; 94°C for 30 sec., 55°C for 30 sec., 72°C for 30 sec. 35 cycles; 94°C for 30

sec., 55°C for 30 sec., 72°C for 2 min. 1 cycle. Products were re-amplified and purified through QIAquick (Qiagen) for DNA sequencing using dye-terminator cycle sequencing using an ABI Prism 373 DNA Sequencer (MOBIX, McMaster University).

A previously described method (Pilgrim et al, 1998) for distinguishing *C. lupus* mtDNA from *C. latrans* was used to identify the presence or absence of gray wolf mtDNA within the historic teeth samples based on a 4 base pair difference between gray wolves and coyotes.

Genetic Analysis

We analyzed allele frequencies at 8 loci among the Algonquin Park and red wolf populations and compared them with the other North American populations of wolves and coyotes (Roy et al. 1994; 1996). Microsatellite alleles were assigned based on size in Roy et al. (1996). Nei's genetic distance (1972) was calculated using the programs SEQBOOT, GENDIST and NEIGHBOR in the computer program PHYLIP (Felsenstein 1993).

An individual index (I_I) was calculated from the DNA profile of each animal using the following equation: $\Sigma \log (p_A/p_B)$, where p_A and p_B are the allele frequencies of a specific allele from population A and B, respectively. If an allele was absent from one of the populations, an allele frequency of one allele in the population (sample size) was used. This LOD score value assesses the origin of the alleles in each animal based on a ratio of the frequencies from two populations. If there are similar allele frequencies in both populations then the I_I values of individuals from both populations would follow a distribution around 0. An increasing positive score indicates an individual originated from population A and a decreasing negative score indicates an individual originated from population B.

A Probability of Identity (POI) measure (Paetkau and Strobeck 1994, Waser and Strobeck 1998) was also calculated to assess whether an individual's genotype was from one of two source populations. The probability of an individual's genotype using the allele frequencies of one source population is summed over all loci. The same calculations are made with respect to the second putative population. The log of the two values for each individual's genotype based on the two source population's allele

frequencies are plotted to produce a scatterplot to assess the population with which the individual has the greatest likelihood of affiliation.

A minimum spanning tree was generated based on data provided by the program MINSPNET (Excoffier, 1992). The phylogenetic relationships of canid mtDNA haplotypes were generated using a neighbor-joining tree with sequence divergence using the program MEGA [Kumar, S. Tamura, K. Nei, N. MEGA: Molecular Evolutionary Genetic Analysis 1.01 (Pennsylvania State University, University Park, PA, 1993)].

RESULTS

The neighbor-joining analysis of genetic distances showed an unexpectedly close relationship among Algonquin Park animals, the red wolf, Minnesota wolves and the southern Quebec wolves (Fig. 1a). To evaluate whether this was because "hybridizing wolves" of southern Quebec and Minnesota and the red wolf contained similar amounts of coyote genetic material (Roy et al. 1994) we determined DNA profiles from captive red wolves and other populations of gray wolves and Texas coyotes. The same relationship between eastern Canadian wolves and captive red wolves was observed when they were compared to gray wolves and Texas coyotes (Fig. 2). In this comparison, the interpretation that eastern Canadian wolves and red wolves shared similar levels of coyote introgression did not seem consistent with the genetic distance between the red wolf and the Texas coyotes, which were the geographically closest coyote source population for the red wolf. The genetic similarity between red wolves and eastern Canadian wolves was not heavily influenced by the introgression of coyote genetic material; alleles that were prevalent in Texas and other coyote populations (Roy et al. 1994) were absent or present at very low frequency in red wolves (Table 1).

We determined a distribution of POI (Fig. 3A) and I_I scores (Fig. 3B) for the captive red wolves using allele frequencies from the Algonquin population representing the eastern Canadian wolf and from the Texas coyote population. The majority of captive red wolves overlapped with the distribution of the eastern Canadian wolf population for both assignment tests. If coyote genetic material resulted in the apparent similarity of these wolves, we would have expected the red wolf to fall within or closer to the

distribution of its geographic neighbor, the Texas coyote population, and not the geographically distant population of Algonquin Park eastern Canadian wolves.

We further assessed the eastern Canadian wolves and captive red wolves in the context of the gray wolf using I_I indices and POI values using allele frequencies from the Algonquin Park eastern Canadian wolves and a gray wolf population from the Northwest Territories. The POI estimates indicated eastern Canadian wolves and red wolves clustered together and distinctly from both the gray wolves and the Texas coyotes (Fig. 4A). The I_I indices from two comparisons (Algonquin wolves vs. Texas coyotes and Algonquin wolves vs. Northwest Territories) were plotted (Fig 4B). The eastern Canadian wolves and red wolves clustered together and away from gray wolves in both I_I comparisons and the two wolves grouped closer to coyotes in the Algonquin/Northwest Territories comparison. The Algonquin wolves and red wolves clustering away from the distribution for gray wolves using both assignment tests suggested little or no gray wolf (*C. lupus*) genetic material in these populations. This finding was inconsistent with the eastern Canadian wolf representing a sub-species of the gray wolf, *C. lupus*, and inconsistent with the gray wolf having a significant contribution in the formation of the red wolf.

Given the apparent absence of gray wolf genetic material, we examined mitochondrial control region sequences from the captive red wolves, from teeth samples collected in Algonquin Park and elsewhere in Ontario during the 1960s and from Texas coyotes. Historic Ontario wolves had approximately 30 years of contact with coyotes and represent the best available natural sample set of the eastern Canadian wolf. We found no gray wolf control region sequences in any red wolf or any historic samples collected in Algonquin Park (n=19) consistent with the microsatellite assignment tests. However, we identified one haplotype (C1) in the park animals and surrounding area that were not found in coyotes and the sequences of which was divergent from those in coyote (Fig. 5A). Among the red wolf samples, we identified a distinct haplotype (C2) not found in coyotes. A third haplotype (C3) was observed in a wolf from Manitoba that grouped with the historic eastern Canadian wolf haplotypes. Phylogenetic analyses grouped the eastern Canadian wolf and red wolf haplotypes (C1-C2) and C3 haplotypes away from the coyote haplotypes in a neighbor-joining analysis (Fig. 5B).

The historic Algonquin Park samples contained the C1 haplotype in 7 of 13 animals we were able to obtain control region sequences and 9 of 12 red wolves contained the C2 haplotype.

The presence of the related C1 and C2 sequences in the geographically separated red wolves and eastern Canadian wolves but not the Texas coyotes, is consistent with a common origin of these two wolves. The remaining samples in this population contained coyote mitochondrial DNA sequences confirming that some level of hybridisation has also occurred.

The sequence divergence between the haplotypes observed in the eastern Canadian wolf and the red wolf haplotype was 2.1%. The intra-specific sequence divergence for coyotes (*C. latrans*) was 1.7 %. A comparison of the eastern Canadian wolf sequence (C1) to coyote sequences indicated 3.2 % sequence divergence and 2.3 % sequence divergence between the red wolf (C2) and coyote haplotypes. The sequence divergence of gray wolf (*C. lupus*) mtDNA from the haplotypes found in eastern Canadian wolves and red wolves was approximately 8.0%, and 10.0% between gray wolf and coyote haplotypes. The sequence difference observed between the eastern Canadian wolf sequences and the coyote sequences is consistent with 150,000-300,000 years separation, using a divergence rate of 1-2% per 100,000 years for the mammalian control region (Stewart and Baker 1994) and is consistent with the 1-2 million year divergence between gray wolves and coyotes (Kurten and Anderson 1980, Wayne 1993, Vila et al. 1997).

DISCUSSION

The similarity between the eastern Canadian wolf and the red wolf has been noted previously and both wolves were described as small eastern wolves long before the eastward expansion of coyotes (Brewster ad Fritts 1995). Neighbor-joining analysis of Nei's genetic distance using previously published data (Roy et al. 1994, Roy et al. 1996) and additional data we obtained from captive red wolf, other gray wolf and coyote populations again grouped the eastern Canadian wolf population and the captive red wolf samples. One interpretation of this relationship was that "hybridizing wolves" of Algonquin, southern Quebec and Minnesota and the red wolf contained similar amounts of coyote genetic material (Roy et al. 1994). This interpretation did not seem consistent with genetic distances between the red wolf and the Texas coyotes, which was the closest coyote source population for the red wolf. The absence of common

coyote alleles within Eastern Canadian wolves Park and the red wolf samples suggested that the close relationship observed between these two wolf populations was the result of a common wolf genetic origin. The application of assignment tests, an Individual Index (I_I) and Probability of Identity (POI), further supported the hypothesis that non-coyote derived parts of the genome were responsible for the similarity between the red wolf and the eastern Canadian wolf.

The presence of distinct control region haplotypes within the eastern Canadian wolves from the historic Algonquin Park population and the fact that captive red wolves clustered closer to coyotes than to gray wolves, supports the evolution of the eastern wolves independent of the gray wolf. These data indicate that, like the nuclear microsatellite DNA, the mtDNA of the eastern Canadian wolf/red wolf is not of gray wolf origin but similar to coyotes because of their relatively recent divergence from a common ancestor. It is unlikely that the eastern Canadian wolf mtDNA haplotypes obtained from the early 1960s represent the total introgression of coyote mtDNA as the Algonquin population would have had only 30 years of contact with the expanding coyote population and would require the replacement of gray wolf (*C. lupus*) mtDNA.

The coyote has been identified as the New World evolved canid species (Nowak 1979, Wayne 1993). Our data indicate a divergence in the North American canis mtDNA lineage of two types: 1) the red wolf and eastern Canadian wolf; and 2) coyote. We propose a model (Fig. 6) in which these two lineages diverged within the mid-Pleistocene, 150,000-300,000 years ago and came into contact in post-settlement time as a result of extensive habitat alteration. Further, the evolution of North American wolves and coyotes occurred independently of the gray wolf, *C. lupus*, that evolved in Eurasia 1-2 million years ago. We suggest the eastern North American wolf adapted to prey such as white-tailed deer within a forested habitat and the western coyote adapted to arid regions and smaller prey. The red wolf mtDNA haplotype, while showing sequence similarity to the eastern Canadian wolf mtDNA, is less divergent from coyote mtDNA and this may reflect continued contact with coyotes.

Several lines of evidence support a common origin for red wolves and eastern Canadian wolves: 1..The historic range of the eastern Canadian wolf overlaps with that of the present day red wolf and both would have existed in southern refugia during the Pleistocene (Nowak 1979, Brewster and Fritts 1995); 2. Pleistocene fossils suggest a small wolf inhabited eastern North America (Nowak 1995); 3. Species that

evolved in the New World and diverged only 150,000-300,000 years ago are more likely to hybridize with each other than with the gray wolf. Lack of introgression of coyote DNA into western and even the Mexican gray wolf, *C. l. baileyi* (Roy et al. 1996, Lehman et al. 1991, Garcia-Moreno 1996) populations sympatric with coyotes suggests that eastern Canadian wolves and red wolves are the only wolves that hybridize readily with coyotes. The fact that the Mexican wolf shows no hybridisation with coyotes suggests that the smaller size of the eastern wolves is not reason for their hybridisation with coyotes.

The predisposition of the eastern North American wolves to hybridize with coyotes may represent an evolutionarily characteristic unique to these wolves, suggesting the red wolf (*C. rufus*) and the eastern Canadian wolf (*C. l. lycaon*) share a common origin. Several additional lines of evidence are consistent with the hypothesis of a common origin between these wolves. First, the historic range of *C. l. lycaon* overlaps with that of the present day *C. rufus*. Further, it has been proposed that these species existed in southern refugia during the Pleistocene (Nowak 1979, Brewster and Fritts 1995). Second, skull morphology comparisons indicate similarities between *C. rufus* and *C. l. lycaon* (Nowak 1979, Lawrence and Bossert 1967, Lawrence and Bossert 1975, Nowak 1995). Algonquin Park wolves have previously been described as a remnant red wolf population, classified as *C. niger* at the time (Stanfield 1970). A common origin also has been suggested by Mech (1971) who stated "if the red wolf is a hybrid between the wolf and coyote, it would be this sub-species (*C. l. lycaon*) of wolf that is involved".

The only evidence contrary to the hypothesis of a North American-evolved wolf is the apparent presence of gray wolf, *C. lupus*, mtDNA haplotypes within 6 red wolf samples collected from the southeastern U.S. and samples from the northwestern Great Lakes region. *Canis lupus* mtDNA haplotypes were identified in 3/6 (Wayne and Jenks 1991) and 3/11 pre-1940s (Roy et al. 1996) red wolves from the historical range of *C. rufus*. We question whether these six samples were red wolves, as the historic range of *C. rufus* has been identified as overlapping with the distribution of the gray wolf sub-species *C. l. nubilus* (Schwartz and Schwartz 1991; Caire et al. 1989) and a Texas range of *C. l. baileyi* (Nowak et al. 1995). Gray wolf mtDNA was also found in 16% of the 77 animals previously analyzed (Wayne and Jenks 1991) from the region where they were selected for the breeding program. Strict morphological criteria were used to classify the animals as red wolf, coyote or red wolf/coyote hybrid and 44 were selected.

Subsequent selection of the most representative red wolf types provided 17 animals were used as founders. We suggest the 12 animals with gray wolf mitochondrial DNA from the original 77 may have been of *C. l. nubilus*, *C. l. baylei* or *C. l. familiaris* origin.

Wayne et al. (1998) stated that "genetically, the historic and recent red wolves were extremely similar suggesting they were derived from a single gene pool" which implies these samples accurately represent red wolves. Nowak and Federoff (1998) expressed concerns about the focus on samples for genetic analyses collected from the historic south central range and not the eastern range of the red wolf. We agree this is a problem, but not for the same reason. Including samples from this region that may represent the sympatric or integradated forms that include gray wolf and hybrid samples within the "red wolf" samples. Although distinct morphological differences exist between the red wolf and the Plains wolf (*C. l. nubilus*), morphologic overlap exists between these two species (Nowak 1979, Lawrence and Bossert 1967, Lawrence and Bossert 1975, Nowak 1995) and pelage color is too variable for specific identification. Therefore, identification of individual specimens based solely on morphology is questionable and a rigorous assessment of samples should be applied in characterizing wolves.

The problem of sympatric ranges of wolf and coyote species also exists in the western Great Lakes region. Northwestern Ontario and Minnesota contain the ranges of eastern Canadian wolves, Plains wolves (*C. l. nubilus*) and coyotes (*C. latrans*). Although the current sub-species distribution of *C. lupus* does not include the eastern Canadian wolf in this region (Nowak 1995), other assessments did (Nowak 1979, Brewster and Fritts 1995) and the presence of a divergent eastern Canadian wolf mtDNA haplotype in Manitoba (C4) supports an extended western range. A number of wolves from the Great Lakes region may have been previously identified with a lycaon/rufus haplotype, although the resolution of the RFLP and cytochrome b markers (Roy et al. 1996, Lehman et al. 1991), would not have resolved it from other coyote haplotypes. Northwestern Ontario, Isle Royale, Minnesota and Manitoba animals contained coyote mtDNA haplotypes not found in extant coyote populations. The original interpretation was that several waves of coyotes expanded into this region, hybridized and then the local coyote population became extinct (Wayne and Lehman 1992); this seems inconsistent with a large panmictic North American coyote population (Roy et al. 1994). These haplotypes are potentially in the same group as the lycaon/rufus

lineage. Similarly, a coyote–like haplotype, that was diagnostic to the red wolf breeding program and not coyotes, was found in 23/30 of the initial animals (Wayne and Jenks 1991).

In summary, much of the Nowak/Wayne debate surrounding the red wolf has focused on the presence of coyote genetic material in red wolves (Wayne et al. 1992, Wayne et al. 1998, Nowak 1992, Nowak and Federoff 1998). However, the main issue stems from the claim that gray wolf mtDNA occurs in red wolves and eastern Canadian wolves. It is generally accepted that the gray wolf, *C. lupus*, evolved in Eurasia (Nowak 1978, Wayne 1993, Vila et al. 1997). Nowak has proposed that a coyote-like progenitor originating in North America diverged on two continents evolving independently into the red wolf and gray wolf. If a wolf evolved in North America then the mtDNA in this canid should be more similar to coyotes, *C. latrans*, than gray wolves, *C. lupus*, which was observed in historic eastern Canadian wolves and the captive red wolf program. Wayne's hypothesis is that gray wolves and coyotes hybridized to form the red wolf. The support for this hypothesis was the absence in red wolves of distinct genetic markers not found in coyotes or gray wolves. We have identified a group of mtDNA control region sequences more closely related to coyotes than gray wolves that are specific to the red wolf and the eastern Canadian wolf (Fig. 5). The mtDNA data support the microsatellite data that indicates a close relationship between the red wolf, *C. rufus*, and eastern Canadian wolf, *C. l. lycaon*. Furthermore, the absence of gray wolf mtDNA and the distribution of assignment test scores away from the gray wolf distribution in captive red wolves and Eastern Canadian wolves support the evolution of a small North American wolf independent of the gray wolf. The data presented leads to the formal rejection of the hypothesis that the red wolf and the eastern Canadian wolf are hybrids of coyotes and gray wolves. Furthermore, we also reject the hypothesis that the eastern Canadian wolf is a sub-species of the gray wolf. At present the red wolf exists as the species *C. rufus*, however, based on historical taxonomic classifications, the eastern North American wolves would require the classification *C. lycaon*.

Assuming the proposed taxonomic revision is accepted, our findings have broader biological, ecological and conservation implications. The present range of the North American-evolved eastern Canadian wolf likely includes northwestern Ontario, Minnesota and Manitoba. These areas may contain two different species of wolves, the eastern Canadian wolf and the gray wolf and it is presently unclear to

what extent these two wolves might interbreed. What is now considered a single population of gray wolves may be two sympatric species or hybrid canids. We are presently examining the amount of inter-breeding between *C. lupus* and *C. lycaon*. Conservation of wolves in North America is dependent on an assessment of population sizes and this can only be made when the species are clearly identified.

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Figure 1. Neighbor-joining tree of Nei's genetic distances for allele frequencies from eight microsatellite loci. With the exception of the Algonquin Provincial Park population, the source of the allele frequencies are from Roy et al. (1994, 1996) ($_1$). Two of ten dinucleotide microsatellite loci, i.e. cxx 344 and 213, from Roy et al. (1994, 1996) were excluded based on our observation of the presence of 1 base pair allele differences not found previously. As a result of the number of alleles differing by one base pair at these two loci, we excluded them from the analysis. Bootstrap values are provided for nodes that were observed in greater than 50% of 1000 bootstrapped data sets. From the 1000 bootstrap re-samplings of the data, Algonquin and captive red wolves were grouped together in 72.4% of trees.

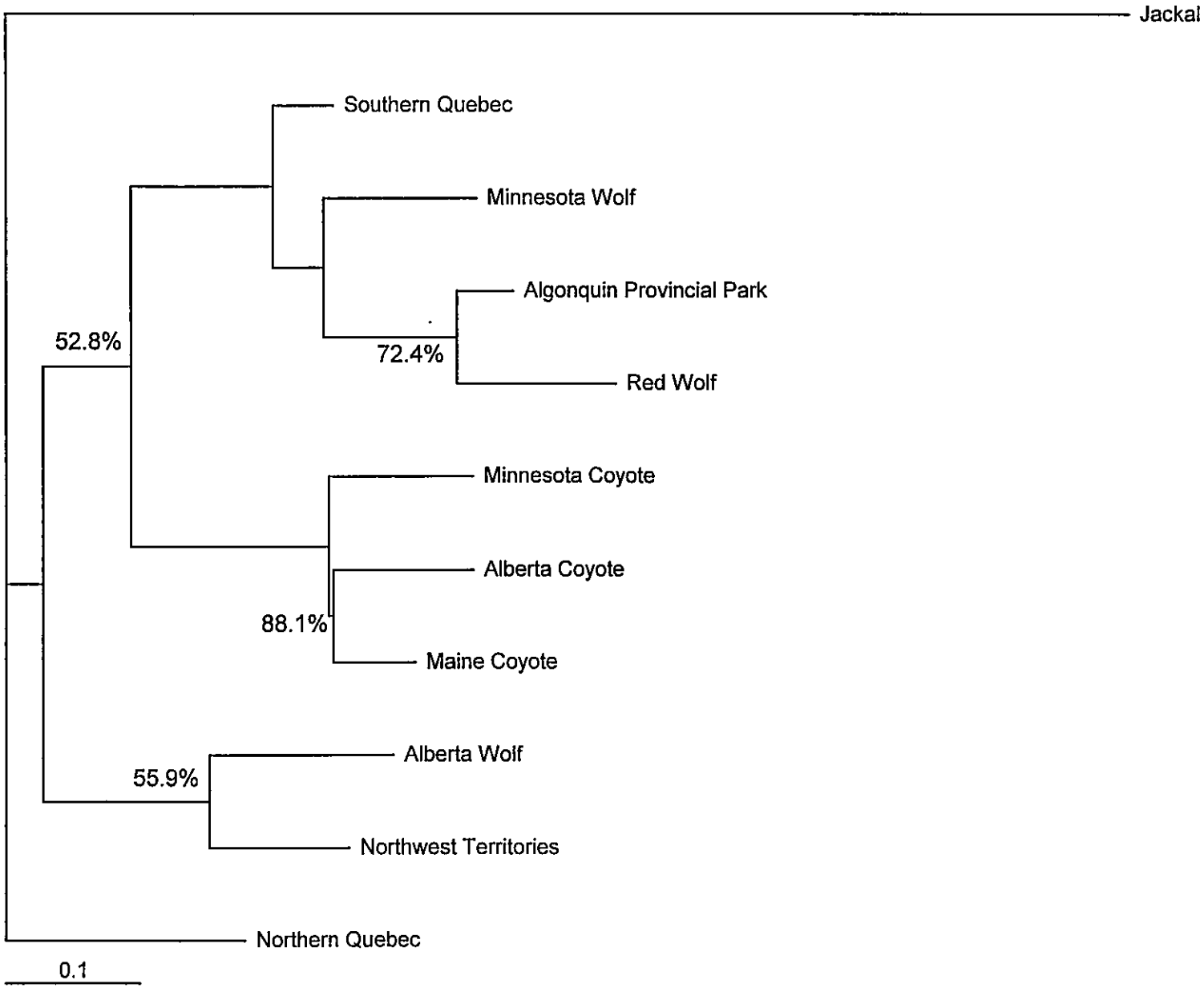

Jackal
Southern Quebec
Minnesota Wolf
Algonquin Provincial Park
52.8%
72.4%
Red Wolf
Minnesota Coyote
Alberta Coyote
88.1%
Maine Coyote
Alberta Wolf
55.9%
Northwest Territories
Northern Quebec
0.1

Figure 2. Neighbor-joining tree of Nei's genetic distances (1972) for allele frequencies from eight microsatellite loci for Eastern Canadian wolves, gray wolf populations and a Texas coyote population. Bootstrap values are provided for nodes that were observed in greater than 50% of 1000 bootstrapped data sets. From the 1000 bootstraps re-sampling the data the Algonquin Park and captive red wolf population were grouped together in 67.8% trees. The neighbor-joining tree gave an approximation of the genetic relationship among these populations and alternative topologies are possible.

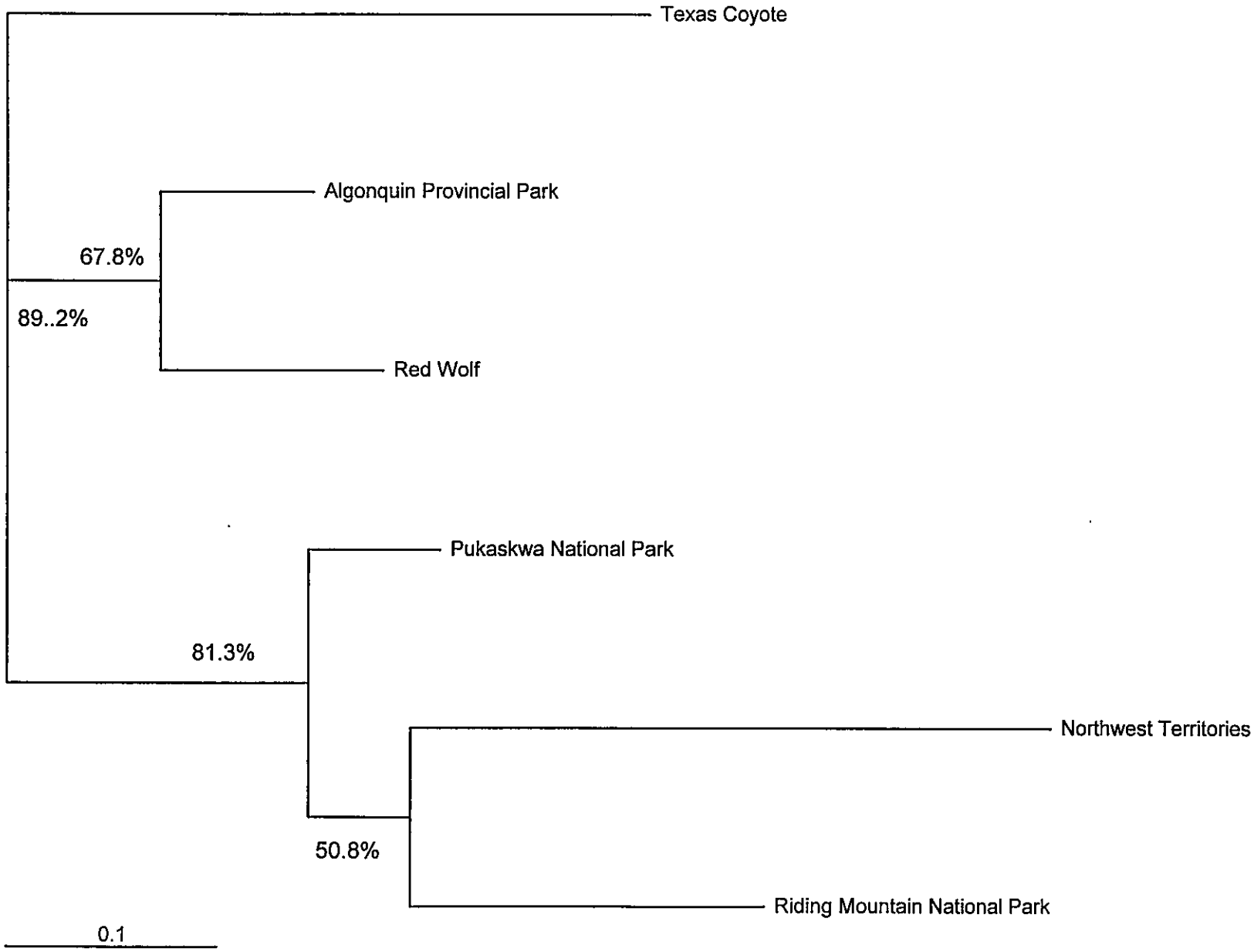
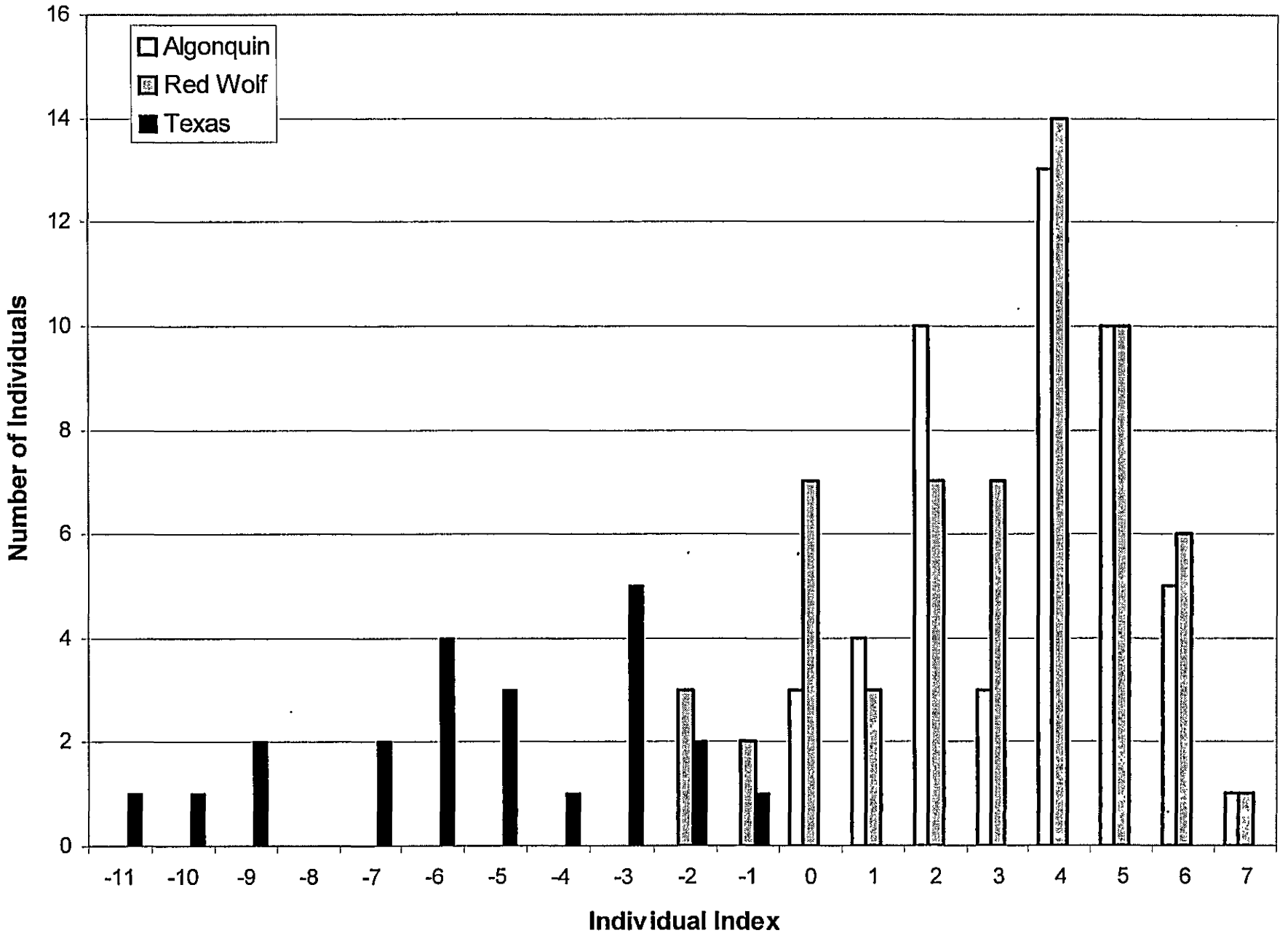

Texas Coyote
Algonquin Provincial Park
67.8%
89..2%
Red Wolf
Pukaskwa National Park
81.3%
Northwest Territories
50.8%
Riding Mountain National Park
0.1

Figure 3. A. Log-likelihood individual indices (I_I) from captive red wolves (n=60) and canids from Algonquin Park (n=49) and Texas (n=22). The I_I were calculated for each individual animal DNA profile at 8 microsatellite loci using the allele frequencies from the Algonquin Park population and Texas coyote population, respectively. **B.** A plot of the log Probability of Identity (POI) values from captive red wolves (n=60) and wolves from Algonquin Park (n=49) and Texas (n=22) using the allele frequencies from the Algonquin Park population and Texas coyote population, respectively.


Algonquin
Red Wolf
Texas
Number of Individuals
0
2
4
6
8
10
12
14
16
-11
-10
-9
-8
-7
-6
-5
-4
-3
-2
-1
0
1
2
3
4
5
6
7
Individual Index

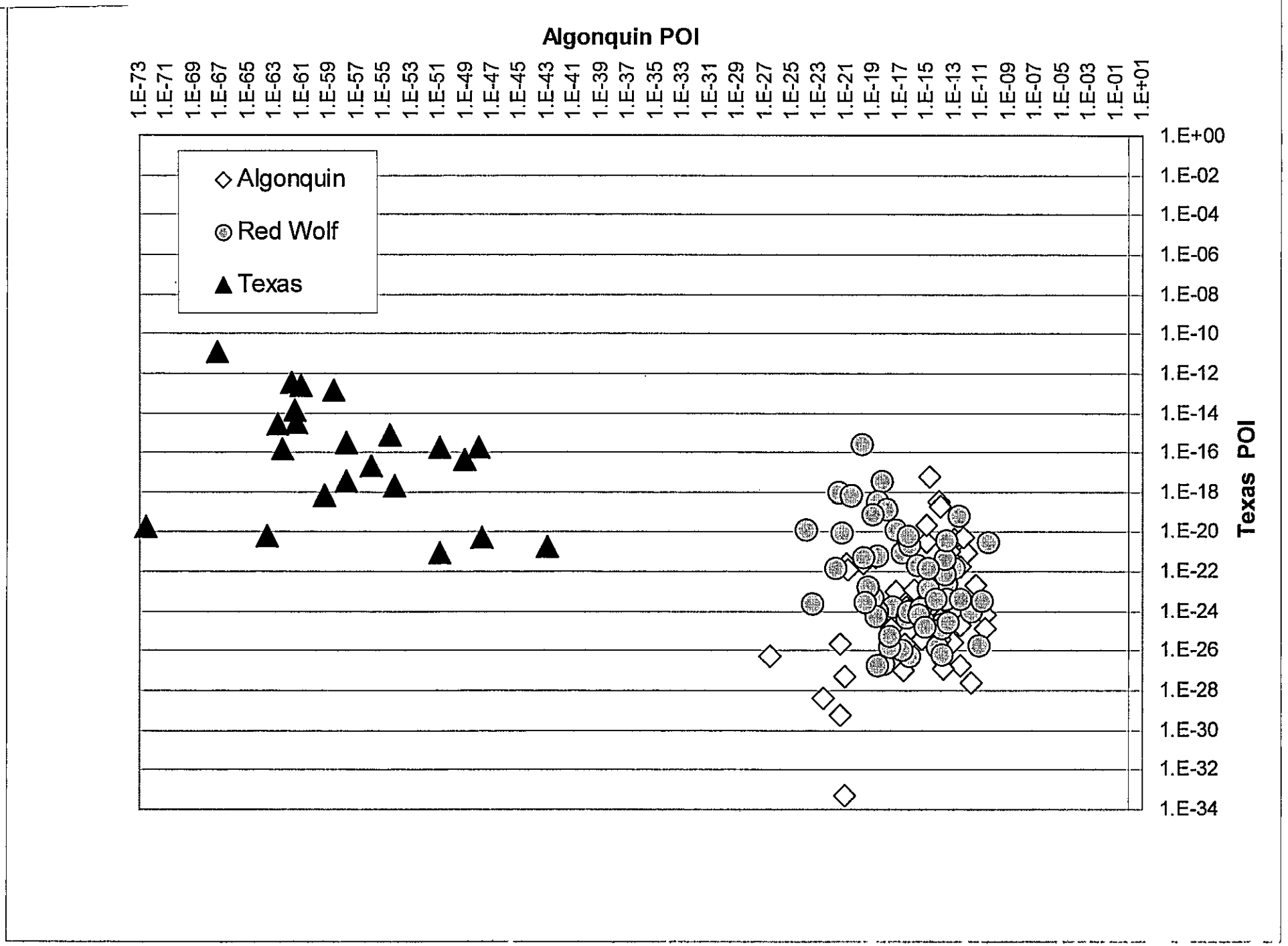
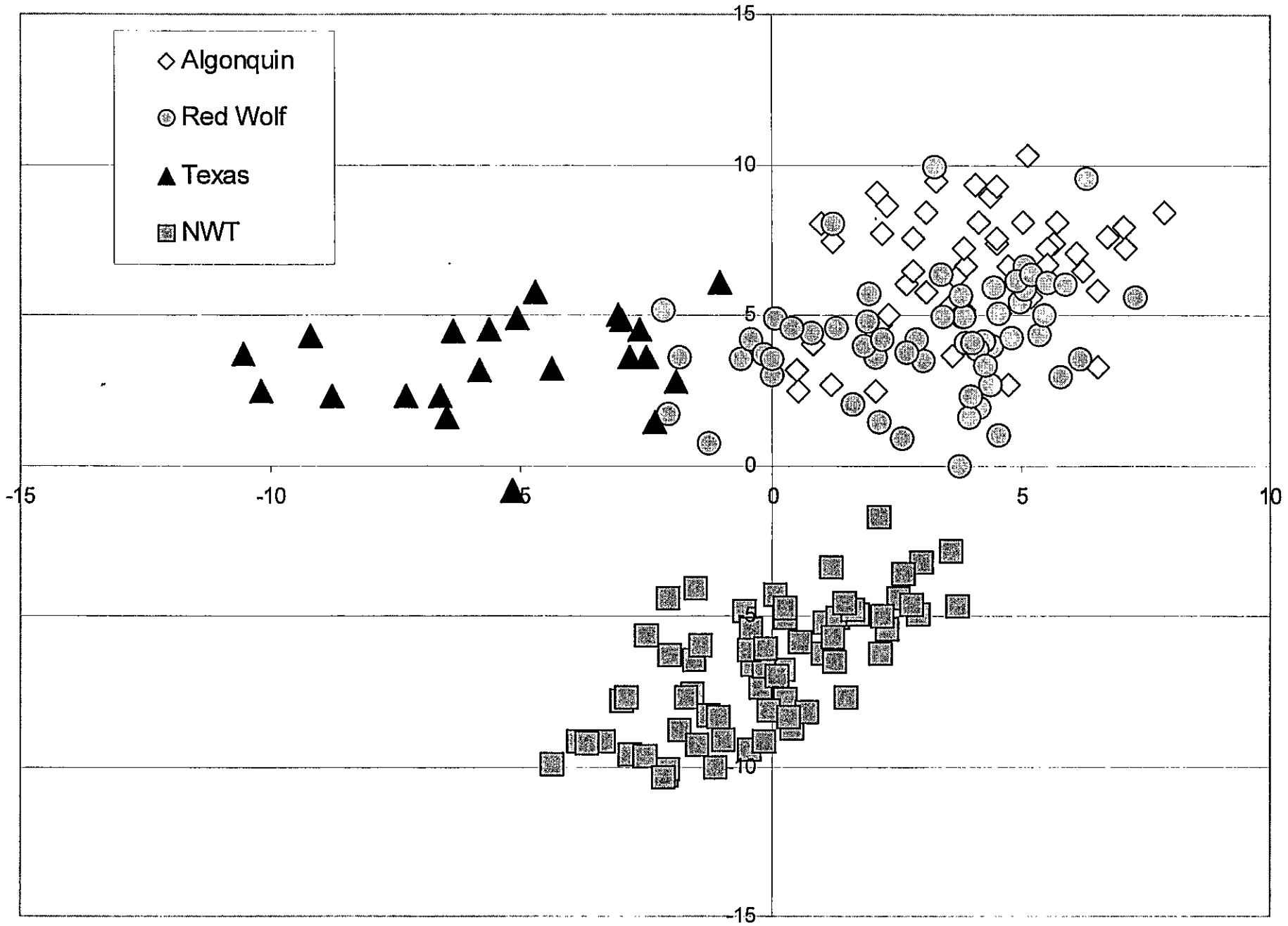

Algonquin POI
1.E-73
1.E-71
1.E-69
1.E-67
1.E-65
1.E-63
1.E-61
1.E-59
1.E-57
1.E-55
1.E-53
1.E-51
1.E-49
1.E-47
1.E-45
1.E-43
1.E-41
1.E-39
1.E-37
1.E-35
1.E-33
1.E-31
1.E-29
1.E-27
1.E-25
1.E-23
1.E-21
1.E-19
1.E-17
1.E-15
1.E-13
1.E-11
1.E-09
1.E-07
1.E-05
1.E-03
1.E-01
1.E+01
Algonquin
Red Wolf
Texas
1.E+00
1.E-02
1.E-04
1.E-06
1.E-08
1.E-10
1.E-12
1.E-14
1.E-16
1.E-18
1.E-20
1.E-22
1.E-24
1.E-26
1.E-28
1.E-30
1.E-32
1.E-34
Texas POI

Figure 4. A. Plot of log-likelihood individual indices (I_I) from captive red wolves (n=60) and wolves from Algonquin Park (n=49), Northwest Territories (n=67) and Texas (n=20). The I_I were calculated for each individual animal DNA profile at 8 microsatellite loci using the allele frequencies from the Northwest Territories wolf population and Texas coyote population, respectively. **B.** A plot of the log of Probability of Identity (POI) values from captive red wolves (n=60) and wolves from Algonquin Park (n=49), Northwest Territories (n=67) and Texas (n=22) using the allele frequencies from the Algonquin Park population and Northwest Territories population, respectively.


Algonquin
Red Wolf
Texas
NWT
15
10
5
0
-5
-10
-15
-15
-10
-5
0
5
10

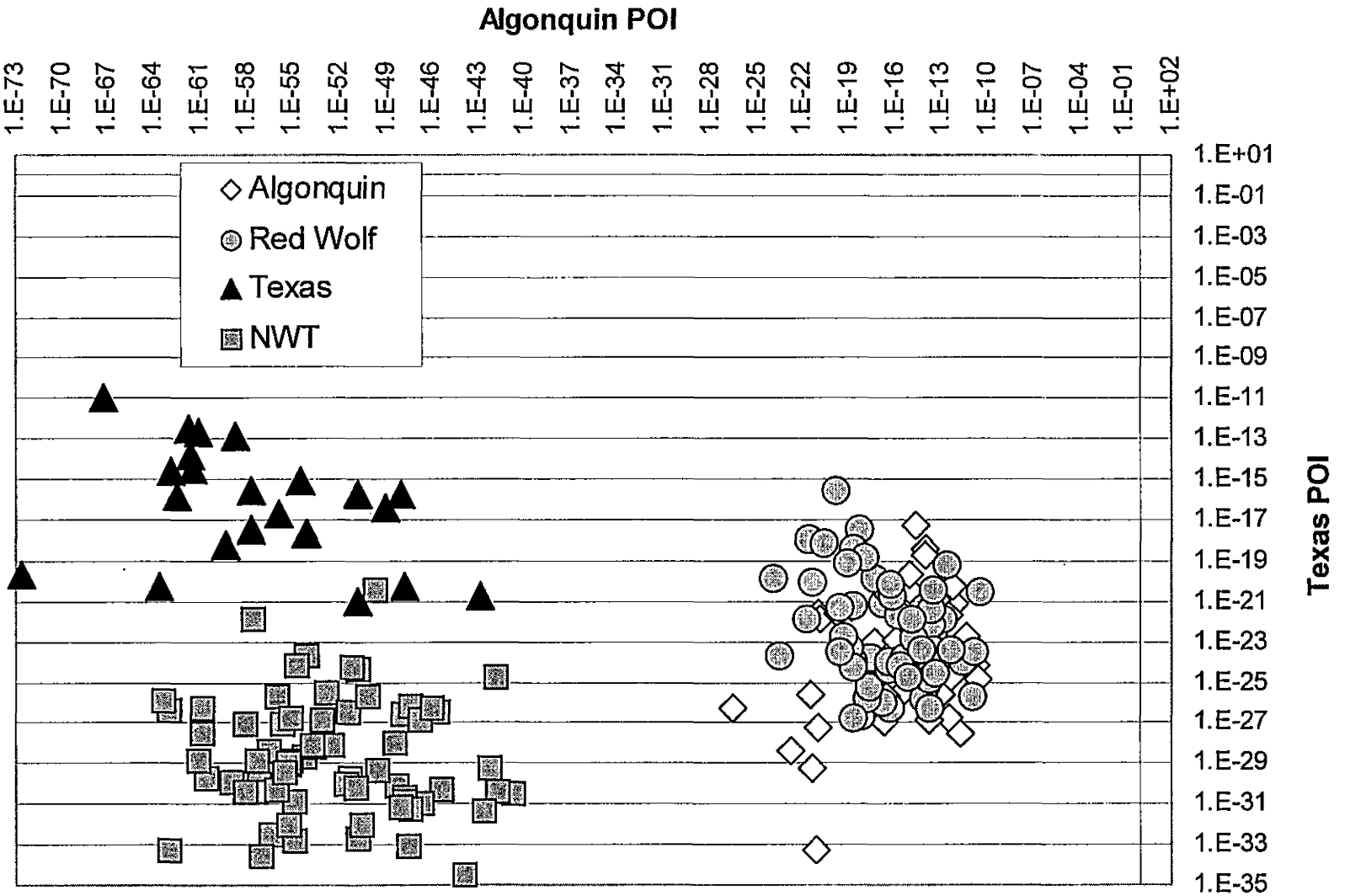
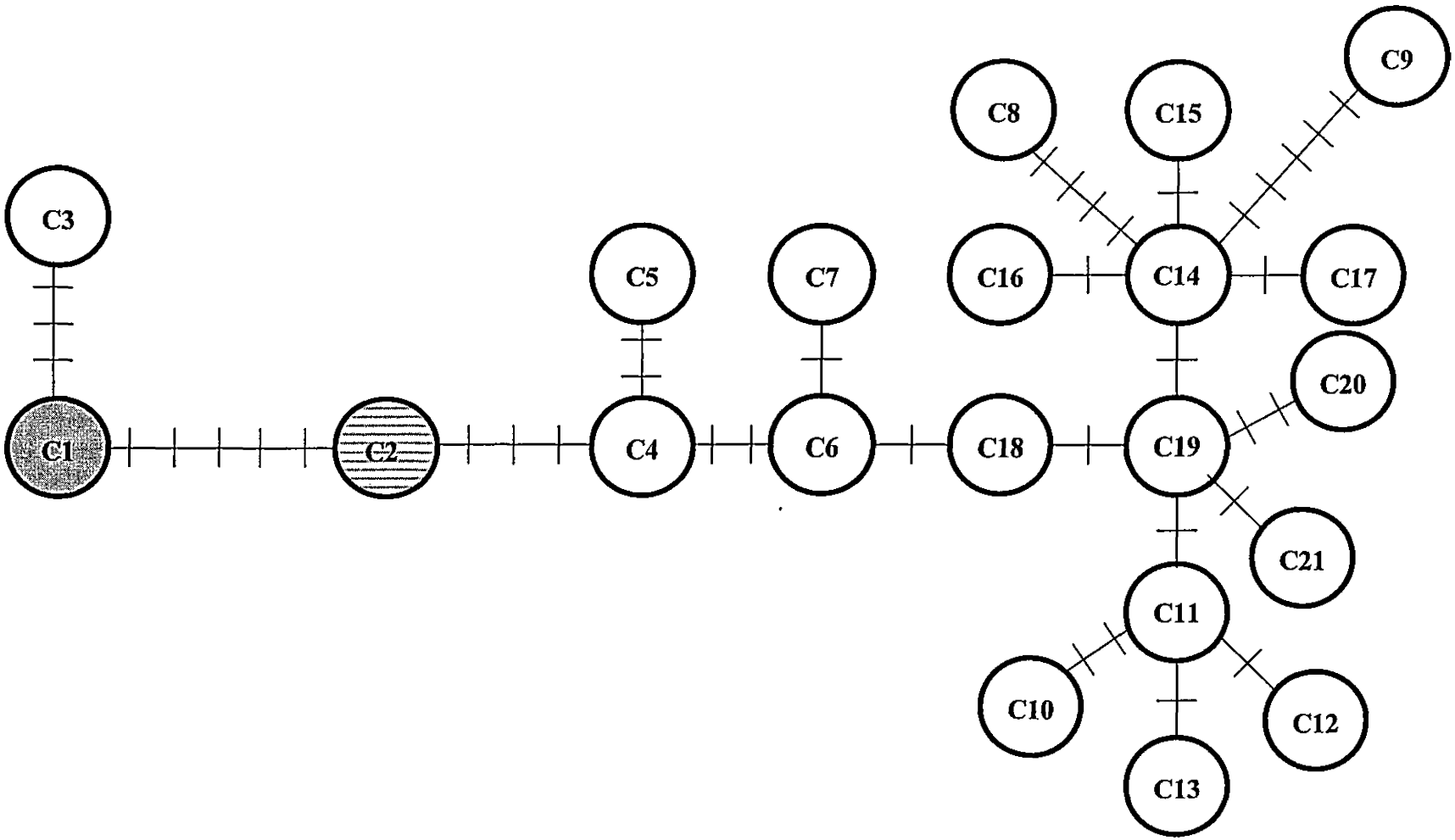

Algonquin POI
1.E-73
1.E-70
1.E-67
1.E-64
1.E-61
1.E-58
1.E-55
1.E-52
1.E-49
1.E-46
1.E-43
1.E-40
1.E-37
1.E-34
1.E-31
1.E-28
1.E-25
1.E-22
1.E-19
1.E-16
1.E-13
1.E-10
1.E-07
1.E-04
1.E-01
1.E+02
Algonquin
Red Wolf
Texas
NWT
1.E+01
1.E-01
1.E-03
1.E-05
1.E-07
1.E-09
1.E-11
1.E-13
1.E-15
1.E-17
1.E-19
1.E-21
1.E-23
1.E-25
1.E-27
1.E-29
1.E-31
1.E-33
1.E-35
Texas POI

Figure 5. A. Minimum-spanning tree for 238 b.p. of control region haplotypes from red wolf, eastern Canadian wolf and coyote. Sequences obtained from this study are labeled with a C designation, i.e. Canis-1 (C1). Gray shaded haplotypes indicate haplotypes found in eastern Canadian wolves (lycaon) and striped haplotypes indicate red wolf (rufus) haplotypes. Dashes between haplotypes indicate the number of base pair substitutions or insertion/deletions. **B.** Neighbor-joining tree of sequence divergence for 238 b.p. of gray wolf, red wolf, eastern Canadian wolf and coyote control region haplotypes. The lycaon/rufus lineage has two nucleotides in the mtDNA control region common with *C. lupus* but different from *C. latrans* which accounts for the proximity of *C. lupus* mtDNA to the lycaon/rufus haplotypes. The scale represents 0.100 or 10.0% sequence divergence. Bootstrap values are provided for nodes that were observed in greater than 50% of 1000 bootstrapped data sets. European wolf haplotypes (W1-W4) (Ellegren 1996) are provided. Sample locations and corresponding haplotypes are as follows: red wolf captive breeding program (C2, n=9, C19, n=3); Algonquin Park and surrounding areas (c. 1960's) (C1, n=7; C9, n=1; C14, n=3; C17, n=1; C19, n=1); southern Ontario (c. 1960's) (C1, n=1; C9, n=1; C14, n=2, C19, n=4); north of Algonquin Park (c. 1960's) (C1, n=1; C16, n=1, C23, n=1); northern boreal region of Ontario (c. 1960's) (C23, n=1); northwestern Ontario (c. 1960's) (C13, n=2; C24, n=1); Manitoba (C3, n=1; C22, n=1; C23, n=1); Ohio (C5, n=1); Texas (C4, n=1, C6, n=2; C7, n=1; C8, n=1; C10, n=1; C11, n=1; C12, n=1; C15, n=1; C18, n=2; C19, n=12; C20, n=2; C21, n=2); northern Quebec (C23, n=1), NWT (C23, n=1), Fort Francis, Ontario (C23, n=1).


C1
C2
C3
C4
C5
C6
C7
C8
C9
C10
C11
C12
C13
C14
C15
C16
C17
C18
C19
C20
C21

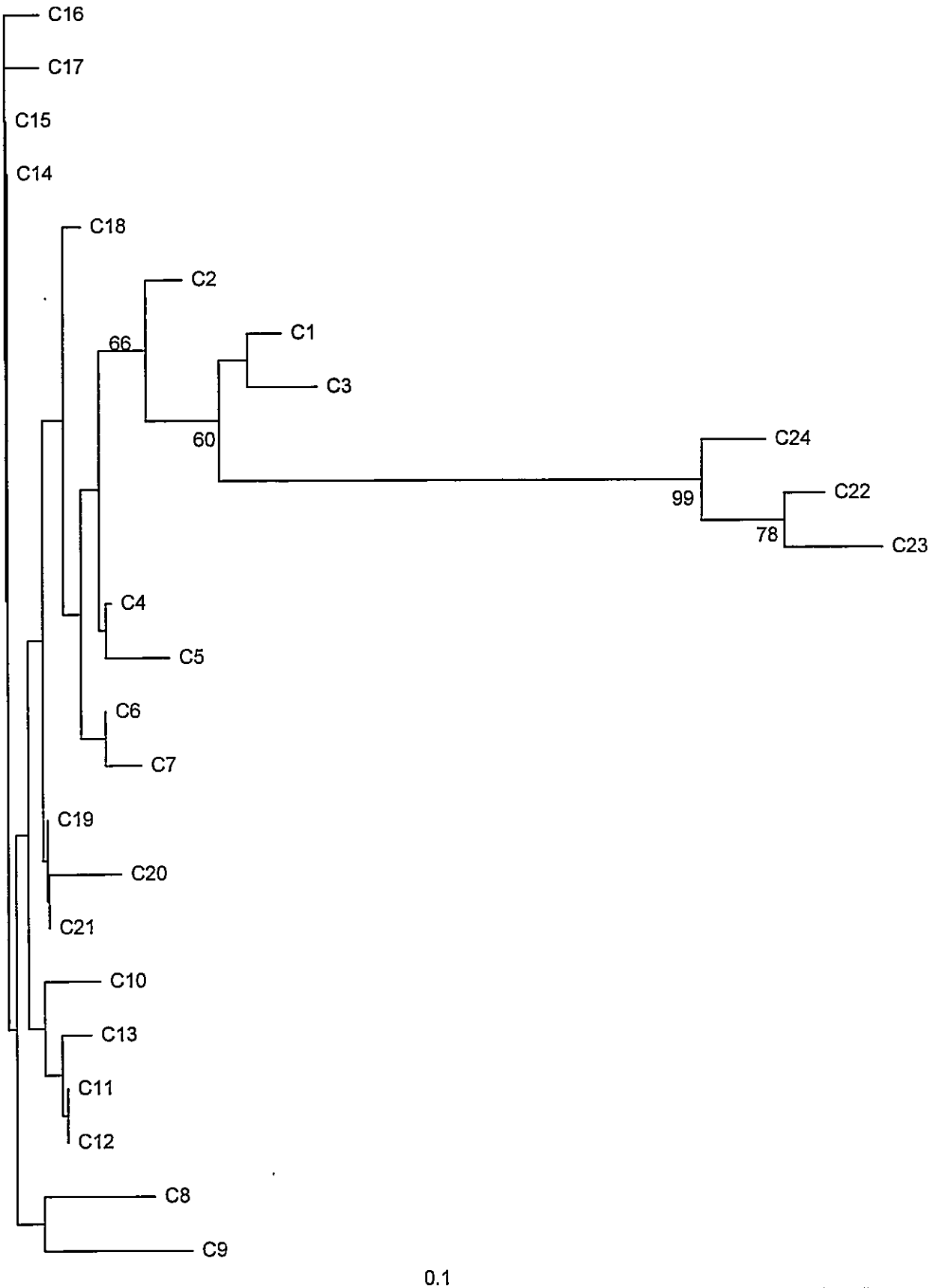

C16
C17
C15
C14
C18
C2
C1
66
C3
60
C24
99
C22
78
C23
C4
C5
C6
C7
C19
C20
C21
C10
C13
C11
C12
C8
C9
0.1

Figure 6. A model for the evolution of North American wolves. The progenitor to *C. lupus*, *C. lycaon* and *C. latrans* is indicated at the top. Divergence from this ancestor is generally accepted to have occurred 1-2 million years ago when the progenitor of *C. lupus* migrated to Eurasia. The North American species diverged 150,000-300,000 years ago into the eastern Canadian wolf /red wolf (*C. lycaon*) and the coyote (*C. latrans*). Recently, *C. lycaon* and *C. latrans* have come into contact and have subsequently hybridized. The Eurasian-evolved *C. lupus* returned to North America within the Pleistocene.

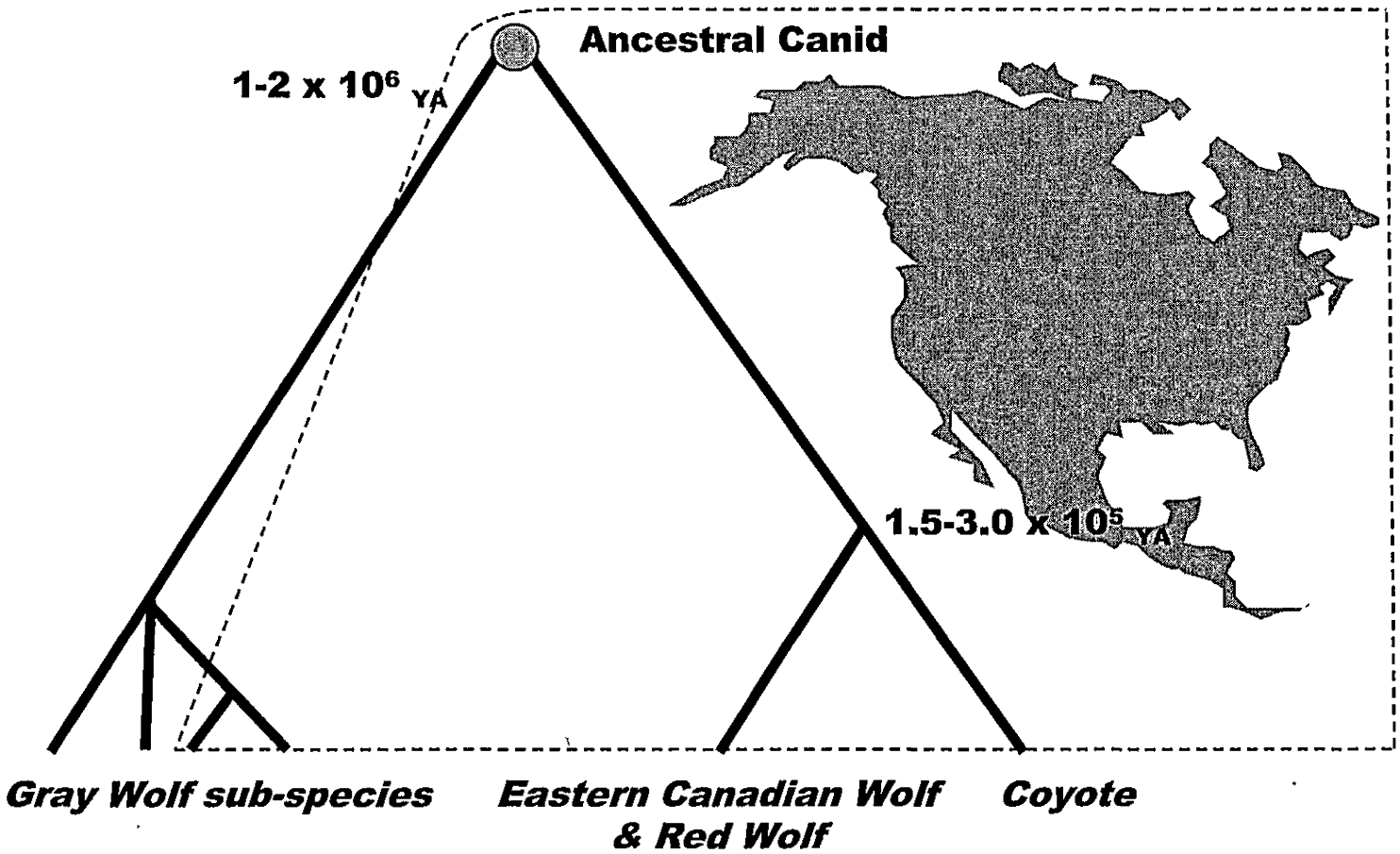

Ancestral Canid
1-2 x 10^6 YA
1.5-3.0 x 10^5 YA
Gray Wolf sub-species
Eastern Canadian Wolf & Red Wolf
Coyote

Table 1. Alleles prevalent in Texas coyotes and other coyote populations that are absent or present at low frequency among captive red wolves. Loci and allele designations and the first red wolf column have been previously described (Roy et al. 1996).

Locus	Allele	Texas coyote	Red wolf (Roy et al. 1996)	Red wolf (this study)
Cxx 225	B	0.239	0.000	0.000
Cxx 225	C	0.500	0.109	0.050
Cxx 109	C	0.395	0.000	0.050
Cxx 172	I	0.167	0.067	0.000
Cxx 250	I	0.348	0.016	0.050
Cxx 123	I	0.146	0.000	0.000
Cxx 123	J	0.104	0.000	0.000

Chapter 6

Mitochondrial DNA extracted from eastern North American wolves killed in the 1800s is not of grey wolf origin.

Mitochondrial DNA extracted from eastern North American wolves killed in the 1800s is not of grey wolf origin.

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ABSTRACT

We have analysed the mitochondrial DNA from two historic eastern North American wolf samples, the last reported wolf killed in northern New York c.1893 and a wolf killed in Maine in the 1880s. These wolves represent eastern wolves, presently classified as the grey wolf sub-species, *Canis lupus lycaon*, which were present well before the expansion of western coyotes into these regions. Here we show the absence of grey wolf mitochondrial DNA (mtDNA) in these wolves. They both contain New World mitochondrial DNA, supporting a North American evolution of the eastern timber wolf (originally classified as *C. lycaon*) and the red wolf (*C. rufus*) independent of the grey wolf that originated in Eurasia. The presence of a second wolf species in North America has important implications for the conservation and management of wolves. In the upper Great Lakes region wolves of both species may be existing in sympatry or interbreeding with each other which impact has impacts on the accuracy of estimates of numbers of wolves of each species within this geographic region. Furthermore the historic distribution of the eastern timber wolf (*C. lycaon*), as revealed by these skin samples, has important implications on the re-introduction of wolves into the northeastern US states, such as New York and Maine.

INTRODUCTION

All of eastern North America was historically inhabited by wolves: the eastern timber wolf (also known as the eastern Canadian wolf), currently designated a subspecies of grey wolf (*C. l. lycaon*) in the north, and the red wolf (*C. rufus*) in the south. Both of these animals are smaller than the grey wolves found in more western and northern regions and taxonomically distinct from western coyotes (*C. latrans*) (Lawrence and Bossert 1967, Lawrence and Bossert 1975, Mech 1971, Nowak 1979, Nowak 1995, Schmitz and Kolenosky 1985). Hybridisation has been identified between eastern timber wolves and coyotes (Lawrence and Bossert 1967, Lawrence and Bossert 1975, Mech 1971, Schmitz and Kolenosky 1985, Lehman et al. 1991, Wayne and Lehman 1992, Roy et al. 1994), and it has been suggested that *C. rufus* is not a valid species but the result of recent extensive hybridisation between grey wolves and coyotes (Wayne and Jenks 1991, Roy et al. 1996).

Recent genetic evidence demonstrates a close relationship between the eastern timber wolf and the red wolf consistent with both being North American-evolved species (Wilson et al. *in press*). A study of the mitochondrial DNA (mtDNA) control region of eastern wolves present in Ontario in the 1960s and captive red wolves identified DNA sequences that were 150,000-300,000 years divergent from those found in western coyotes. These sequences and DNA profiles at microsatellite loci have led to the proposal that the eastern wolf diverged from the western coyote at about that time. It is generally accepted that the grey wolf, *C. lupus*, evolved in Eurasia (Old World) and returned to North America approximately 300,000 years ago and is 1-2 million years diverged from the coyote, *C. latrans*, (Kurten and Anderson 1980; Wayne 1993, Vila et al. 1997).

In order to test the hypothesis for a North American-evolved wolf, we analysed samples from wolves that were present in eastern North America prior to the arrival of western coyotes. The first prediction is that if the eastern timber wolf is truly a subspecies of the grey wolf then the mtDNA, prior to the arrival of coyotes, would be *C. lupus* in origin. The second prediction is that if the eastern timber wolf is a North American-evolved wolf, more closely related to the coyote, the mtDNA sequences would be closely related to *C. latrans* as a result of evolutionary history and not the result of recent hybridisation. Two historic skin samples collected from New York (c. 1983) and Maine (c. 1880s) provided by the

Adirondack Museum and the Museum of Comparative Zoology, Harvard University, respectively, were used to test this hypothesis. Coyote-like animals were not reported in New York until the 1920s and the 1930s in Maine.

MATERIALS & METHODS

Sample Collection

The Maine historic sample (1880s) (Harvard Museum Accession Number 50518) had been reported as a wolf collected by bounty in Penobscot County Maine State. The historic New York sample (c. 1893) from the Adirondack Museum has been described as the last wolf killed within the State. The mounted New York wolf demonstrates a wolf-like morphology based on observation. Both hide samples were extracted using a modified QIAmp (Qiagen) extraction protocol using a lysis buffer 1x lysis buffer (4 M urea, 0.2 M NaCl, 0.5% n-lauroyl sarcosine, 10 mM CDTA (1,2-cyclohexanediamine), 0.1 M Tris-HCl pH8.0).

Control region Sequencing and Sequence Analysis

The following primers were used to amplify the control region of the mitochondrial DNA.

Primer 1 5'-GAA GCT CTT GCT CCA CCA ATC-3'

Primer 2 5'-GGG CCC GGA GCG AGA AGA GGG AC-3'

The control region was amplified in a total reaction volume of 20µl per tube using 25ng of genomic DNA, 200 µM dNTPs, 1x amplification buffer, 2.0 mM $MgCl_2$, primers 1 and 2 (0.2 mM) and 0.5 units of *Taq* polymerase (BRL). Products were amplified under the following conditions: 94°C for 5 min., 55°C for 30 sec., 72°C for 30 sec. 1 cycle; 94°C for 30 sec., 55°C for 30 sec., 72°C for 30 sec. 35 cycles; 94°C for 30 sec., 55°C for 30 sec., 72°C for 2 min. 1 cycle. Products were re-amplified and purified through QIAquick (Qiagen) for DNA sequencing using dye-terminator cycle sequencing using an ABI Prism 373 DNA Sequencer. The phylogenetic relationships of canid mtDNA haplotypes were generated using a neighbor-joining tree with Kimura's two-parameter DNA distance (2:1 transition-to-transversion ratio) using the PHYLIP programs DNADIST and NEIGHBOR (Felsenstein 1993).

RESULTS & DISCUSSION

The Maine sample contained a mtDNA with a sequence that clustered with previously identified eastern wolf and red wolf-specific mtDNA sequences (Fig. 1). The mtDNA control region of the New York hide sample did not cluster with this group (Wilson et al. *in press*), but with sequences found in modern western coyotes (Fig. 1). The presence of a coyote-like sequence in the New York wolf suggests there may have been contact between coyotes and eastern wolves during the Pleistocene, following the their original separation 150,000-300,000 years ago (Wilson et al. *in press*). The absence of grey wolf mtDNA in eastern wolves prior to the expansion of western coyotes into New York and Maine does not support its present classification as a subspecies of grey wolf (*C. lupus lycaon*). The presence of a sequence similar to that found in modern eastern and red wolves is consistent with the hypothesis of a parallel evolution of a North American wolf and its original classification as *C. lycaon* (Brewster and Fritts 1995).

The original presence of a North American-evolved wolf in large areas of Canada and the U.S. has significant conservation ramifications for management programs and proposed re-introduction efforts. Assuming the proposed taxonomic revision is accepted, the present range of the North American-evolved eastern timber wolf likely includes northern Ontario, Quebec, Michigan Minnesota, Manitoba and Wisconsin. These areas may contain two different species of wolves, the eastern timber wolf and the grey wolf and it is presently unclear to what extent these two wolf species might interbreed. What is now considered a single population of grey wolves may be two sympatric species or hybrid canids. We are presently examining the levels of inter-breeding between *C. lupus* and *C. lycaon*. Conservation and management of wolves in North America is dependent on an assessment of population sizes and this can only be made when the species are clearly identified.

The presence of the eastern timber wolf, *C. lycaon*, within Maine and New York in the 1880s has implications on re-introduction efforts as to the specific wolf species to move into these states. The genetic evidence supports the re-introduction of the eastern timber wolf based on historic distribution. However, the feasibility of this will depend on the risk of hybridisation with the existing eastern coyote, representing

a *C. lycaon* x *C. latrans* hybrid (Paquet et al. 1999, Wilson et al. submitted). The end result of hybridisation between re-introduced eastern timber wolves and the inhabiting eastern coyotes will ultimately be the already present *C. lycaon* x *C. latrans* hybrid. Alternative consideration may be given to introducing predominantly *C. lycaon* animals that have been impacted by interbreeding with *C. lupus* from regions in Ontario and Quebec – an animal that may be less likely to hybridise with coyotes. Certainly as only two historic samples from these states have been analysed to date, other historic specimens may reveal the presence of grey wolves, *C. lupus*, within areas such as the north woods of Maine suggesting their potential re-introduction into these regions.

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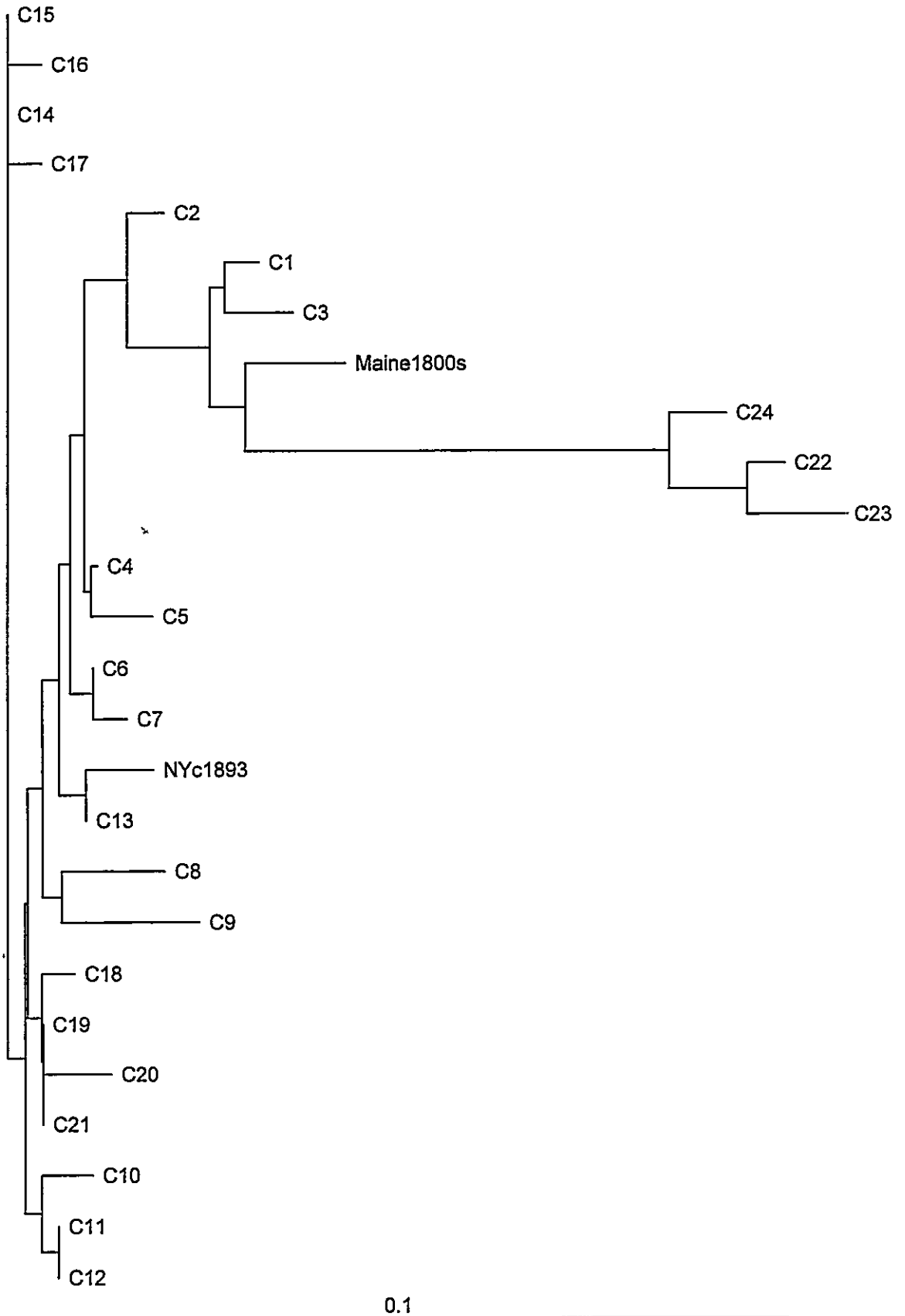
Figure 1. A neighbor-joining tree of Kimura's two-parameter DNA distance measure (2:1 transition-to-transversion ratio) for an approximately 238 b.p. region of coyote, grey wolf, eastern Canadian wolf and red wolf control region haplotypes. European wolf haplotypes (Ellegren et al. 1997) (W1-W4) and haplotypes Canis-24-26 represent grey wolf mtDNA and haplotypes Canis 5-23 represent sequences found in Texas coyotes as well as eastern timber wolves and red wolves. Canis 1-4 haplotypes were found in historic eastern timber wolves (1960s) and captive red wolves (Wilson et al. *in press*).


C15
C16
C14
C17
C2
C1
C3
Maine1800s
C24
C22
C23
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C5
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NYc1893
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C20
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0.1

Chapter 7

Genetic evidence for the origin of the eastern coyote by hybridisation between western coyotes and the eastern timber wolf.

Genetic evidence for the origin of the eastern coyote by hybridisation between western coyotes and the eastern timber wolf.

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ABSTRACT

The eastern coyote has been described as a larger form of its western counterpart and there has been considerable debate over the role that hybridisation with wolves has had on its origin. Previous studies

have demonstrated that the "Tweed wolves" in the Frontenac Axis region of Ontario are hybrids between the eastern Canadian wolf and the western coyotes. The origin of three eastern coyote populations, from Cortlandville, New York, Adirondack State Park, NY, and Kouchibouguac National Park, New Brunswick, was assessed using DNA profiles at 8 microsatellite loci and the mitochondrial DNA control region. Assignment indices (I_I), using microsatellite loci allele frequencies from eastern Canadian wolves, coyotes and "Tweed wolves", were consistent with eastern coyotes being hybrids. The I_I scores for red wolf/coyote hybrids from the Alligator River Refuge red wolf re-introduction program further supported the hybrid origin of eastern coyotes. In addition the eastern coyote populations contained an allele at one microsatellite locus that was common in eastern Canadian wolves but absent in Texas coyotes. There were no gray wolf/dog mtDNA haplotypes in "Tweed wolves" and eastern coyote populations but there were sequences found only in eastern Canadian wolves and western coyotes. The nuclear and mitochondrial DNA profiles support the origin of eastern coyotes through symmetrical mating between western coyotes and eastern Canadian wolves. The presence of a large coyote/wolf hybrid in eastern North America has implications for wolf management and the proposed re-introduction of eastern Canadian wolves into northeastern US states.

INTRODUCTION

The coyote (*Canis latrans*) has expanded its geographic range over North America significantly over the past 150 years in response to human activities and the reduction of wolf numbers throughout the United States and Canada (Wayne *et al.* 1992, Moore and Parker 1992). Following a 40-year period during which few, if any, large wild canids were known to occur in New York, a coyote-like animal was reported in the St. Lawrence Valley area in 1920. Reports of large coyote-like animals continued to increase in the early 1930s in Ontario and as early as 1936 in Maine. Coyotes were considered to be common in the Adirondacks of New York and New England during the 1950s and by 1970s these animals had extended their range across southeastern Canada and the northeastern states, even reaching Newfoundland in 1987 (Moore and Parker 1992).

This large coyote-like canid has been described as the "eastern coyote" and has been considered intermediate between western coyotes and gray wolves in body size and skull characteristics (Gaskin 1975, Lawrence and Bossert 1975, Nowak 1979). In addition, the behaviour of eastern coyotes has been described as more aggressive than western coyotes (Silver and Silver 1969) with the highest percentage of the diet being white-tailed deer (*Odocoileus virginianus*) at levels between 75-90% (Chambers 1987, Messier *et al.* 1986, Pekins 1992).

Four main hypotheses have been proposed to account for the larger size and intermediate wolf-like characteristics of the eastern coyote. First, the eastern coyote represents a "coydog" resulting from dog (*C. l. familiaris*) and coyote interbreeding (Mengel 1971). Secondly, the western coyote has undergone selection for a larger body size as a response to adapting to larger prey, specifically white-tailed deer (Kolenosky and Stanfield 1975, Schmitz and Kolenosky 1985, Schmitz and Lavigne 1987) within its eastern range. Thirdly, the eastern coyote is a phenotypic response to enhanced food supply in the eastern range of coyotes (Thurber and Peterson 1991). Lastly, the eastern coyote is the result of hybridisation between gray wolves (*C. lupus*) and coyotes (Silver and Silver 1969, Mengel 1971, Lawrence and Bossert 1975, Schmitz and Kolenosky 1985, Nowak 1979, Lariviere and Crete 1993).

There appears to be little evidence for successful coyote/dog inter-breeding resulting in hybrid coydog populations. Breeding experiments between coyotes and dogs (Silver and Silver 1969, Mengel

1971) revealed breeding asynchrony between coyotes and the F1 hybrids, little or no parental care from male coydog, and a high degree of congenital deformities. It was the conclusion of these authors that viable coydog populations were not likely under natural conditions in the wild. Recent genetic evidence suggests that even wolves and dogs, which are the same species (*C. lupus*), do not readily breed under natural conditions (Vila and Wayne 1999). One additional line of evidence against the viability of natural coydog populations is based on geography as the larger morphology of the coyote is observed in specific eastern regions of North America. One would predict the ubiquitous presence of dogs throughout the US and Canada could result in similar hybridisation elsewhere.

There has been some debate over the hypothesis of adaptation to larger prey size and the hypothesis of phenotypic response to food supply (Kolenosky and Stanfield 1975, Schmitz and Kolenosky 1985, Schmitz and Lavigne 1987, Thurber and Peterson 1991, Lariviere and Crete 1993). The debate stems from the role that genetic selection may have on the morphology of the eastern coyote and whether hybridisation with wolves is necessary to explain the increased size of eastern coyotes. Laviviere and Crete (1993) identify a number of critical points in the approach taken by Thurber and Peterson (1991) in their suggestion that food supply in the absence of genetic selection can account for the large size observed in the eastern coyote. One point of agreement in the debate is the need to apply genetic markers to confirm or refute the presence of wolf genetic material in the eastern coyote.

Previous genetic analysis of wolves in Minnesota and eastern Canada (Lehman *et al.* 1991, Wayne and Lehman 1992, Roy *et al.* 1994) suggested that wolves and coyotes hybridised in these regions. One of their conclusions was that hybridisation did not affect coyote populations, because the introgression of genetic material was only from coyote to gray wolf. Genetic results have also been used to propose that the origin of the red wolf is the result of hybridisation of gray wolves (*C. lupus*) and coyotes (*C. latrans*) (Wayne and Jenks 1991, Roy *et al.* 1994, Roy *et al.* 1996). Recent genetic evidence supports a close evolutionary history between the eastern Canadian wolf (presently *C. l. lycaon*) and the red wolf (*C. rufus*) that is independent of the gray wolf (Wilson *et al. in press*) suggesting the eastern wolves represent a separate species. Under this model, the eastern wolves evolved in North America and shared a common ancestor with coyotes 150,000-300,000 years ago with both *C. l. lycaon*/*C. rufus* and *C. latrans* being 1-2

million years divergent from the gray wolf (*C. lupus*). Based on the existing genetic evidence, we have suggested that the eastern Canadian wolf retain its original species designation, *C. lycaon*, which, based on precedent, may also be applied to the red wolf. We use the terminology eastern Canadian wolf based on our frame of reference but these wolves represent the once larger distribution of the eastern timber wolf that occupied the eastern portion of North America (Brewster and Fritts 1995).

Hybridisation of coyotes and wolves appears limited to eastern North America based on field observations (Thurber and Peterson 1991, Paquet 1989, Paquet 1991, Paquet 1992) and genetic studies (Lehman *et al.* 1991, Wayne and Lehman 1992, Roy *et al.* 1994, Forbes and Boyd 1998). The geographic range of wolf/coyote hybridisation is consistent with the historic range of both the red wolf and the eastern Canadian wolf (Brewster and Fritts 1995, Nowak *et al.* 1995). In a model of a North American-evolved eastern wolf (Wilson *et al. in press*) this hybrid zone would represent inter-breeding of closely related species.

A genetic study of Ontario wolf-like canids using microsatellite loci and the mtDNA control region identified a wolf/coyote hybrid south of Algonquin Park in the Frontenac Axis (Grewal *et al.* in prep). This animal had earlier been described as a small wolf called the "Tweed wolf" and has previously been proposed to represent a hybrid between the "Algonquin" type wolf, i.e. eastern Canadian wolf (*C. lycaon*), and coyotes (Schmitz and Kolenosky 1985). Additional morphological studies examining these animals and eastern coyotes have also suggested wolf/coyote hybridisation as the origin of the eastern coyote (Lawrence and Bossert 1975, Richens and Hugie 1974).

We examined the mtDNA control region and 8 microsatellite loci in eastern coyote populations to determine if eastern Canadian wolf genetic material has been introgressed into eastern coyote populations. We compared the Algonquin wolf and the hybrid "Tweed wolf" from the Frontenac Axis to eastern coyote populations based on the recently proposed model of a North American evolved eastern wolf. We also compared wild born red wolf/coyote hybrid offspring from the Alligator River Refuge, N.C. population with the above populations to further assess the origin of the eastern coyote.

MATERIALS & METHODS

Samples and DNA Extraction

We analysed samples from populations of eastern Canadian wolves (*C. lycaon*), Tweed "wolves" and eastern coyotes. Algonquin Provincial Park, Ontario (n=49) represented eastern Canadian wolves with the Frontenac Axis, Ontario (n=74) representing "Tweed wolves" Schmitz and Kolenosky 1985. Eastern coyotes from Adirondack State Park, NY (n=66) and Cortlandville, NY (50 miles southwest of Syracuse) (n=24) were *a priori* considered as different populations representing a forested park ecosystem and agriculturally developed area, respectively. Eastern coyote samples from Kouchibouguac National Park, New Brunswick (n=20) were also analysed. Texas coyotes (n=24) were included to represent western *C. latrans*. Captive red wolves (n=60) and hybrid red wolf/coyote hybrids (n=15) collected from the wild population at the Alligator River Refuge, North Carolina and included as a comparative population with corresponding hybrids based on the close genetic similarity between eastern Canadian wolves and red wolves. DNA was extracted following a modified Qiagen$_{TM}$ extraction protocol using the lysis buffer described in Guglich *et al.* (1994) from frozen tissue samples (liver, heart, kidney, or muscle).

Mitochondrial DNA Analysis

Sizing Assay for the Identification of New World (C. lycaon/C.latrans) and Old World (C. lupus) Control region Sequences. A previously described method (Pilgrim *et al.* 1998) for distinguishing gray wolf (*C. lupus*) mtDNA from coyote (*C. latrans*) was modified to identify the presence or absence of gray wolf mtDNA within eastern coyote samples. The following primers were used to amplify a section of the control region of the mitochondrial DNA:

Primer 1 5'-GAA GCT CTT GCT CCACCA ATC-3' (Pilgrim *et al.* 1998)

Primer 2 5'-CAA ACC ATT AAT GCA CGA CG-3'

The control region was amplified in a total reaction volume of 10µl per tube using 25ng of genomic DNA, 200 µM dNTPs, 1x amplification buffer, 2.0 mM $MgCl_2$, primers 1 and 2 (0.2 mM) and 0.5 units of *Taq* polymerase (BRL). Products were amplified under the following conditions: 94°C for 5 min., 55°C for 30

sec., 72°C for 30 sec. 1 cycle; 94°C for 30 sec., 55°C for 30 sec., 72°C for 30 sec. 35 cycles; 94°C for 30 sec., 55°C for 30 sec., 72°C for 2 min. 1 cycle. Amplified products were separated by size using a 6% denaturing acrylamide gel. A phage M13 DNA sequencing reaction was run as a sizing marker.

Control region Sequence Analysis & Haplotype Identification.

The following primers were used to amplify the control region of the mitochondrial DNA for specific haplotype identification:

Primer 1 5'-GAA GCT CTT GCT CCACCA ATC-3' (Pilgrim *et al.* 1998)

Primer 2 5'-GGG CCC GGA GCG AGAAGA GGG AC-3'

The control region was amplified in a total reaction volume of 20µl per tube using 25ng of genomic DNA, 200 µM dNTPs, 1x amplification buffer, 2.0 mM $MgCl_2$, primers 1 and 2 (0.2 mM) and 0.5 units of *Taq* polymerase (BRL) and radioactively labeled (γ ^{33}P-dATP (ICN)) primers were added in addition to the unlabeled primers. Products were amplified under the following conditions: 94°C for 5 min., 55°C for 30 sec., 72°C for 30 sec. 1 cycle; 94°C for 30 sec., 55°C for 30 sec., 72°C for 30 sec. 35 cycles; 94°C for 30 sec., 55°C for 30 sec., 72°C for 2 min. 1 cycle. Products were identified as to specific haplotypes using single-stranded conformational polymorphism (SSCP) analysis. Amplified products were electrophoresed through a non-denaturing acrylamide Model S2 gel (GIBCOBRL) (5% acrylamide [59 acrylamide:1 bisacrylamide], 10% glycerol and 0.5 X TBE) for 16 hours at 4°C.

Microsatellite Analysis

Eight microsatellite loci (Ostrander *et al.* 1993, Roy *et al.* 1994, 1996) were amplified using 4.6 pmol λ^{33}P T4 polynucleotide kinase (Boehringer-Mannheim) end labeled primer ATP in a total reaction volume of 10µl per tube using 25ng of genomic DNA, 200 µM dNTPs, 1x amplification buffer, 2.0 mM $MgCl_2$, unlabelled primer (0.2 mM), labeled primer, 1.0 µg of Bovine Serum Albumin (BSA) (BRL) and 0.5 units of *Taq* polymerase (BRL). Products were amplified under the following conditions: 94°C for 5 min., 55-65°C for 30 sec., 72°C for 15 sec. 1 cycle; 94°C for 15 sec., 55°C for 30 sec., 72°C for 15 sec. 30

cycles; 94°C for 15 sec., 55°C for 30 sec., 72°C for 2 min. 1 cycle. Products were then mixed with 0.4 volume of formamide loading buffer and were heated at 95°C for 5 minutes before loading onto a 6% sequencing gel containing 50% (w/v) urea. A control sequencing reaction of phage M13 DNA was run adjacent to the samples to produce size markers for the microsatellite alleles.

Genetic Analysis

We analyzed allele frequencies at 8 loci among Algonquin Provincial Park wolves, Tweed wolves from the Frontenac Axis, Texas coyotes and eastern coyote populations from NewYork and New Brunswick. Microsatellite alleles were assigned based on allele sizes in Roy *et al.* (1996). An individual index (I_I) (Wilson *et al. in press*) was calculated from each individual animal's DNA profile using the following equation: $\Sigma \log (p_A/p_B)$, where p_A and p_B are the allele frequencies of a specific allele from population A and B, respectively. In the event an allele was absent from one of the populations, an allele frequency equivalent to that of one allele being found in the sample was used. This LOD score value assesses the origin of the alleles in each animal based on a ratio of the frequencies from two populations. If the populations have similar allele frequencies then the I_I values of individuals from both populations would follow a distribution around 0. An increasing positive score indicates an individual originated from population A and a decreasing negative score indicates an individual originated from population B.

Nei's unbiased genetic distance (Nei 1978) was calculated using the programs GENDIST and NEIGHBOR in the computer program PHYLIP (Felsenstein 1993). Population genetic structure was estimated using F_{ST} (Weir and Cockerham 1984) and R_{ST} (Slatkin 1995) from the computer program GENEPOP.

RESULTS

The "Tweed wolves" of the Frontenac Axis have previously been classified wolf/coyote hybrids (Schmitz and Kolenosky 1985, Grewal *et al.* in prep.). We assessed this classification by comparing the distributions of Individual indices, (I_I), of "Tweed wolves" with those of Algonquin Provincial Park

wolves (eastern Canadian wolves) and Texas coyotes. This demonstrated a distribution separate from the coyotes and overlapping those of the eastern Canadian wolves (Figure 1). This supports the presence of eastern Canadian wolf genetic material within the "Tweed wolves". The introgression of nuclear genetic material was also observed in "Tweed wolves" by the presence of allele 154 at locus cxx.172 that was first identified in Algonquin Provincial Park animals and which is absent in western coyotes and other wolf populations (Table 1).

The introgression of eastern Canadian wolf genetic material into the "Tweed wolves" is further supported by the mtDNA control region sequence analysis. Although the majority of animals within Algonquin Park have coyote-like control region mtDNA haplotypes, a distinct and divergent mtDNA lineage was identified in skull samples from animals killed in the 1960s (Wilson *et al. in press*). Similar distinct and divergent mtDNA sequences were also observed in red wolves. Approximately 25% of the "Tweed wolves" contained the eastern Canadian wolf mtDNA sequence and the remaining animals contained coyote-like sequences. The presence of wolf mtDNA in hybrid populations and coyote mtDNA in the Algonquin wolf population (Lehman *et al.* 1991, Wayne and Lehman 1992, Wilson *et al. in press*) suggests the bi-directional movement of mtDNA into and from eastern wolf and coyote populations. In Algonquin Park, wolves with coyote mtDNA haplotypes have I_I values consistent with eastern Canadian wolves indicating introgression through a maternal contribution followed by female hybrids back-crossing with male wolves. Reciprocally, the eastern Canadian wolf-specific mtDNA haplotype within Frontenac Axis animals had I_I scores supporting female hybrids back-crossing to coyote or coyote/wolf hybrid males.

We then compared eastern coyote populations to eastern Canadian wolves, western coyotes and "Tweed wolves". We assessed the genetic similarity among "Tweed wolves" and eastern coyote populations using genetic distances calculated from the microsatellite allele frequencies (Nei, 1978). A neighbor-joining tree of the populations indicates that the "Tweed wolves" are more divergent from Texas coyotes than from Algonquin Provincial Park wolves (Table 2, Fig. 2). The eastern coyote populations in New York and New Brunswick show a close genetic similarity to "Tweed wolves" and appear nearly equally distant from both Texas coyotes and Algonquin wolves. This intermediate position is consistent with the presence of genetic material from both of these two species. The 154 allele at locus cxx. 172

which was observed at low frequency in the "Tweed wolves" was also present in the eastern coyote populations from New Brunswick, the Adirondacks and Cortlandville, NY (Table 1). This further supports a bi-directional flow of genetic material.

To further assess the level of coyote/wolf genetic material in eastern coyotes we plotted I_I values using the Texas western coyote/"Tweed wolf" allele frequencies (Table 1) and the "Tweed wolf"/Algonquin Park eastern Canadian wolf allele frequencies (Fig 3). The first comparison evaluated animals as western coyotes or "Tweed wolves" and the second evaluated the animals as "Tweed wolves" or eastern Canadian wolves. The I_I values of animals from the Adirondacks and Cortlandville, NY, showed very similar distributions to the "Tweed wolves" (Fig. 3). The Adirondack and Cortlandville populations have fewer eastern Canadian wolf genotypes within their populations than the "Tweed wolves". The distribution of the New Brunswick animals is similar to the "Tweed wolves" and the New York populations.

Sixty captive red wolves were genotyped at the 8 microsatellite loci to identify the complete complement of alleles within the original breeding program. Animals collected from the Alligator River Refuge were confirmed as coyote/wolf hybrids based on the presence of coyote alleles. The overlapping distribution of the red wolf/coyote hybrids and eastern coyote populations support the hybrid origin of eastern coyote populations (Fig. 3).

A total of 109 eastern coyotes were examined from New York and New Brunswick for the presence of gray wolf (*C. lupus*) mtDNA. None of the animals contained mtDNA specific to gray wolf or dog. The presence of eastern Canadian wolf mtDNA was examined using single-stranded conformational polymorphism (SSCP) analysis. A total of 16 of 66 (24.2%) Adirondack animals contained this mtDNA as did 3 of 24 (12.5%) Cortlandville animals and 8 of 19 (42.1%) New Brunswick animals. The I_I scores for these individual animals were consistent with coyote/wolf hybrids. Conclusions about hybrids back-crossing to coyotes or coyote/wolf hybrids in the Frontenac Axis similarly apply to eastern coyote populations.

An analysis of population structuring among eastern coyote populations using F_{ST} and R_{ST} estimates are shown in Table 3. The F_{ST} and R_{ST} values are consistently high between the "Tweed

wolves"/eastern coyote populations in pair-wise comparisons with the Texas coyotes. Previous estimates of gene flow among coyote populations indicated a panmictic population among North American coyote populations (Roy *et al.* 1994). The F_{ST} values correspond to geographical distances among the populations with the two New York populations demonstrating less structuring than the New York to "Tweed wolf" comparisons and with the New Brunswick population demonstrating higher F_{ST} values to both the "Tweed wolves" and New York populations.

The R_{ST} values demonstrate show less geographic relationship with the Adirondack and New Brunswick populations having a relatively low value. R_{ST} examines variance based on differences in specific allele sizes in a step-wise mutation model (Slatkin 1995) while F_{ST} examines variance based on heterozygosity under an infinite-allele model (Weir and Cocherham 1984). As a result, R_{ST} would be predicted to be more dependent on the specific alleles that were contributed from a founding population. This difference between F_{ST} and R_{ST} has been observed elsewhere (Bossart and Pashley-Prowell 1998) and may reflect the sensitivity of the values to gene flow, population history and selection.

DISCUSSION

The "Tweed wolves" of Ontario have very similar genetic profiles at eight microsatellite loci to the eastern coyotes found in New York and New Brunswick. The "Tweed wolves" have been shown to be hybrids between eastern Canadian wolves and coyotes. The names "Tweed wolf" and eastern coyote appears dependent on whether one views the hybrids as small wolves or large coyotes. The genetic data from the introduced Alligator River red wolf population supports the hypothesis of introgression of wolf genetic material into eastern coyote populations as red wolf/coyote hybrids have similar DNA profiles. Previous genetic studies have concluded that hybridisation between wolves and coyotes resulted in the movement of coyote genetic material into wolf populations but not the reverse. These conclusions were initially based on mitochondrial DNA (Lehman *et al.* 1991, Wayne and Lehman 1992) and then analysis of nuclear microsatellite loci (Roy *et al.* 1994). Earlier conclusions about the absence of wolf mtDNA in coyote populations were based on the assumption that the "hybridising wolves" of eastern North America were gray wolves (*C. lupus*) (Lehman *et al.* 1991, Wayne and Lehman 1992, Roy *et al.* 1996). The North American-evolved eastern wolf (Wilson *et al. in press*) does not have gray wolf mtDNA and therefore

could not have transmitted this molecule to coyote populations. Another bias in the examination of introgression of wolf genetic material into coyote populations was the *a priori* classification of animals as strictly wolf or coyote in analyses using nuclear microsatellite loci (Roy *et al.* 1994).

An eastern Canadian wolf mtDNA was previously identified based on its presence at high frequency in 1960s Algonquin wolf samples and the divergence of this sequence from coyote mtDNA haplotypes (Wilson *et al. in press*). The presence of this mtDNA haplotype in "Tweed wolves" and eastern coyotes could be interpreted as evidence against this sequence originating from eastern Canadian wolves. However its divergence from coyote sequences is consistent with it originating in a North American - evolved wolf. The presence of a coyote-like mtDNA in a historic New York wolf hide (c.1893) and a second divergent *C. lycaon* mtDNA sequence in a Maine wolf sample (1880s), both pre-dating the arrival of western coyotes further supports a New World evolution of eastern wolves (Wilson *et al. in prep*).

A comparison of mtDNA data with individual-specific microsatellite genetic scores indicated that eastern wolf mtDNA has been transmitted to eastern coyote populations and I_I values support back-crossing with coyotes or coyote/wolf hybrids. A hybridisation study examining wolf/coyote inter-breeding (Kolenosky 1971) involved mating a female Algonquin eastern Canadian wolf with a male coyote, but the reciprocal cross was not successful in this study. Back-crosses of individuals from these hybrid litters were successful.

In addition to back-crossing, selection may maintain the nuclear genotypes of animals within specific habitats. While the view that selection alone acted on these coyotes in the absence of wolf gene introgression is not supported by this study, the hypotheses of selection in combination with hybridisation can not be rejected. The introgression of eastern Canadian wolf/red wolf genes into eastward expanding coyotes could have provided composite genomes that would facilitate selection for larger prey. The "Tweed wolf" and eastern coyote may represent animals selected in more developed areas such as the Frontenac Axis and Cortlandville, NY. This may explain the success of the eastern coyote in expanding through developed regions to colonize the Maritime Provinces and New England States.

There may be a limit to the effect of selection on eastern coyotes as the animals inhabiting areas such as Kouchibouguac National Park, which has a high density of deer, are smaller than eastern Canadian

wolves (Sears 1999, Dumond and Villard 2000). This may further be reflected by the similar genetic profiles and gene flow between the agriculturally developed Cortlandville region and Adrirondack State Park coyotes suggesting little differentiation based on the two landscapes. The "wolf-like" characteristics of eastern expanding hybrids would be limited in the amount of eastern Canadian wolf genetic material due to the near or complete extirpation of wolves in these regions. The adaptable eastern coyote may represent a model to examine the interaction between genetic profiles (wolf-like vs. coyote-like) and morphological adaptations to different landscapes and habitat. We are presently analysing a number of coyote-like animals from southern Ontario that are bounded by the Frontenac Axis and the Adirondacks to determine if landscape and the amount of human development have influenced the genotypes.

In this study we found no evidence supporting "coydogs" in the eastern coyote populations based on the absence of *C. lupus* control region mtDNA. Our findings appear consistent with captive breeding studies examining coyote/dog hybridisation (Silver and Silver 1969, Mengel 1971).

We conclude that the "Tweed wolf" and the eastern "coyote" represent hybrids between western coyotes and eastern wolves (*C. latrans/C. lycaon*) as previously proposed (Silver and Silver 1969, Mengel 1971, Lawrence and Bossert 1975, Schmitz and Kolenosky 1985, Nowak 1979, Lariviere and Crete 1993). The major difference between eastern coyotes and their western counterparts is their larger size (Richens and Hugie 1974, Lawrence and Bossert 1975). This hybrid is a highly adaptable animal that inhabits a range of habitats from forested regions in New York and New Brunswick with a predominantly deer diet to more agriculturally developed areas such as Cortlandville, NY.

Southeastern Canada and northeastern US have a parallel history with the southeastern US with respect to the expansion of western coyotes that hybridised with surviving eastern wolves. This hybridisation is limited to the eastern regions of Canada and the US while in the west, wolves and coyotes exist together but do not hybridise (Thurber and Peterson, Roy *et al.* 1994, Paquet 1989, Paquet 1991, Paquet 1992, Forbes and Boyd 1998). This characteristic of the eastern wolves of hybridising with coyotes distinguishes them from the western gray wolf, *C. lupus*. This characteristic is also a major source of concern for maintaining the genetic integrity of eastern wolf populations.

The presence of hybrid eastern coyotes has implications on the re-location of eastern wolves from Canada into the northeastern US states. At present, the coyote/wolf hybrids inhabiting New York State are serving a functional role as a top end predator preying on white-tailed deer. Specific questions need to be addressed as to the role the eastern Canadian wolf would add to ecosystems such as the Adirondacks. A comparison of the ecological differences between eastern Canadian wolves and eastern coyotes should be undertaken to determine what differences exist between the two predators. If the re-introduction of the eastern Canadian wolf is intrinsically important, because it historically existed in the northeastern US and was extirpated as a result of human activities, then the feasibility of maintaining such a population of *C. lycaon* in proximity to populations of eastern coyotes must be addressed. It is unlikely that the relocation of eastern Canadian wolves into the northeastern US would result in the cohesion of sufficient packs to prevent the breeding with eastern coyotes. There are two biological systems that may provide answers – the Algonquin Provincial Park population and the red wolf recovery program. A genetic analysis of Algonquin wolves and neighboring "Tweed wolves" has identified limited gene flow (Grewal *et al.* in prep). Contrary to this is the red wolf population at the Alligator River refuge, which is readily hybridising with coyotes. Consideration of the Algonquin model and the factors influencing the barriers to gene flow from "Tweed wolves" is important in assessing management strategies for maintaining eastern wolves (*C. lycaon* and *C. rufus*) within eastern US ecosystems. Potential factors that may be limiting inter-breeding between eastern Canadian wolves and eastern coyotes include pack cohesion, population size and habitat. These factors and others should be analysed in a comparative study to make the appropriate recommendations for re-introduction and recovery programs.

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Table 1. Allele frequencies at 8 microsatellite loci for eastern Canadian wolves in Algonquin Provincial Park, eastern coyote populations and Texas coyote population. The common Algonquin allele at locus cxx.172 is provided in bold.

Locus/ Allele	Algonquin Park ON	Frontenac Axis ON	Adirondack NY	Cortland-ville NY	New Brunswick	Texas
cxx.377						
144	0.051	0.020	0.121	0.022	0.250	0.000
146	0.000	0.007	0.008	0.000	0.075	0.000
148	0.010	0.000	0.000	0.000	0.000	0.000
150	0.000	0.020	0.159	0.065	0.150	0.207
154	0.306	0.162	0.000	0.109	0.000	0.188
156	0.194	0.054	0.046	0.000	0.100	0.083
158	0.000	0.020	0.030	0.022	0.025	0.063
160	0.051	0.027	0.083	0.174	0.050	0.042
162	0.082	0.122	0.038	0.088	0.000	0.021
164	0.163	0.047	0.023	0.044	0.000	0.104
166	0.133	0.393	0.401	0.390	0.150	0.125
168	0.000	0.000	0.000	0.022	0.050	0.104
170	0.000	0.000	0.008	0.000	0.000	0.063
172	0.000	0.027	0.083	0.000	0.000	0.000
174	0.010	0.074	0.000	0.065	0.150	0.000
176	0.000	0.027	0.000	0.000	0.000	0.000
cxx.225						
163	0.112	0.054	0.076	0.083	0.075	0.239
165	0.143	0.115	0.099	0.104	0.000	0.500
167	0.500	0.304	0.152	0.063	0.225	0.196
169	0.143	0.115	0.226	0.353	0.100	0.065
171	0.040	0.034	0.000	0.063	0.025	0.000
177	0.031	0.054	0.129	0.188	0.200	0.000
179	0.031	0.324	0.318	0.146	0.375	0.000
cxx.109						
141	0.000	0.000	0.000	0.000	0.000	0.063
143	0.674	0.385	0.378	0.650	0.292	0.104
145	0.112	0.047	0.000	0.000	0.000	0.395
147	0.051	0.101	0.205	0.150	0.104	0.354
149	0.041	0.068	0.053	0.000	0.083	0.063
151	0.071	0.284	0.296	0.200	0.333	0.021
153	0.010	0.068	0.030	0.000	0.042	0.000
155	0.041	0.047	0.038	0.000	0.146	0.000
cxx.172						
144	0.316	0.388	0.371	0.357	0.425	0.666
154	**0.163**	**0.054**	**0.045**	**0.071**	**0.025**	**0.000**
156	0.031	0.014	0.008	0.000	0.050	0.000
158	0.235	0.270	0.182	0.286	0.375	0.000
160	0.255	0.273	0.394	0.286	0.125	0.104
162	0.000	0.000	0.000	0.000	0.000	0.063
164	0.000	0.000	0.000	0.000	0.000	0.167
166	0.000	0.000	0.000	0.000	0.000	0.000
168	0.000	0.000	0.000	0.000	0.000	0.000
174	0.000	0.000	0.000	0.000	0.000	0.000

Locus/ Allele	Algonquin Park ON	Frontenac Axis ON	Adirondack NY	Cortlandvil leNY	New Brunswick	Texas
cxx.250						
129	0.000	0.007	0.000	0.000	0.000	0.022
131	0.072	0.082	0.068	0.167	0.000	0.022
133	0.112	0.062	0.015	0.000	0.050	0.261
135	0.561	0.288	0.106	0.125	0.100	0.087
137	0.082	0.171	0.121	0.167	0.100	0.087
139	0.102	0.226	0.394	0.437	0.300	0.346
141	0.061	0.075	0.205	0.083	0.275	0.044
143	0.000	0.007	0.000	0.000	0.025	0.044
145	0.010	0.082	0.091	0.021	0.150	0.087
cxx.123						
139	0.000	0.000	0.078	0.000	0.000	0.000
143	0.112	0.041	0.078	0.021	0.050	0.063
145	0.000	0.007	0.078	0.042	0.000	0.063
147	0.276	0.385	0.346	0.146	0.200	0.353
149	0.306	0.472	0.530	0.540	0.575	0.229
151	0.000	0.007	0.100	0.188	0.125	0.146
153	0.306	0.088	0.000	0.063	0.050	0.104
155	0.000	0.000	0.000	0.000	0.000	0.021
157	0.000	0.000	0.000	0.000	0.000	0.021
cxx.200						
205	0.021	0.000	0.000	0.000	0.000	0.000
207	0.021	0.155	0.038	0.063	0.025	0.000
209	0.000	0.108	0.273	0.290	0.200	0.500
211	0.125	0.169	0.189	0.104	0.100	0.250
213	0.021	0.014	0.106	0.125	0.050	0.000
215	0.406	0.188	0.242	0.188	0.550	0.125
217	0.052	0.095	0.068	0.063	0.075	0.042
219	0.000	0.034	0.008	0.000	0.000	0.000
221	0.292	0.169	0.046	0.125	0.000	0.083
223	0.021	0.000	0.000	0.000	0.000	0.000
225	0.000	0.000	0.000	0.000	0.000	0.000
227	0.031	0.068	0.030	0.042	0.000	0.000
229	0.010	0.000	0.000	0.000	0.000	0.000
cxx.204						
200	0.847	0.784	0.871	0.958	0.975	0.771
202	0.122	0.169	0.106	0.042	0.025	0.229
206	0.031	0.027	0.000	0.000	0.000	0.000
208	0.010	0.000	0.023	0.000	0.000	0.000
216	0.000	0.020	0.000	0.000	0.000	0.000

Figure 1. Log-likelihood individual indices (I_l) of Tweed wolves from the Frontenac Axis (n=74), Algonquin Provincial Park wolves (n=48) and Texas coyotes (n=22). I_l scores were calculated for each individual animal DNA profile at 8 microsatellite loci using the allele frequencies from the Algonquin wolves and Texas coyote population.

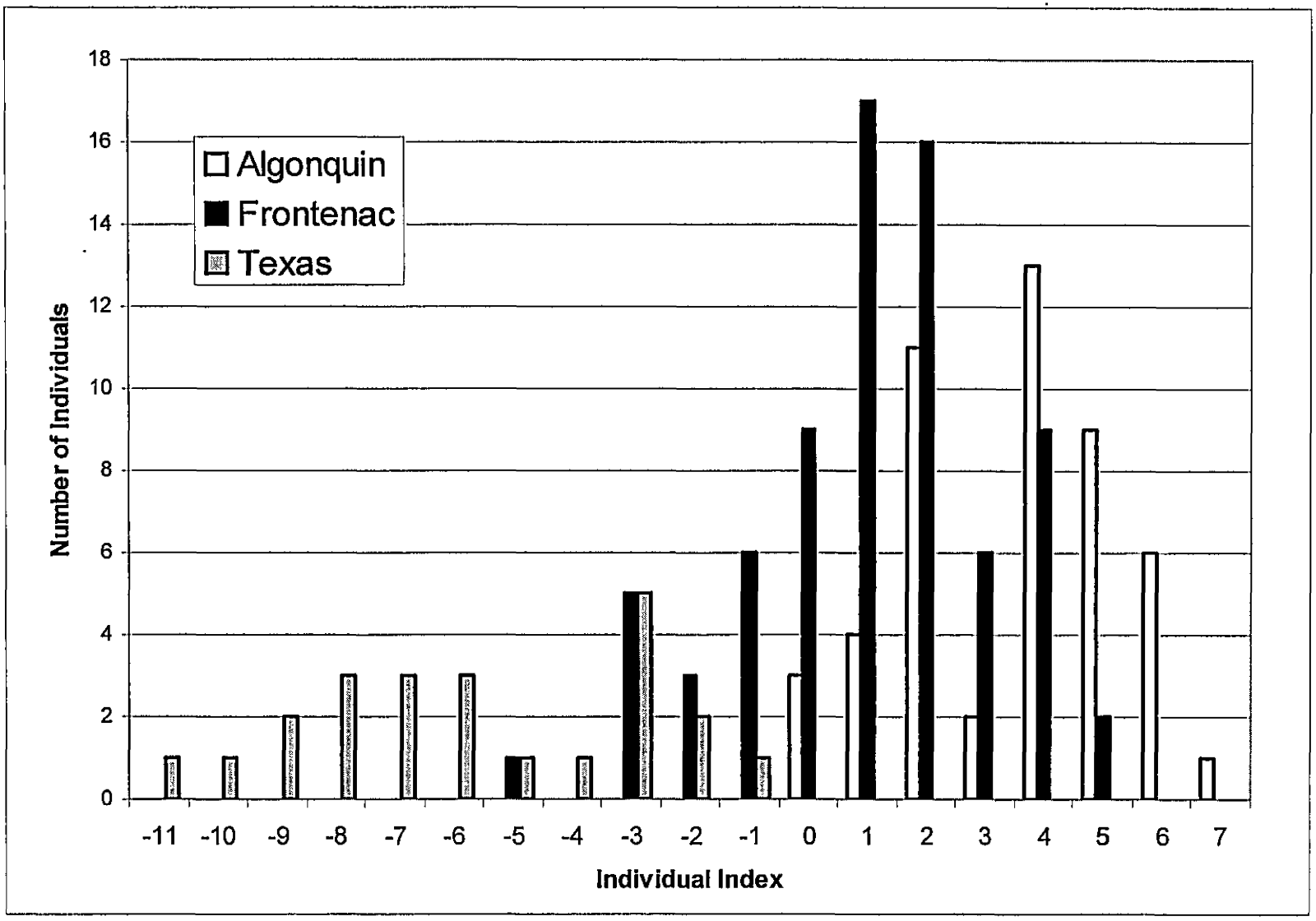

Algonquin
Frontenac
Texas
Number of Individuals
Individual Index
0
2
4
6
8
10
12
14
16
18
-11
-10
-9
-8
-7
-6
-5
-4
-3
-2
-1
0
1
2
3
4
5
6
7

Figure 2. Neighbor-joining tree (unrooted) of Nei's genetic distances for allele frequencies for eight microsatellite loci from Algonquin Provincial Park eastern Canadian wolves, eastern coyote populations and Texas coyotes.

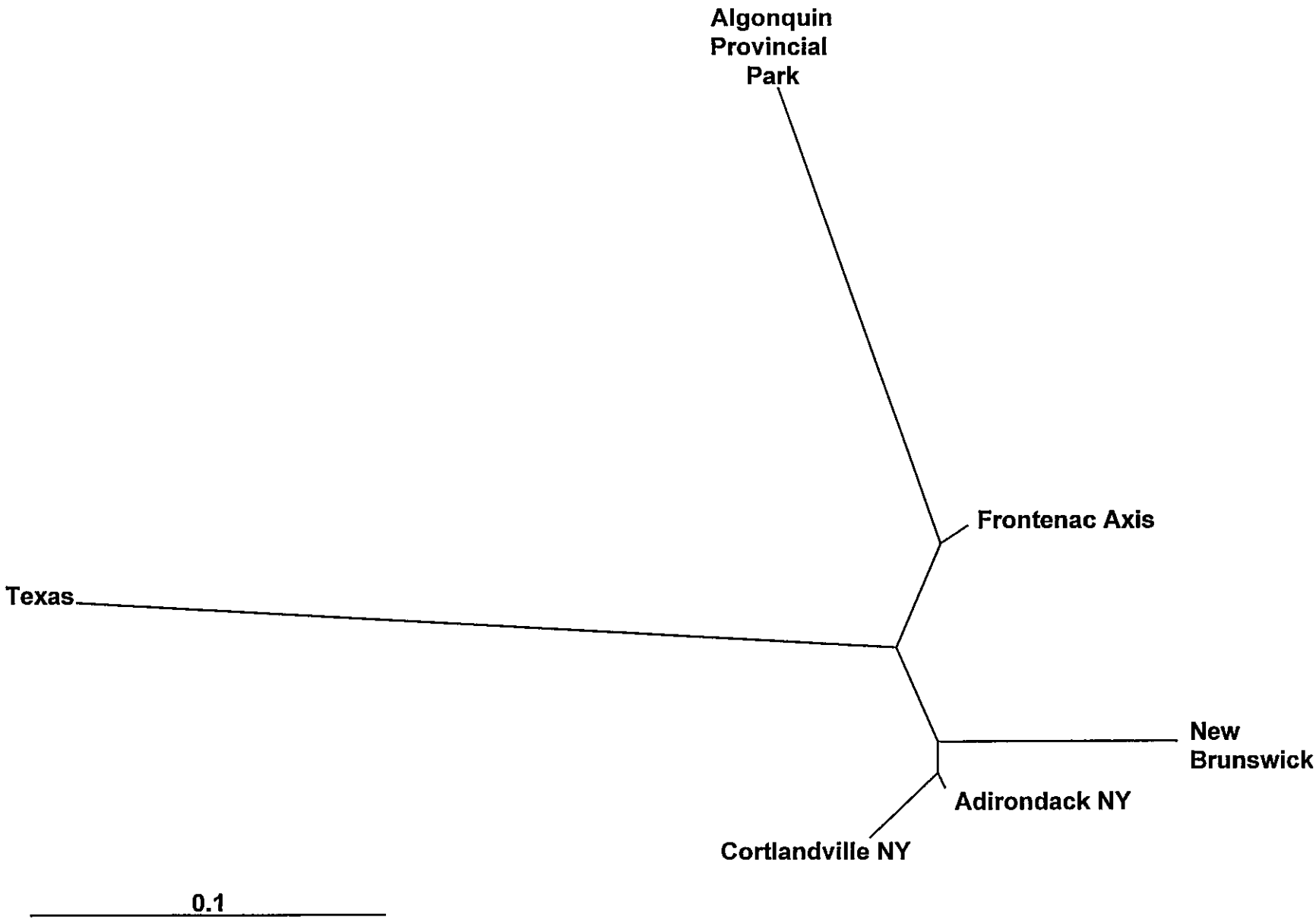
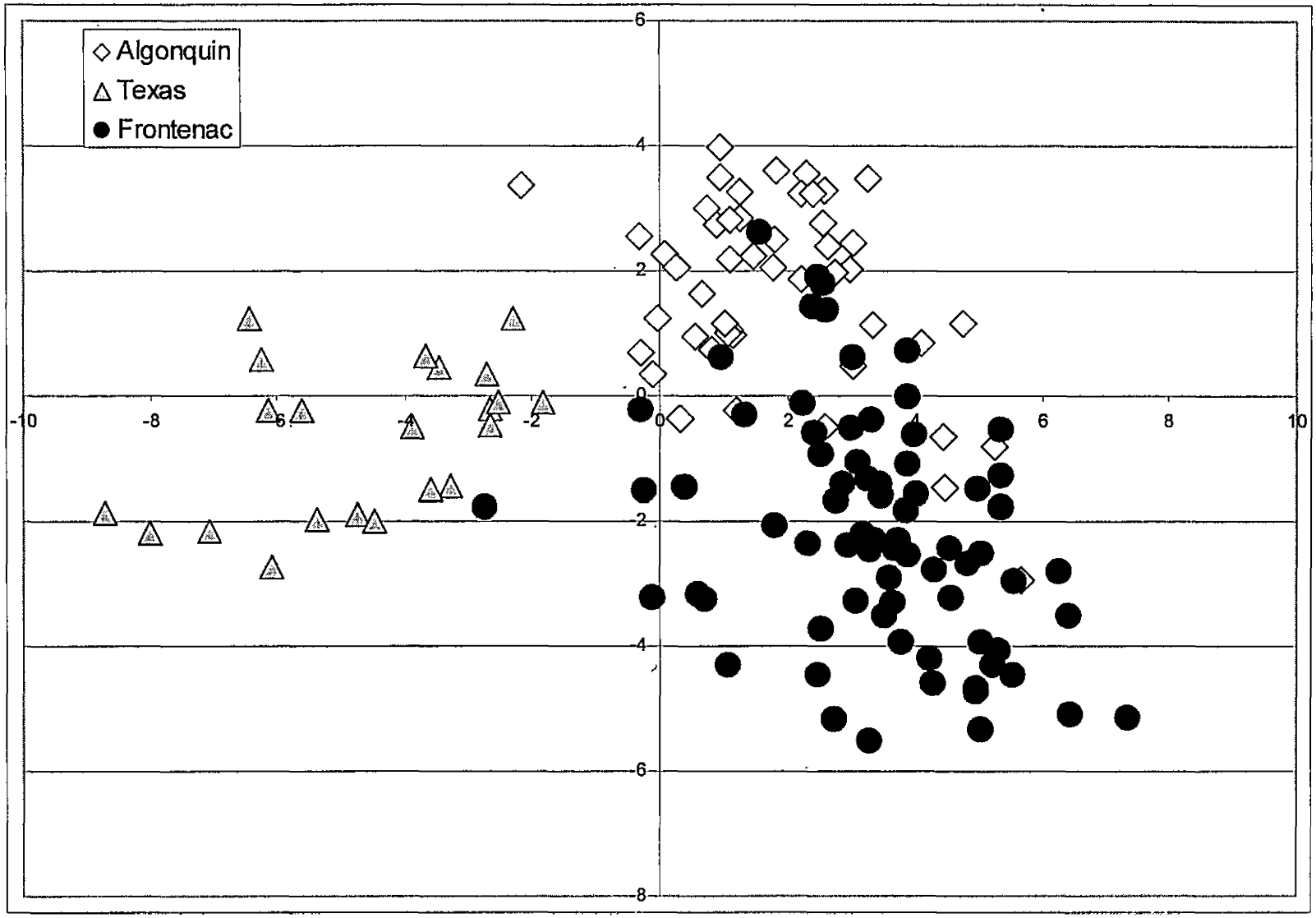
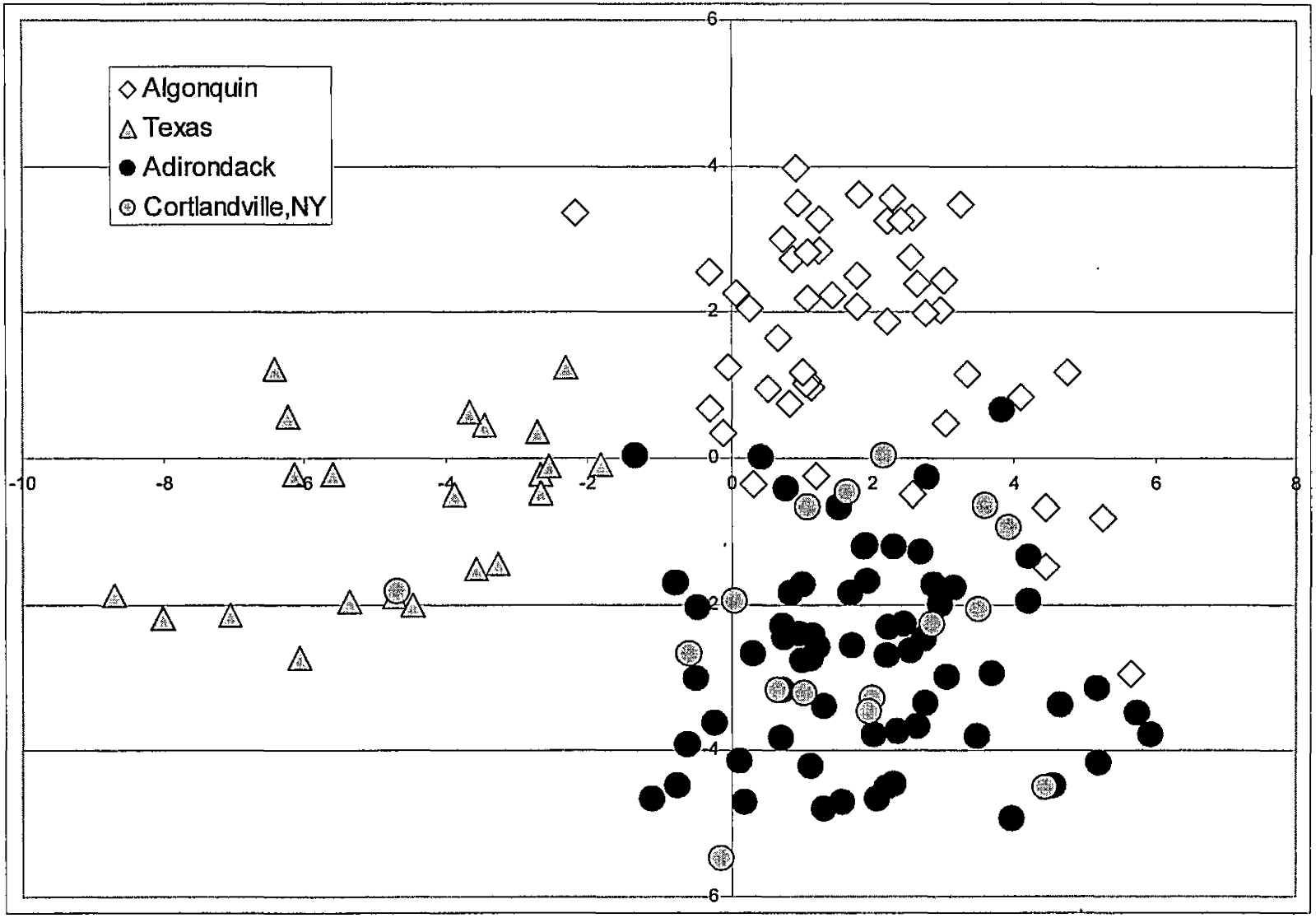
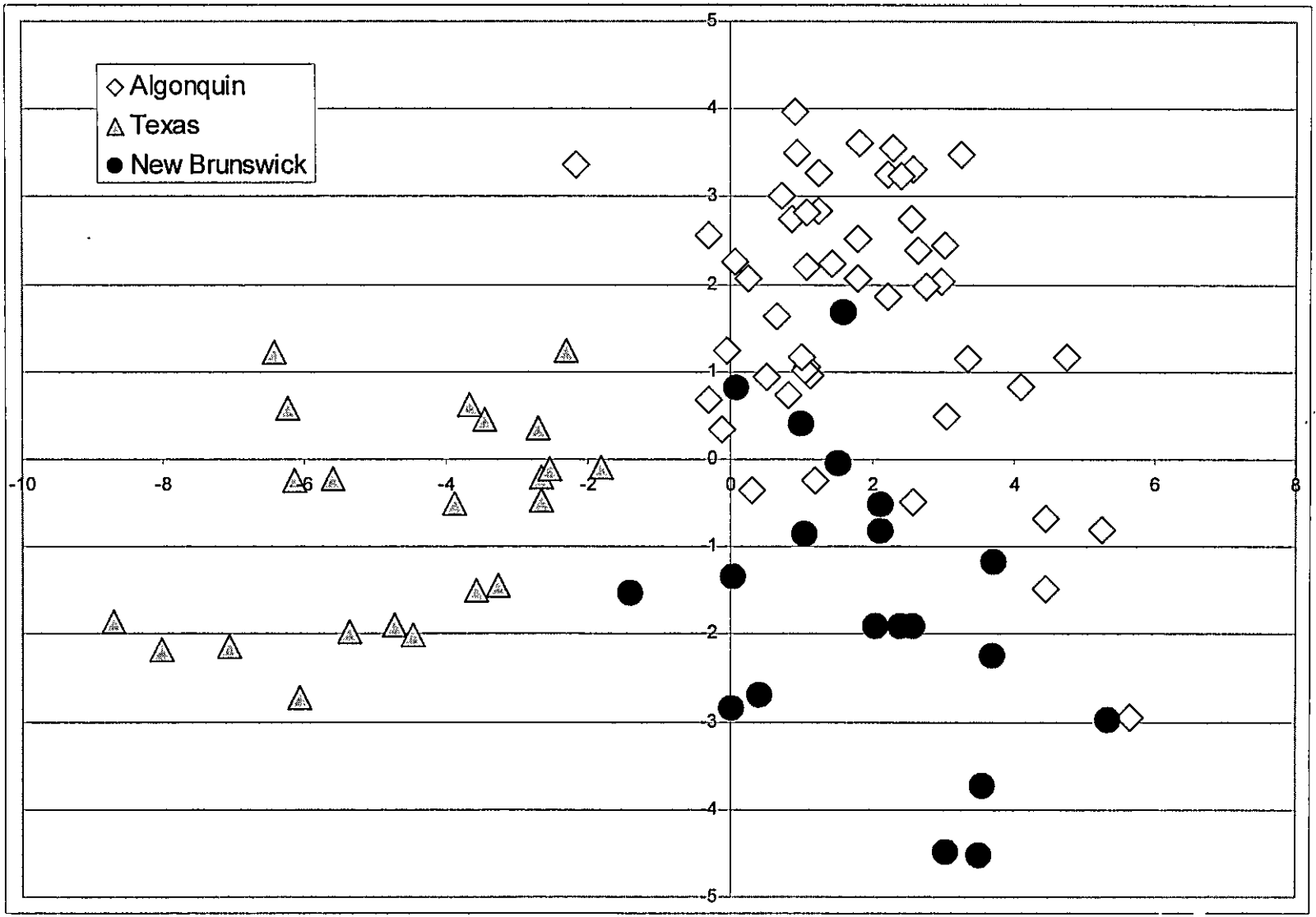

Algonquin
Provincial
Park
Frontenac Axis
Texas
New
Brunswick
Adirondack NY
Cortlandville NY
0.1

Figure 3. Two-dimensional Individual-Index plots of two log-likelihood individual index (I_I) estimations for Frontenac Axis Tweed wolves (n=74), eastern coyotes, Texas coyotes (n=22), eastern Canadian wolves (Algonquin Provincial Park) (n=48), captive red wolves, and wild caught red wolves from Alligator River Refuge, North Carolina. The I_I were calculated for each individual animal DNA profile at 8 microsatellite loci using the allele frequencies from the Frontenac Axis population compared independently to the Texas coyote and Algonquin wolf allele frequencies. **2A.** Frontenac Axis Tweed wolves. **2B.** New York animals from the Adirondacks (n=65) and Cortlandville County (n=16). New Brunswick animals (n=20) from Kouchibouguac National Park. **2D.** Captive red wolves (n=60) and wild caught red wolves identified as red wolf/coyote hybrids (n=14).


◇ Algonquin
△ Texas
● Frontenac
6
4
2
0
-2
-4
-6
-8
-10
-8
-6
-4
-2
0
2
4
6
8
10


Algonquin
Texas
Adirondack
Cortlandville,NY
6
4
2
0
-2
-4
-6
-10
-8
-6
-4
-2
0
2
4
6
8


Algonquin
Texas
New Brunswick
5
4
3
2
1
0
-1
-2
-3
-4
-5
-10
-8
-6
-4
-2
0
2
4
6
8

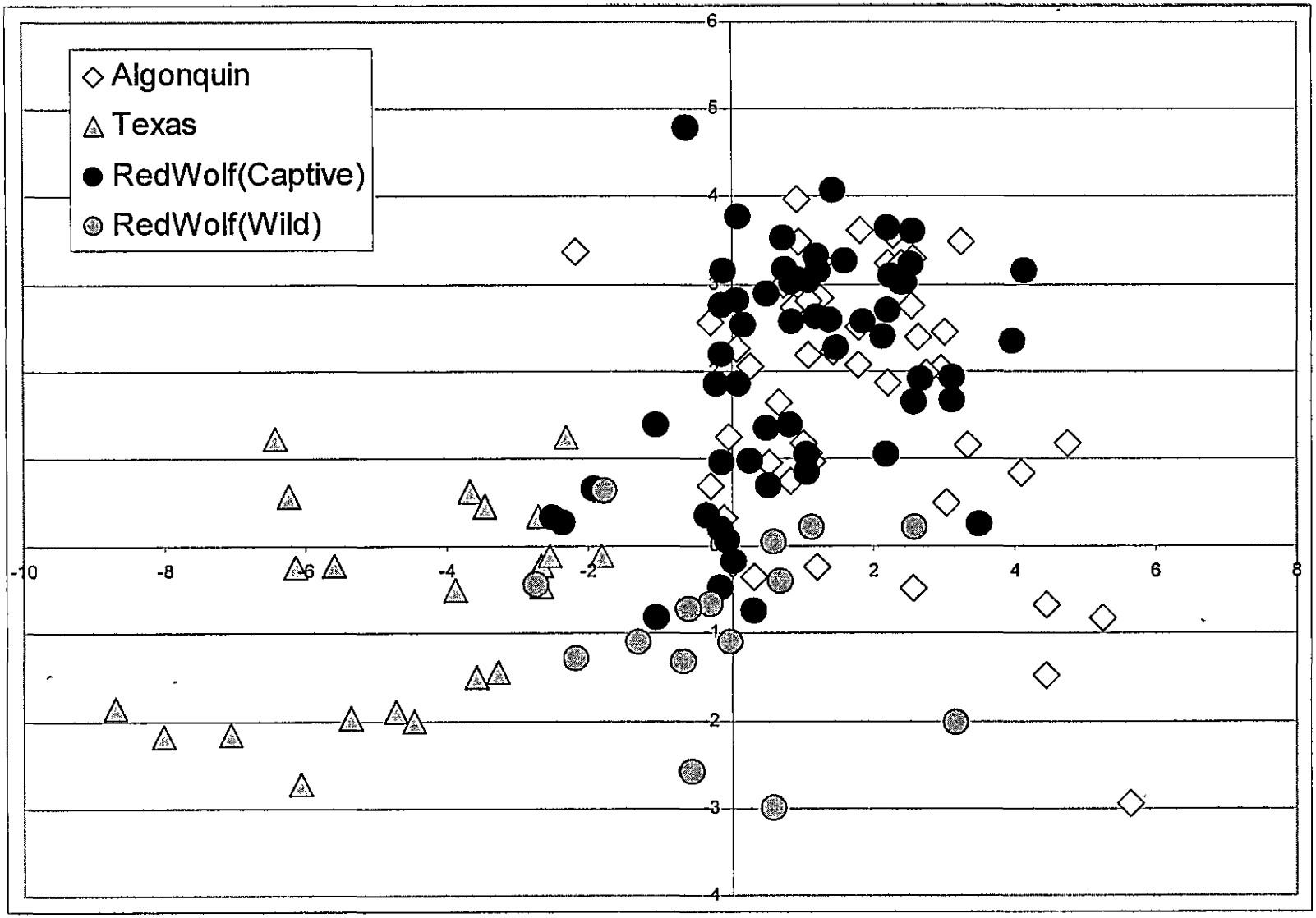

Algonquin
Texas
RedWolf(Captive)
RedWolf(Wild)
6
5
4
3
2
1
-1
-2
-3
-4
-10
-8
-6
-4
-2
2
4
6
8

Table 2. Nei's unbiased genetic distance for eastern coyote populations and Texas coyote population.

Population	Algonquin Park	Frontenac Axis	Adirond.	Cortland-ville NY	New Brunswick	Texas
Algonquin Park	-					
Frontenac Axis	**0.129**	-				
Adirondacks	**0.265**	**0.050**	-			
Cortlandville, NY	**0.264**	**0.074**	**0.022**	-		
New Brunswick	**0.203**	**0.114**	**0.066**	**0.111**	-	
Texas	**0.376**	**0.280**	**0.250**	**0.274**	**0.363**	-

Table 3. Pair-wise F_{ST} (above diagonal) and R_{ST} (below diagonal) estimates for Algonquin Provincial Park eastern Canadian wolves, eastern coyote populations and Texas coyotes.

Population	Algonquin	Frontenac Axis	Adirond.	Cortland-ville NY	New Brunswick	Texas
Algonquin	-	**0.0545**	**0.1057**	**0.1055**	**0.0939**	**0.1376**
Frontenac Axis	**0.1354**	-	**0.0215**	**0.0293**	**0.0511**	**0.0952**
Adirondacks	**0.1435**	**0.0343**	-	**0.0095**	**0.0326**	**0.0953**
Cortlandville, NY	**0.1803**	**-0.0015**	**0.0215**	-	**0.0524**	**0.0996**
New Brunswick	**0.1495**	**0.1346**	**0.0398**	**0.1294**	-	**0.1393**
Texas	**0.1446**	**0.1574**	**0.1260**	**0.1686**	**0.1686**	-

Chapter 8

Characterization of wolves across Ontario using mitochondrial and microsatellite DNA profiles

Characterization of wolves across Ontario using mitochondrial and microsatellite DNA profiles.

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ABSTRACT

Four "types" or "races" of wolves have been previously described in Ontario. (1) A subspecies of the gray wolf (*Canis lupus hudsonicus*) inhabiting the sub-arctic tundra. (2) A "race" ("Ontario type") of a second subspecies of the gray wolf, the eastern Canadian wolf (*C. l. lycaon*) that inhabits the boreal forests and much of the Hudson Bay Lowlands. (3) A second "race" ("Algonquin type") of C. *l. lycaon* that inhabit the deciduous forests of the upper Great Lakes. (4) A small wolf ("Tweed type") that has been proposed to be a hybrid between the "Algonquin type" wolf and the expanding population of western coyotes, *C. latrans*. Using mitochondrial control region sequences and 8 microsatellite loci, we developed DNA profiles for 345 wolves collected from across Ontario between 1960 and 1999. We assessed the relationship of wolves in six geographic regions using the R_{ST} statistic and Nei's genetic distance. Assignment tests indicated that the "Tweed wolves" in this region are hybrids between the western coyote and *C. lycaon* and are representative of the eastern coyote. In both the northwestern and northeastern Ontario upper Great Lakes regions the wolves also appear to be primarily *C. lycaon* and low population differentiation among these regions and Algonquin Park suggests a larger metapopulation. Pukaskwa National Park maintains a small population of wolves, which are genetically closer to the gray wolves of

the Northwest Territories than the surrounding *C. lycaon*. We suggest these represent an isolated remnant population of the "Ontario type", which is a gray wolf, *C. lupus*. The *C. lycaon* in the upper Great Lakes region contain gray wolf mitochondria and represent products of historic and/or continuing hybridisation between *C. lycaon* and *C. lupus*.

INTRODUCTION

Central Ontario is inhabited by a mixture of wolf "types" and the area has been described as containing "Canis soup". Some of this complexity has been attributed to wolf hybridisation with the coyotes, *Canis latrans*, which began colonizing Ontario in the early 1900s (Kolenosky and Stanfield 1975). Although a similar expansion of coyotes into northwestern North America occurred there has been no similar formation of "Canis soup" in areas inhabited by western wolves. In 1975, Kolenosky and Standfield recognized four "types" or "races" of wolves in Ontario (Fig. 1a). In the sub-arctic tundra along the coasts of James and Hudson Bay, the subspecies of gray wolf, *C. lupus hudsonicus,* was identified. In the boreal forest of the Hudson Bay lowlands they recognized a "race" of another subspecies of gray wolf, *C. l. lycaon* and referred to it as the "Ontario type". In the deciduous forests of the upper Great Lakes, they differentiated a race of the same gray wolf subspecies, the "Algonquin type". The fourth type termed the "Tweed wolf" appeared to have resulted from hybridisation of the "Algonquin type", *C. l. lycaon,* with coyotes, *C. latrans.*

North American wolf taxonomy has undergone a series of revisions in the past century. The gray wolf, *C. lupus*, is thought to have originated in the Old World and migrated to the New World via the Bering Land bridge during the Illinoian period of the Pleistocene glaciation, some 300,000 years ago (Nowak 1979, Kurten and Anderson 1980). The wide variation in color, size and weight in North American wolves was noted by many early authors and Miller (1912) attempted to provide a taxonomic framework to the morphological complexity. In eastern North America, he recognized 5 species including *C. lycaon* (eastern Canada) and *C. floridanus*, *C. lupus* var rufus and *C. frustror* that were later recognized as subspecies of the red wolf, *C. rufus*. Pockock (1935) recognized many of the species of Miller (1912) as subspecies of the gray wolf, *C. lupus,* but maintained the eastern Canadian wolf as *C. lycaon.* Following a number of revisions, Goldman (1944) produced a comprehensive treatment that considered the eastern Canadian wolf as a subspecies of the gray wolf (*C. l. lycaon*) and the only one present in Ontario. Hall and Kelson (1959) recognized *C. l. hudsonicus* along the coastal area of Hudson Bay in northern Ontario (Fig. 1b); however, Nowak (1983) and Mulders (1997) concluded *C. l. hudsonicus* should be reversed to *C. l. occidentalis*. Nowak further suggested grouping North American wolves into 5 subspecies based on

Pleistocene refugia, with three of these occurring in Ontario based on similarities of skull measurements, *C. l. occidentalis, C. l. nubilis and C. l. lycaon* (Nowak 1995) (Fig.1c). Based on this proposed distribution most of Ontario was inhabited by *C. l. nubilis,* a subspecies originally assigned to the central Plains of the US.

There has been little consideration of the barriers to gene flow that originally must have been present to cause and maintain the differences among the "types" or subspecies. The Pleistocene ice sheets clearly had a major impact on the distribution of wolves and their ungulate prey in North America. Since the arrival of the Europeans, human impacts such as deforestation, farming, trapping and bounty hunting, extirpated wolves throughout most of the continent providing opportunities for the expansion of the coyote and the subsequent breakdown of the reproductive barriers between coyotes and eastern wolves. If the eastern wolves, *C. lycaon* and *C. rufus*, are North American-evolved wolves (Wilson et al. in press, Nowak, 1983), a further level of reproductive isolation between them and the Eurasian-evolved gray wolf, *C. lupus*, would be expected. Standfield and Kolenosky (1975) described the northern limits of the "Algonquin type" wolves coinciding with the limits of white-tailed deer (*Odocoileus virginianus*) and deciduous forest. Their description of an absence of a cline between the "Ontario" and "Algonquin type" indicates recognition of a barrier to gene flow and is consistent with the theory that the boundary between the gray wolf and the eastern wolf was prevalent until the 1960s. There is some evidence that this frontier may have been south of the St Lawrence River in the mid 19th century as the Natural History of the Adirondacks describes the presence of two types of wolves similar (De Kay 1842) to the "Algonquin" and "Ontario" types of Standfield and Kolenosky (1975). The northward movement of the "Algonquin" type in Ontario likely paralleled the northward movement of the white-tailed deer.

Earlier genetic studies of wolves in the Great Lakes region concluded that there are "hybridizing" wolf populations in northwest Ontario, Minnesota as well as in Algonquin Provincial Park and extending east in southern Quebec (Lehman et al. 1991, Wayne et al. 1992; Wayne and Lehman 1992). This conclusion was based largely on the presence of both gray wolf and "coyote" mitochondrial DNA (mtDNA) in wolves in these areas. A recent study has proposed that the eastern Canadian wolf in Algonquin Provincial Park is closely related to the red wolf, *C. rufus*, and that both diverged from the

coyote 300,000 years ago, while the gray wolf diverged more than one million years ago (Wilson et al. in press). It was further suggested that this wolf should retain its original species designation of *C. lycaon* (Pockock 1935, Peterson 1966) rather than the presently accepted gray wolf subspecies designation of *C. l. lycaon*. The proposed evolutionary relationship of *C. rufus* and *C. lycaon* to the coyote, *C. latrans*, is consistent with the presence of sister-species hybridizing in eastern North America and the absence of hybridisation in western North America (Roy et al. 1994, Boyd and Forbes 1998).

In this study, we analyzed the control region of the mtDNA and eight microsatellite loci in 345 Ontario wolf samples that were collected between 1965 and 1999. The primary objective of the study was to understand the genetic relationships of the four wolf "types" identified by Kolenosky and Standfield (1975) in the context of the coyote (*C. latrans*) and two distinct wolf species, the gray wolf (*C. lupus*) and the eastern Canadian wolf/red wolf (*C. lycaon*).

MATERIALS & METHODS

Sample Collection and DNA Extraction

We analysed 269 samples from the 1990s from 6 geographic regions within Ontario (Figure 2): the Frontenac Axis (n=74); the Magnetawan Region (n=26); Algonquin Provincial Park (n=92); Northeastern Ontario, north of the French River and south of Highway 11 (n=34) (includes animal from Fort Severn); Northwestern Ontario (n=30); and Pukaskwa National Park (n=13) (Table 1). In addition, three of the 6 geographic regions were represented by 76 teeth samples from the 1960s: Algonquin Provincial Park (n=19); Northeastern Ontario (n=46); and Northwestern Ontario (n=11) (Table 1). DNA from present day blood and tissue samples was extracted by standard phenol-chloroform extraction methods described in Guglich et al. (1994). Both the hide samples and the teeth from the 1960s were extracted using a modified QIAmp (Qiagen) extraction protocol.

Mitochondrial DNA Analysis

*Sizing Assay for the Identification of New World (*C. lycaon/C. latrans*) and Old World (*C. lupus*) Control Region Sequences.* A previously described method (Pilgrim et al. 1998) for distinguishing *C. lupus* mtDNA from *C. latrans* was modified to identify the presence or absence of gray wolf mtDNA within the 6 geographic regions. A 343-347 bp product of the mtDNA control region was amplified using primers described in Wilson et al. (*in press*). The control region was amplified in a total reaction volume of 10µl per tube using 25ng of genomic DNA, 200 µM dNTPs, 1x amplification buffer, 2 mM $MgCl_2$, primers 1 and 2 (0.2 µM) and 0.5 units of *Taq* polymerase (BRL). Products were amplified under the following conditions: 94°C for 5 minutes, 55°C for 30 seconds, 72°C for 30 seconds (1 cycle); 94°C for 30 seconds, 55°C for 30 seconds and 72°C for 30 seconds (35 cycles); 94°C for 30 seconds, 55°C for 30 seconds and 72°C for 2 minutes (1 cycle). Products were then mixed with 0.4 volume of formamide loading buffer and were heated at 95°C for 5 minutes before loading onto a 6% sequencing gel containing 50% (w/v) urea. A control sequencing reaction of phage M13 DNA was run adjacent to the samples to produce size markers. The bands were visualized by autoradiography.

Microsatellite Analysis

Eight microsatellite loci (Ostrander et al. 1993, Roy et al. 1994, 1996) were amplified in a total reaction volume of 10µl using 25ng of genomic DNA, 200 µM dNTPs, 1x amplification buffer, 2 mM $MgCl_2$, unlabeled primers R and F (0.2 µM and 0.18 µM), radioactively labeled γ^{33}P-dATP (ICN) F primer (0.02 µM), 1 µg of Bovine Serum Albumin (BSA) (Pharmacia) and 0.5 units of *Taq* polymerase (BRL). Products were amplified under the following conditions: 94°C for 5 minutes, 55-65°C for 30 seconds, 72°C for 15 seconds (1 cycle); 94°C for 15 seconds, 55-65°C for 30 seconds and 72°C for 15 seconds (30 cycles); 94°C for 15 seconds, 55-65°C for 30 seconds and 72°C for 2 minutes (1 cycle). Products were then mixed with 0.4 volume of formamide loading buffer and were heated at 95°C for 5 minutes before loading onto a 6% sequencing gel containing 50% (w/v) urea. A control sequencing reaction of phage M13 DNA

was run adjacent to the samples to produce size markers for the microsatellite alleles. The bands were visualized by autoradiography.

Genetic Analysis

We analyzed allele frequencies at 8 loci among the 6 geographic regions. Nei's unbiased genetic distance (Nei 1978, Takezaki and Nei 1996) was calculated using the program GENETIX 3.3 (Belkhir et al.1998) and a neighbor-joining tree was constructed in the computer program PHYLIP (Felsenstein 1993). Genetic structuring between populations was estimated using R_{ST} (Slatkin 1995) from the computer program R_{ST}CALC (Goldman 1997). Levels of significance for the pairwise R_{ST} values were calculated following 1000 bootstraps and permutations of the data. R_{ST} is applied to loci undergoing a stepwise mutation process permitting homoplasy where two alleles of the same size can occur independently in two populations. This is best applied to our data set as we are assessing two lineages, *C. lupus* (Old World) and *C. lycaon/C. latrans* (New World) which diverged 1-2 million years ago.

An individual index was calculated for each individual animal DNA profiles over all eight loci using the following equation: I_I (each individual) = Σ log (Xp_A/Xp_B) where p_A and p_B are the allele frequencies of allele X from population A and B respectively. The log of the frequency of allele X in population A is taken over the frequency of the same allele X in population. In the event an allele was absent from one population, the frequency of 1 allele in the population, i,e, sample size, was used. Once the log of all the ratios for every allele over all loci are calculated, the sum of the log values is calculated to give a LOD score which is designated as an Individual Index score. This LOD score value assess the origin of the alleles in each animal based on a ratio of frequencies from two populations. If the populations have similar allele frequencies then the I_I values of individuals from both populations would follow a distribution around zero. An increasing positive score indicates an individual originated from population A and an increasing negative score indicates an individual originated from population B.

RESULTS

Mitochondrial control region analysis

To broadly assess the distribution of *C. lycaon* and *C. lupus* and the impact of *C. latrans* introgression in certain regions of Ontario, we screened a short amplified segment of the control region sequence for size differences. This allowed us to differentiate between animals with *C. lupus* mtDNA (Old World-evolved) and animals with *C. lycaon/C. latrans* mtDNA (New World-evolved) (Table 2). Surprisingly there was little evidence of major changes over the last 30 years with no gray wolf (Old World) mtDNA in Algonquin Park samples from the 1960s and only 4 in the 92 samples from the 1990s. These represented four full siblings, of which had microsatellite profiles similar to the other Algonquin animals which had New World mtDNA. There were only New World mtDNA in animals from both the Frontenac Axis and Magnetawan regions. The animals in both northeastern and northwestern Ontario represent a mixture of New and Old World mtDNA and this does not appear to have changed significantly in the last 30 years. Eleven of the 13 animals from within Pukaskwa National Park had Old World mtDNA.

Microsatellite loci analyses

We genotyped 345 wolf samples at 8 microsatellite loci. The samples were grouped into 6 areas (Table 1) (Fig. 2) that approximately corresponded to the distribution of the "types" identified by Kolenosky and Standfield (1975) and allele frequencies were determined (Appendix I). The allele profiles from 49 of the 92 wolves from Algonquin were used to represent this population following the removal of known parent-offspring relationships. Population structuring was assessed using R_{ST} (Slatkin 1995) to determine levels of gene flow among these regions (Table 3a). Limited gene flow was detected between the *C. lycaon* from Algonquin Park found in the Park and "Tweed wolves" found to the south and east (Frontenac Axis region) of the park. Less structuring was apparent between those animals found to the west and northwest of the Park (Magnetawan region) and the Algonquin Park animals. Surprisingly the animals from northeastern Ontario, in the area north of the French River and east of Georgian Bay and Lake Superior showed marked structuring with the gray wolves found in the adjacent Pukaskwa National Park, but little structuring with the more distant Algonquin Park *C. lycaon* population. Even more

surprising was the lack of structuring of the northwestern Ontario animals and the animals in Algonquin Provincial Park. In both northwestern and northeastern Ontario there was little structuring between animals with *C. lupus* (Old World) or *C. lycaon/C. latrans* (New World) mtDNA suggesting a single population with both types of mtDNA segregating (Table 3b).

Allele F at microsatellite locus cxx172 was first seen in Algonquin Park animals, having not been observed in previous analyses of North American wolf and coyote populations (Wilson et al. *in press*, Roy et al. 1994). This allele was seen at its highest frequency (0.357) in animals from Algonquin Provincial Park in the 1960s (Table 4). It was found in animals from both northeastern and northwestern Ontario where its frequency may have declined since the 1960s and was absent in gray wolves found in Pukaskwa National Park. These data and the structuring analysis are consistent with the northeastern and northwestern animals being more related to Algonquin Provincial Park *C. lycaon* than the Pukaskwa National Park *C. lupus.*

In order to further assess the relationships of the animals in the six regions we estimated Nei's unbiased genetic distance using the microsatellite loci allele frequencies (Table 4, Fig. 3). This indicated a separation of the northeastern and northwestern animals from those in Algonquin Provincial Park, the Magnetawan region and the Frontenac Axis.

Assignment Analysis

In order to assess the relationships of the groups and the homogeneity of the animals within the groups, we used an assignment procedure that was based on the likelihood of a genotype originating from one of two taxonomic groups (Wilson et al. *in press*). We established databases to represent eastern Canadian wolves/red wolves (*C. lycaon*), eastern coyotes (*C. latrans*) and gray wolves (*C. lupus*). The *C. lycaon* database consisted of 10 random samples from each of Algonquin Provincial Park, northeastern Ontario and the captive red wolf program. Red wolves were included in the representative *C. lycaon* database based on a previous analysis (Wilson et al. *in press*). Neighbouring northeastern Ontario animals were included based on the low R_{ST} values and to provide a representative sample of the potentially northward movement of *C. lycaon.* As Algonquin Park animals have been impacted by coyote

introgression (Wilson et al. *in press*), the northeastern Ontario animals would represent either "purer" *C. lycaon* genomes or *C. lycaon* animals impacted by *C. lupus* genetic material. The eastern coyote database contained 10 random samples from Cortlandville, NY, the Adirondacks and the Frontenac Axis based on similarities observed between the "Tweed" wolf and the eastern coyote (Wilson et al. *in preparation*). The gray wolf database consisted of 10 samples from Pukaskwa with complete genotypes at all 8 microsatellite loci and 10 random samples from both Riding Mountain National Parks and the Northwest Territories.

Algonquin wolves showed a different distribution of index values from Adirondack eastern coyotes using the *C. lycaon* and *C. latrans* as the comparative databases (Fig. 3). The "Tweed wolves" of the Frontenac Axis showed considerable overlap with the coyote-like animals of the New York Adirondacks (Fig. 4) with minimal overlap with Algonquin Provincial Park animals, which is consistent with the structuring analysis (Table 3a). Animals in the Magnetawan region appear to be a mixture of those in the Frontenac Axis "Tweed wolf" and the *C. lycaon* of Algonquin Provincial Park, which is also consistent with the structuring values (Fig. 5).

To assess the relationship of animals in northern Ontario, we compared samples to the representative *C. lycaon* and *C. lupus* databases. Algonquin wolves showed an overall different distribution of values from Northwest Territories wolves (Fig. 6), consistent with previous findings (Wilson et al. *in press*). The 10 animals from Pukaskwa National Park with complete genotypes at all 8 loci showed overlap with the NWT gray wolf (Fig. 6). For animals in northeastern Ontario (Fig. 7) and northwestern Ontario (Fig. 8), although distributions were different, some animals have genotypes resembling *C. lycaon* of Algonquin Provincial Park, although some appear to originate from *C. lupus* populations genetically similar to the Pukaskwa gray wolves. Low R_{ST} scores between Algonquin Provincial Park, northeastern and northwestern Ontario provides evidence of gene flow (Table 3) consistent with the assignment analysis where animals from these regions appeared very similar. Unfortunately, we had few samples from northern Ontario to assess whether the Pukaskwa National Park animals were representative of gray wolves of the Hudson Bay Lowlands. One animal from the coastal region of Hudson Bay (Fort Severn) had a genetic profile similar to animals in Pukaskwa National Park.

Taxonomic Designation of Ontario Wolves

Although the data presented above suggests gene flow between the "types" of wolves, for management and conservation purposes it is important to classify Ontario wolves and estimate the numbers of each type. Genetic data show remarkable congruence with the "races" or "types" described by Kolenosky and Standfield (1975). We used the representative eastern Canadian wolf (*C. lycaon*), eastern coyote (*C. latrans*) and gray wolf (*C. lupus*) allele frequency databases used in the assignment analysis to attempt a genetic classification.

Using the index distributions, we developed criteria to distinguish *C. latrans / C. lycaon* hybrids (Tweed wolf) from *C. lycaon* (Algonquin type). Any animal with a score of 0 or greater was classified as *C. lycaon* and any individual falling below an index value of 0 was classified as a *C. latrans / C. lycaon* hybrid (Table 5). The nature of the mitochondrial DNA was not used in this assessment, as only 4/214 animals in the Magnetawan, Algonquin Park and Frontenac Axis regions contained Old World mtDNA and mtDNA can be introgessed into populations and maintained despite back-crossing.

A similar approach was used to classify *C. lupus* and *C. lupus / C. lycaon* hybrids from *C. lycaon* in Ontario (Table 5). Animals with a score of less than or equal to –1 with an Old World mtDNA were classified as *C. lupus*, while animals with scores in this range with New World mtDNA were classified as *C. lycaon / C. lupus* hybrids. Animals with a score greater than -1 with a New World haplotype were classified as *C. lycaon*, while animals with scores in this range with an Old World mtDNA were also classified as hybrids. We recognize a continuum of genotypes exits rather than discrete taxonomic units but these criteria allow us to provide some assessment of numbers of each "type".

Based on these criteria (Table 5) 86% of the animals of the Frontenac Axis classify as 'Tweed wolves" while 14% were classified as the "Algonquin type", *C. lycaon*. In contrast, 86% of Algonquin Provincial Park animals were classified as *C. lycaon* and 14% as "Tweed wolves". West and north of Algonquin Provincial Park (Magnetawan), 42% of the animals were classified as "Tweed" like animals, 54% were identified as "Algonquin type" animals and another 4% were classified as *C. lupus / C. lycaon* hybrids (Table 6).

North of the Magnetawan region across the French River, in northeastern Ontario, 38% of the animals were classifed as *C. lycaon*, 29% as *C. lycaon / C. lupus* hybrids, 24% as the "Ontario type" *C. lupus* and 9% were classified as *C. lycaon / C. latrans* hybrids. Of the "Ontario type" animals, one of these was from Fort Severn on the Hudson Bay coast and two were from the northern part of the region. In general the northeastern Ontario animals are predominantly *C. lycaon* that has had some gray wolf genetic introgression, but less coyote introgression than those in the south. Within the northern limits of the northeastern Ontario region there was a small population of "Ontario type", *C. lupus* in Pukaskwa National Park, although one animal within the national park pack structure was classified as a *C. lycaon / C .lupus* animal. The Algonquin Provincial Park allele F at locus cxx 172 is present at a frequency of 0.20 in northeastern Ontario but is completely absent in the population of Pukaskwa National Park (Table 4).

In northwestern Ontario, 27% of animals have *C. lupus* (Old World) mtDNA and the "Algonquin type" allele F at locus cxx 172 is present at a frequency of 0.083 (Table 4). However, 34% of the animals were classified as *C. lycaon*, 60% as hybrids between *C. lycaon / C. lupus* and 3% as the *C. lupus* "Ontario type" (Table 6). One animal (3%) was classified as a *C. lycaon/C. latrans* hybrid, suggesting only a small amount of coyote introgression in this area similar to northeastern Ontario.

DISCUSSION

Prior to European settlement, wolves occupied all of Ontario (Bates 1958) and primarily preyed on larger ungulates such as elk (*Cervus elaphus*), caribou (*Rangifer tarandus*) and moose (*Alces alces*). Forested ecosystems were substantially altered as a result of logging and agriculture. These activities resulted in the decline of large ungulates such as elk and woodland caribou and also their gray wolf (*C. lupus*) predators and allowed the northern advancement of deer and eastern wolves (*C. lycaon*) and eventually coyotes (*C. latrans*). Changes in prey and habitat finally resulted in the elimination of wolves in southern Ontario (Standfield 1970). It is interesting to speculate that areas such as Algonquin Provincial Park were originally dominated by gray wolves preying on elk, caribou and moose and that the logging and associated human killing drove out the gray wolves and eliminated or reduced these large ungulates. As white-tailed deer moved into these areas they were followed by *C. lycaon* possibly originating from the

Adirondacks and moving through the Frontenac Axis or from southern Ontario. The reduction of wolves from southern Ontario also allowed the spread of the coyote throughout the newly created farmland and the subsequent hybridisation with *C. lycaon.*

The genetic data presented supports the hypothesis that the "Tweed wolf" as described by Kolenosky and Standfield (1975) and Kolenosky and Schmitz (1985) is a hybrid between the "Algonquin" type wolf and the coyote (*C. lycaon* x *C. latrans*). However, in contrast to Kolenosky and Standfield, recent genetic evidence (Wilson et al. *in press*) suggests that these hybrids originated from inter-breeding between two North American evolved Canis species, *C. lycaon*, representing the eastern Canadian wolf and red wolf, and the coyote, *C. latrans.* The absence of the gray wolf in this hybridisation explains the anomaly of the lack of inter-breeding between western coyotes and gray wolves (Roy et al. 1994, Wilson et al. *in press*). As Kolenosky and Schmitz (1985) alluded to, the absence of a "pure" coyote in southern Ontario is apparent immediately south of Algonquin Park and into the Frontenac Axis. This hybrid eastern wolf/coyote is extremely adaptable to both agricultural and low-density forested habitats. Despite the high numbers of the "Tweed" animals, southeast of Algonquin Park, evidence suggests barriers to gene flow exists by maintaining larger wolf-like animals within the park proper. Despite limited gene flow from the Frontenac Axis, the high level of genetic variation in Algonquin Park (Appendix I) is supported by the gene flow from the Magnetawan region, northeastern Ontario and potentially from Quebec. Although the Algonquin Park population numbers less than 200, evidence suggests it is part of a larger metapopulation that includes animals from northeastern and northwestern Ontario and Quebec. The low R_{ST} values between the northeast and northwest animals suggest there is substantial gene flow between both regions of the province. This supports the conclusion the population of *C. lycaon* in Ontario is large, numbering in the thousands rather than the hundreds. The "Algonquin" type *C. lycaon* ranges in size from smaller animals in Algonquin Provincial Park to larger animals in northeastern and northwestern Ontario (Kolenosky and Stanfield 1975). This cline is likely related to the introgression of more coyote genetic material in the south and more gray wolf genetic material in northern Ontario. Introgression of genes into *C. lycaon* animals may further be influenced by selection based on factors such as prey size (Hillis 1990, Mulders 1997). *Canis lycaon* within Algonquin Park prey predominantly on white-tailed deer and beaver

(Forbes and Theberge 1996). With the ecological changes in Algonquin Park from a high density of deer in the 1960s to the present lower densities and the highest moose densities in the province (Whitlaw and Lankester 1994), a selection for larger animals that can utilize moose more effectively might occur in the future. The connectivity of the Algonquin Park population to the northern animals may facilitate this natural evolution.

Pukaskwa National Park maintains a small population of gray wolves (*C. lupus*) that prey on moose, and appear to be surrounded by the larger "Algonquin type" animals in patchy habitat that contains moose and white-tailed deer. The high structuring value between the "Ontario type" wolves in Pukaskwa and the "Algonquin type" individuals suggests a predominantly *C. lupus* population with limited gene flow to surrounding *C. lycaon*. One animal in Pukaskwa National Park was assigned as a *C. lupus* / *C. lycaon* hybrid and one as *C. lycaon* suggesting that these gray wolves may be at the early phase of hybridisation with surrounding *C. lycaon*.

The broad band across northeastern and central Ontario, which Kolenosky and Standfield (1975) described as the area where the "Ontario" and "Algonquin" types meet, but in which interbreeding was apparently absent, now appears to contain hybrid wolves (Table 6). However, the hybrids still appear to be primarily "Algonquin" genotypes suggesting the hybridisation between *C. lupus* and *C. lycaon* is more restricted than that between *C. lycaon* and *C. latrans* to the south of Algonquin Park.

Due to our reliance on fur samples from commercial fur houses for many of our more northern samples we have few samples to allow us to assess animals in the Hudson Bay lowlands and the coastal regions of Hudson and James Bay. Of interest is the single animal from Fort Severn on the Hudson Bay coast that was assigned as originating from Pukaskwa National Park. This suggests there may not be a separate gray wolf subspecies, *C. l. hudsonicus* in the Hudson and James Bay coastal areas and is consistent with the Pukaskwa population representing a remnant population of gray wolves, while most of the population moved further north or was extirpated.

In northwestern Ontario, populations appear to be genetically related to animals in Algonquin Provincial Park. There appears to have been less hybridisation with coyotes and an absence of Tweed wolves in this area (Table 5). Animals have been classified genetically as *C. lycaon* at microsatellite DNA

profiles but the presence of a number of animals with gray wolf mtDNA haplotypes (Table 2) is evidence of past and perhaps present hybridisation with *C. lupus*. The Minnesota and Wisconsin wolves are probably the same as the animals in northwestern Ontario. Increases in wolf numbers in Minnesota have led to moves to de-list the gray wolf as endangered in the U.S. We would urge caution until classification of these wolves is clarified.

In summary, the genetic data support the hypothesis that the "Tweed wolf" is a hybrid between the coyote and eastern Canadian wolf (*C. latrans* and *C. lycaon*). The eastern Canadian wolf appears to be a North American-evolved species closely related to the red wolf and represents the "Algonquin" type described by Kolenosky and Standfield (1975). We suggest it retain its original taxonomic designation of *C. lycaon.* In northeastern and northwestern Ontario, *C. lycaon* has hybridized with the gray wolf (*C. lupus*) and is larger than the animals found in Algonquin Provincial Park. The populations in northeastern and northwestern Ontario appear to be genetically connected with the Algonquin Provincial Park population and Quebec populations and the total number of animals may number in the thousands. Pukaskwa National Park contains a small isolated population of *C. lupus* that might represent the original "Ontario type" described by Kolenosky and Standfield (1975). As a result of poor sampling in northern Ontario we have not resolved the genetics of the wolves in Hudson Bay lowlands or the coastal regions of Hudson and James Bay. The single animal from the Hudson Bay coast resembled animals from Pukaskwa suggesting that there may be only one gray wolf, *C. lupus*, subspecies in Ontario. The higher number of *C. lupus* animals in northeastern Ontario suggests the beginning of the present-day boundary between *C. lycaon* and *C. lupus* in this region. The equivalent boundary in northwestern Ontario may lie farther north as fewer *C. lupus* animals were detected in this region. The absence of a "*Canis* soup" in western North America appears to be attributed to the absence of *C. lycaon,* which readily hybridizes with coyotes and can hybridize with gray wolves, thus mediating gene flow among the three species.

Acknowledgements.

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Table 1. Wolf sample information including geographic location, number of samples, type of biological material and the source of the submitted material.

Area[1]	Number	type	Source
Frontenac Axis	74	muscle	University of Waterloo[2]
Algonquin Provincial Park (1960s)	19	teeth	OMNR
Algonquin Provincial Park (1990s)	92	muscle and blood	University of Waterloo[2]
Magnetawan Region	26	muscle	University of Waterloo[2]
Northeastern Ontario (1960s)	46	teeth	OMNR
Northeastern Ontario (1990s)	34*	hide	North Bay Fur House
Pukaskwa National Park	13	blood	Parks Canada[3]
Northwestern Ontario (1960s)	11	teeth	OMNR
Northwestern Ontario (1990s)	30	hide	Laurentian University[4]
Total	345		

[1] Location of area shown in Figure 2.
[2] University of Waterloo samples – see Theberge et al. 1999 and Sears et al. 1999
[3] provided by Dr P. Paquet and F. Burrows.
[4] Laurentian University samples provided by Dr F. Mallory.
*Ft. Severn animal included in Northeastern Ontario sample set. Only used in assignment analysis.

Table 2. Distribution of New World and Old World mitochondrial DNA control region in Ontario.

Population	Frontenac Axis	Magnetawan Region	Algonquin Provincial Park		Northeastern Ontario		Northwestern Ontario		Pukaskwa National Park
	1990	1990	1960	1990	1960	1990	1960	1990	1990
Old World	0	0	0	4	22	18	1	9	11
New World	74	26	19	88	24	14	10	21	2
Total	n=74	n=26	n=19	n=92	n=46	n=33	n-11	n=30	n=13

Table 3. A. R_{ST} values for each pairwise comparison of six Ontario geographic regions.

Population	Frontenac Axis	Magnetawan Region	Algonquin Provincial Park	Northeastern Ontario	Northwestern Ontario	Pukaskwa National Park
Frontenac Axis	-	0.040	0.112	0.099	0.116	0.292
Magnetawan Region		-	0.056	0.015	0.054	0.199
Algonquin Provincial Park			-	0.037	0.020	0.203
Northeastern Ontario				-	0.028	0.154
Northwestern Ontario					-	0.141
Pukaskwa National Park						-

Table 3B. R_{ST} values for each pairwise comparison of New World and Old World in northeastern and northwestern Ontario.

Population	Northeastern Ontario (New World)	Northeastern Ontario (Old World)	Northwestern Ontario(New World)	Northwestern Ontario (Old World)
Northeastern Ontario (New World)	-	0.049	0.041	0.033
Northeastern Ontario (Old World)		-	0.035	0.002
Northwestern Ontario (New World)			-	0.028

Table 4. Allele frequency distributions at microsatellite locus cxx. 172 in 6 geographic regions of Ontario.

Locus cxx.172	Frontenac Axis	Magnetawan Region	Algonquin Provincial Park		Northeastern Ontario		Northwestern Ontario		Pukaskwa National Park
	1990	1990	1960	1990	1960	1990	1960	1990	1990
A.....	0.385	0.269	0.179	0.316	0.340	0.280	0.182	0.283	0.115
F*.....	.0054	0.154	0.357	0.163	0.045	0.020	0.136	0.083	0.000
G.....	0.014	0.308	0.036	0.031	0.045	0.180	0.136	0.067	0.346
H.....	0.270	0.212	0.071	0.235	0.364	0.400	0.455	0.517	0.423
I.....	0.277	0.000	0.357	0.255	0.205	0.120	0.091	0.050	0.115
	n=74	n=26	n=14	n=49	n=22	n=33	n=11	n=30	n=13

Note: Each allele is represented by an alphabetic letter with A representing the smallest allele.
The * represents the *lycaon*-specific marker.

Table 5. Criteria for distinguishing four different Wolf "types" in Ontario.

Classification	**mtDNA**	**Index** *C. lycaon* **vs.** *C. latrans*	**Index** *C. lycaon* **vs.** *C. lupus*
C. lupus	**OW**	-	≤ -1
C. lycaon / C. lupus	**OW**	-	> -1
C. lycaon / C. lupus	**NW**	-	≤ -1
C. lycaon	**NW**	> 0	> 1
C. lycaon / C. latrans	**NW**	≤ 0	-

Table 6. Distribution of Wolf "types" in Ontario.

Population[1]	Frontenac Axis	Magnetawan Region	Algonquin Provincial Park	Northeastern Ontario	Northwestern Ontario	Pukaskwa National Park
C. lupus	0	0	0	8	1	9
C. lupus / C. lycaon	0	1	0	10	18	1
C. lycaon	10	14	42	13	10	0
C. lycaon / C. latrans	64	11	7	3	1	0
Total	n=74	n=26	n=49	n=34	n=30	n=10

Table 7. Nei's (1978) genetic distance for animals in the 6 Ontario geographic regions.

Population	Frontenac Axis	Magnetawan Region	Algonquin Provincial Park	North-eastern Ontario	North-western Ontario	Pukaskwa National Park	Northwest Territories
Frontenac Axis	-	0.062	0.129	0.238	0.313	0.351	0.874
Magnetawan Region		-	0.043	0.151	0.167	0.212	0.622
Algonquin Provincial Park			-	0.180	0.175	0.244	0.691
Northeastern Ontario				-	0.045	0.146	0.395
Northwestern Ontario					-	0.127	0.454
Pukaskwa National Park						-	0.332
Northwest Territories							-

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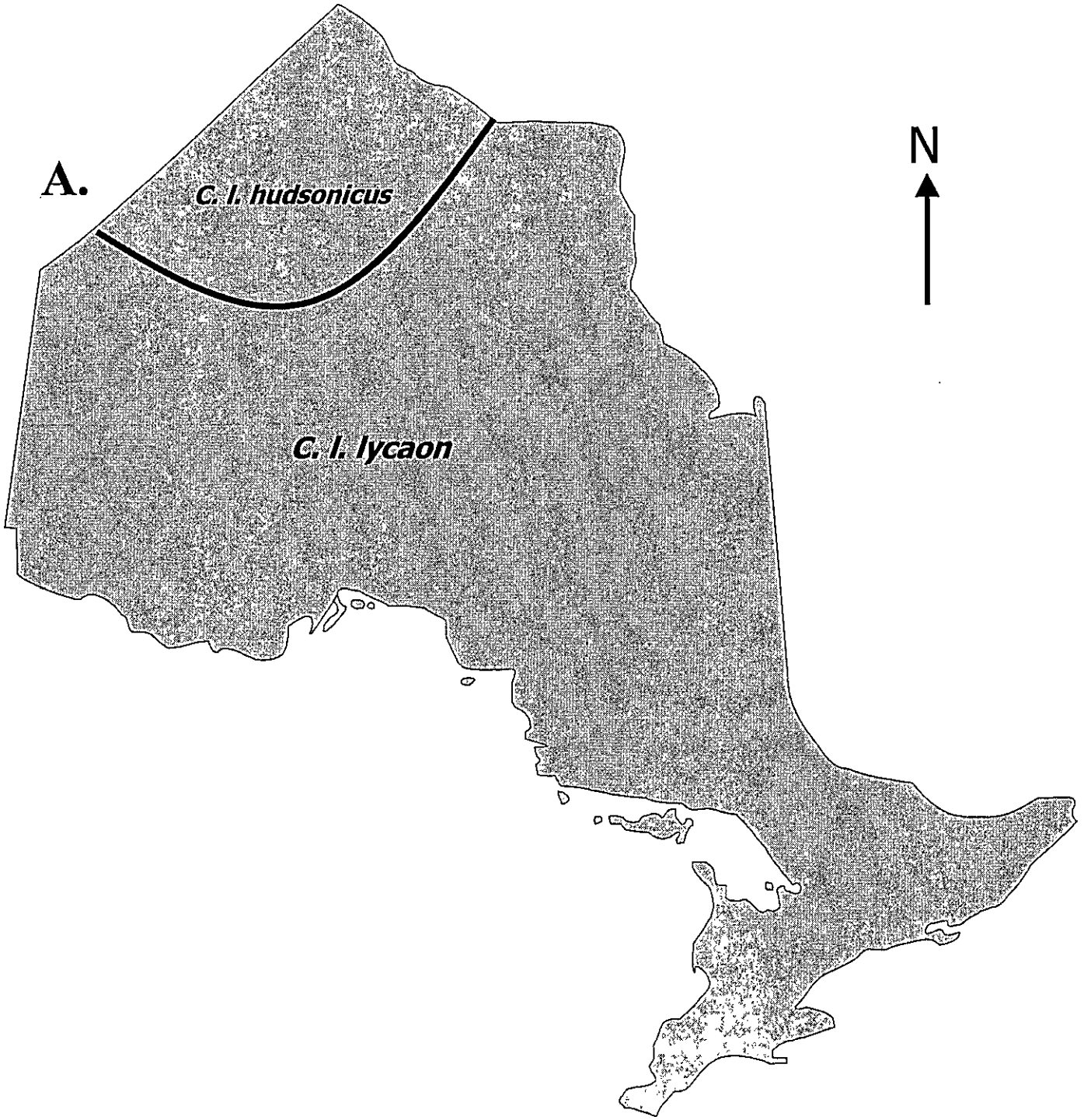
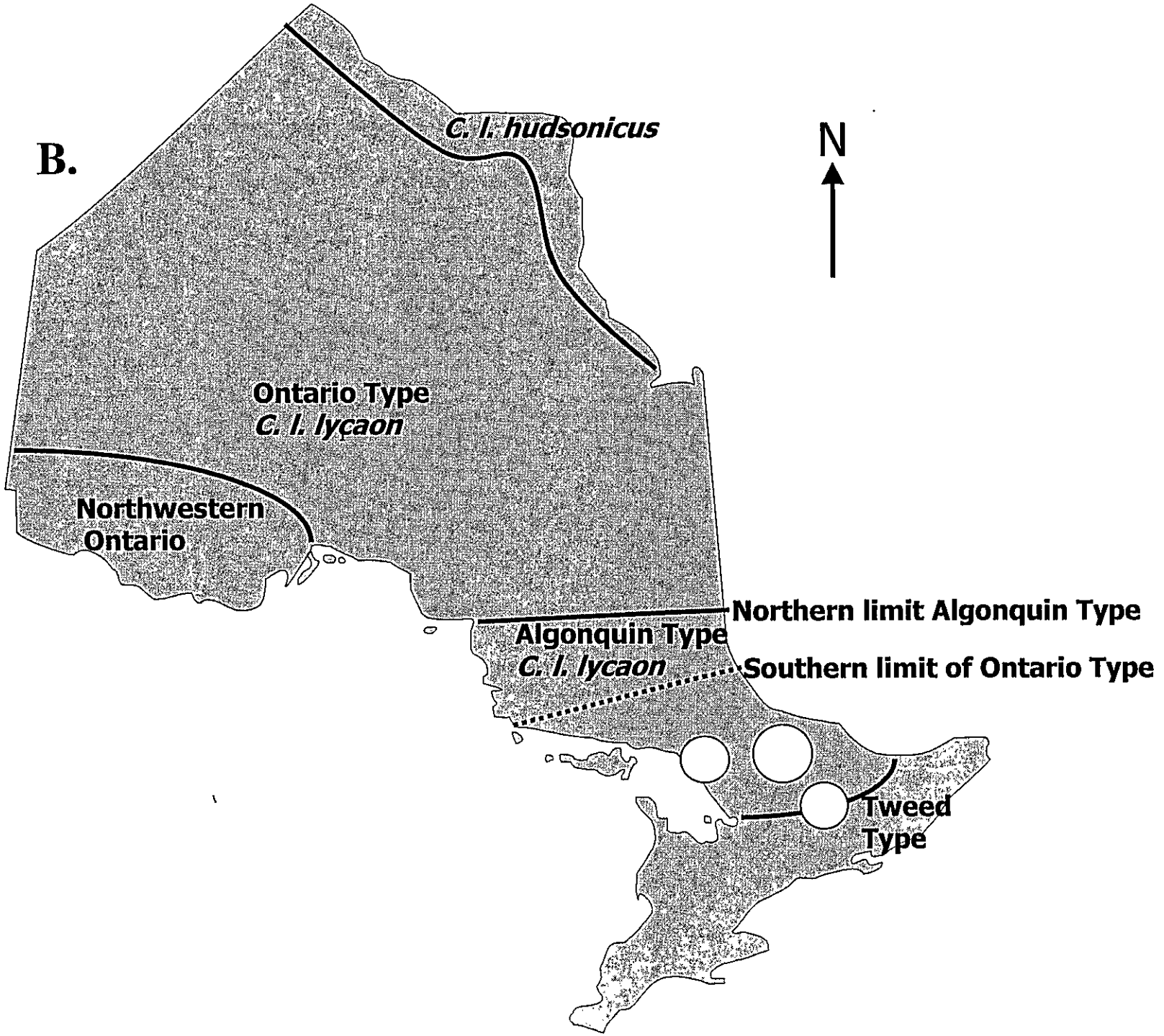
Figure 1. Previous assessments of the distribution of wolf types in Ontario. **A.** Hall and Kelson 1959. **B.** Stanfield and Kolenosky, 1975 showing the estimated northern limit of the "Algonquin type" and southern limit of the "Ontario type" in central Ontario. The larger circle represents Algonquin Provincial Park and the smaller circles are locations of "Tweed" wolves. **C.** Nowak, 1995. Showing most of Ontario occupied by the gray wolf sub-species *C. l. nubilus* that he also placed throughout the Plains States of the US.


A.
C. l. hudsonicus
C. l. lycaon
N


B.
C. l. hudsonicus
N
Ontario Type
C. l. lycaon
Northwestern
Ontario
Northern limit Algonquin Type
Algonquin Type
C. l. lycaon
Southern limit of Ontario Type
Tweed
Type

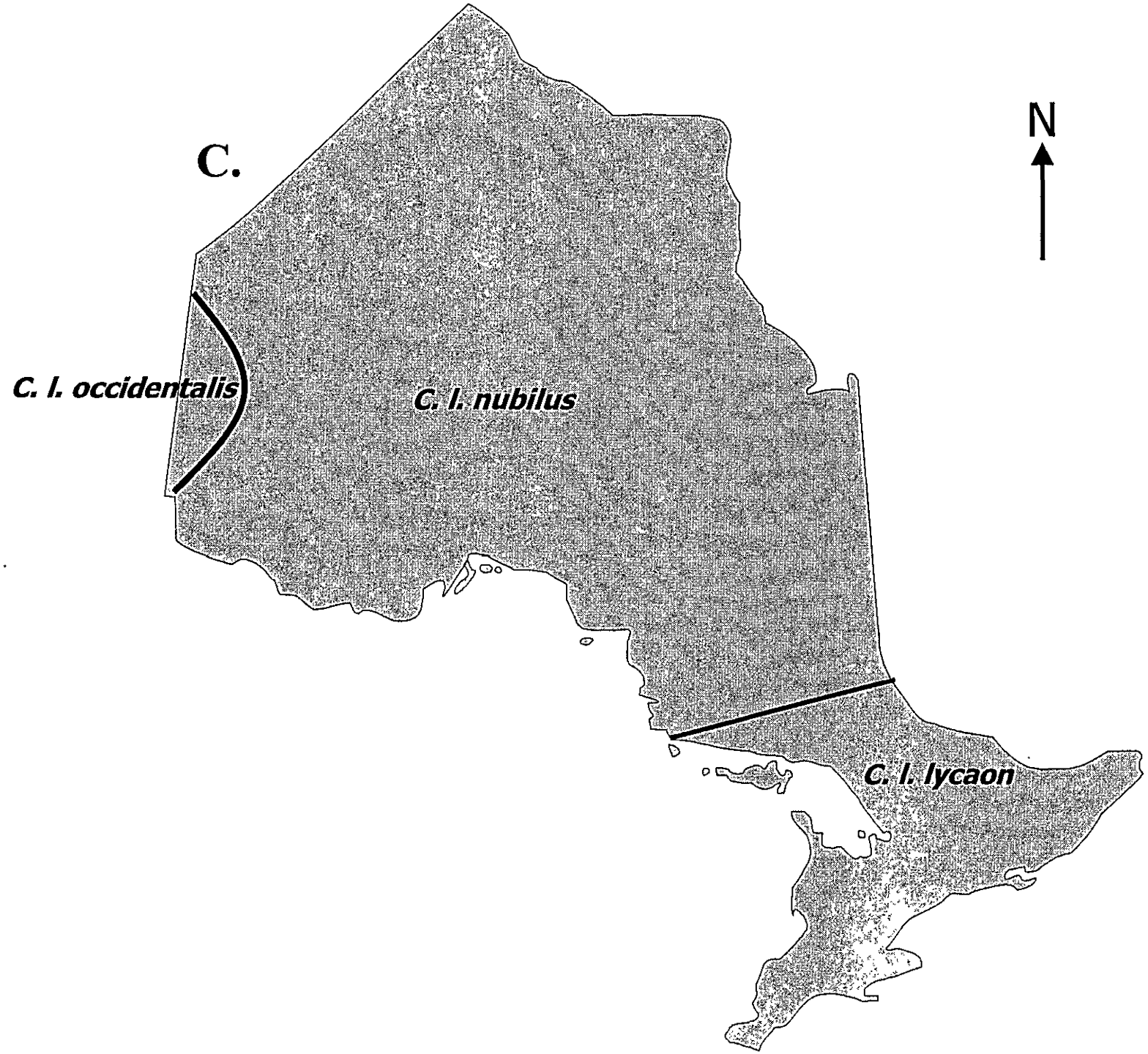

C.
N
C. l. occidentalis
C. l. nubilus
C. l. lycaon

Figure 2. Map showing location of wolf samples from across Ontario.The samples (Table 1) were grouped into six regions in order to examine the types described by Kolenosky and Stanfield, 1975. Northwest Ontario, northeast Ontario, Pukaskwa National Park, Algonquin Provincial Park, Magnetewan region to the west and north of Algonquin Provincial Park and the Frontenac Axis to the west and south of Algonquin Provincial Park. One animal came from Fort Severn (indicated by star) on the shore of Hudson Bay.

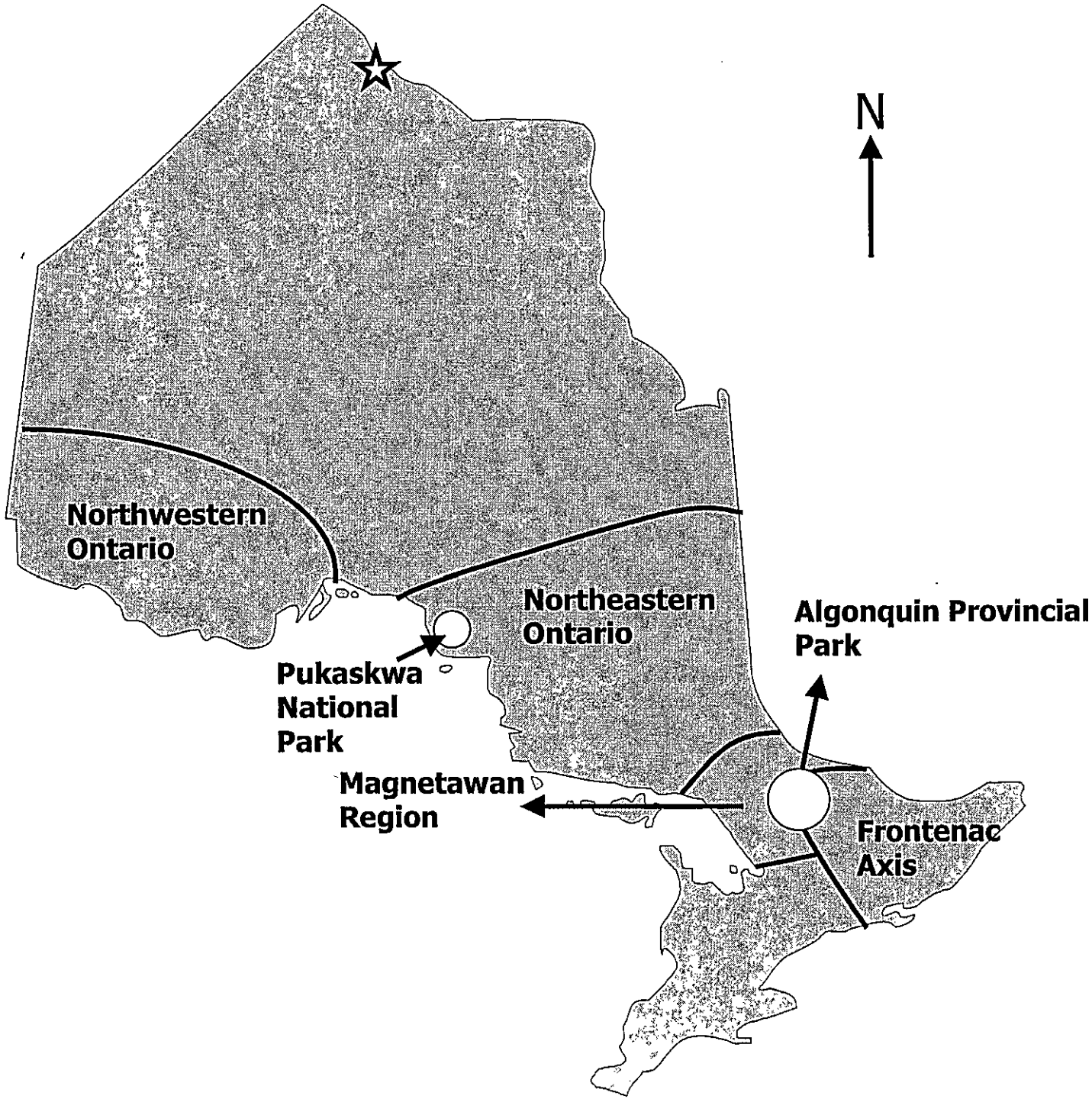

N
Northwestern Ontario
Northeastern Ontario
Algonquin Provincial Park
Pukaskwa National Park
Magnetawan Region
Frontenac Axis

Figure 3: Distribution of Individual Indicies of Algonquin and Adirondack animals based on microsatellite allele frequencies from representative *C. lycaon* and eastern coyotes (*C. latrans*) samples. The log likelihood (Individual Index, Ii) of a genotype originating from Algonquin Provincial Park compared to the Adirondacks was determined in these areas.

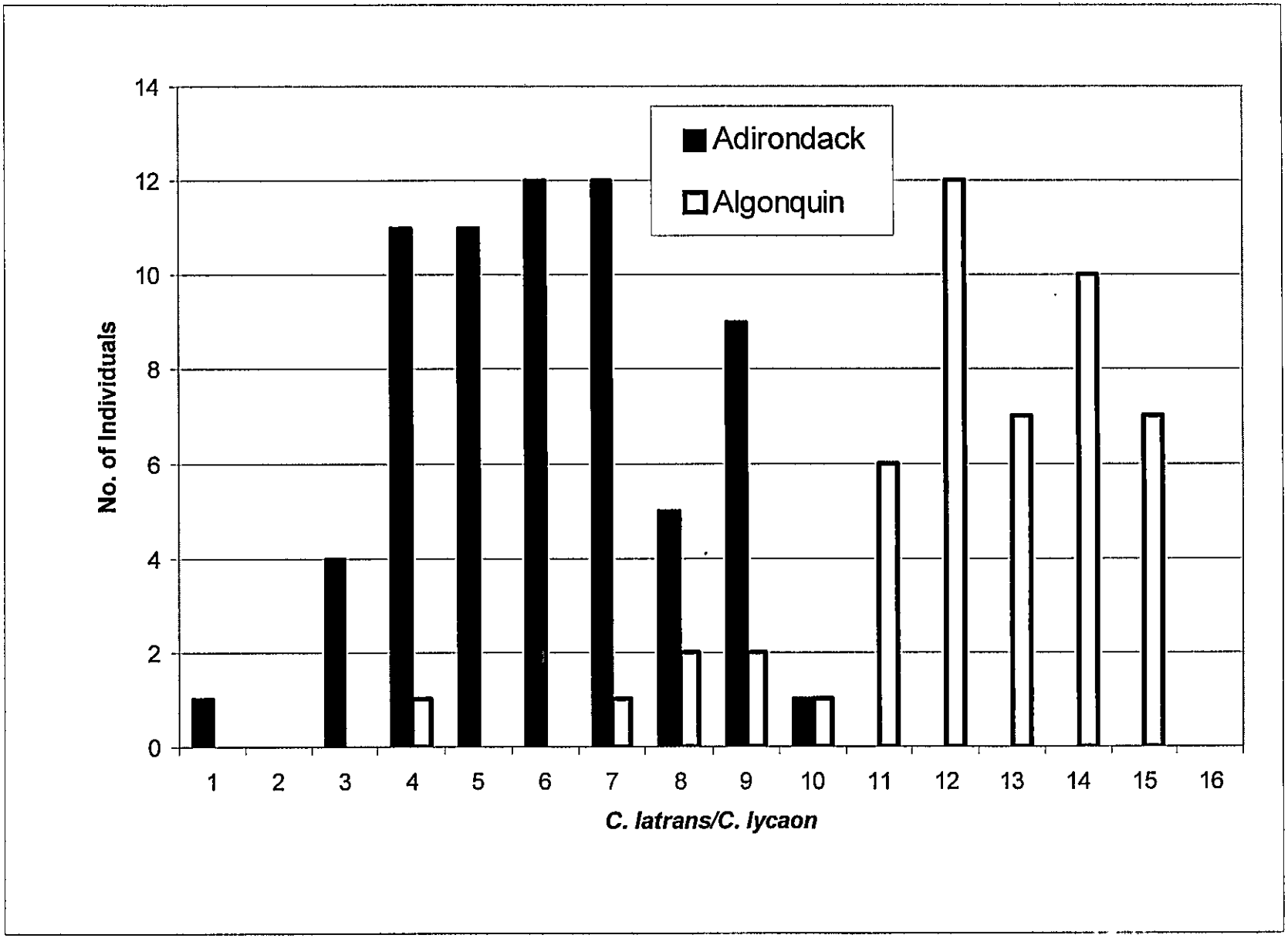

Adirondack
Algonquin
No. of Individuals
0
2
4
6
8
10
12
14
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
C. latrans/C. lycaon

Figure 4. Distribution of Individual Indices based on allele frequencies from representative *C. lycaon* and eastern coyotes (*C. latrans*) samples. The log likelihood of a genotype originating from Algonquin Provincial Park compared to the Adirondacks in northern New York State was determined for animals in these areas and the Frontenac Axis region.

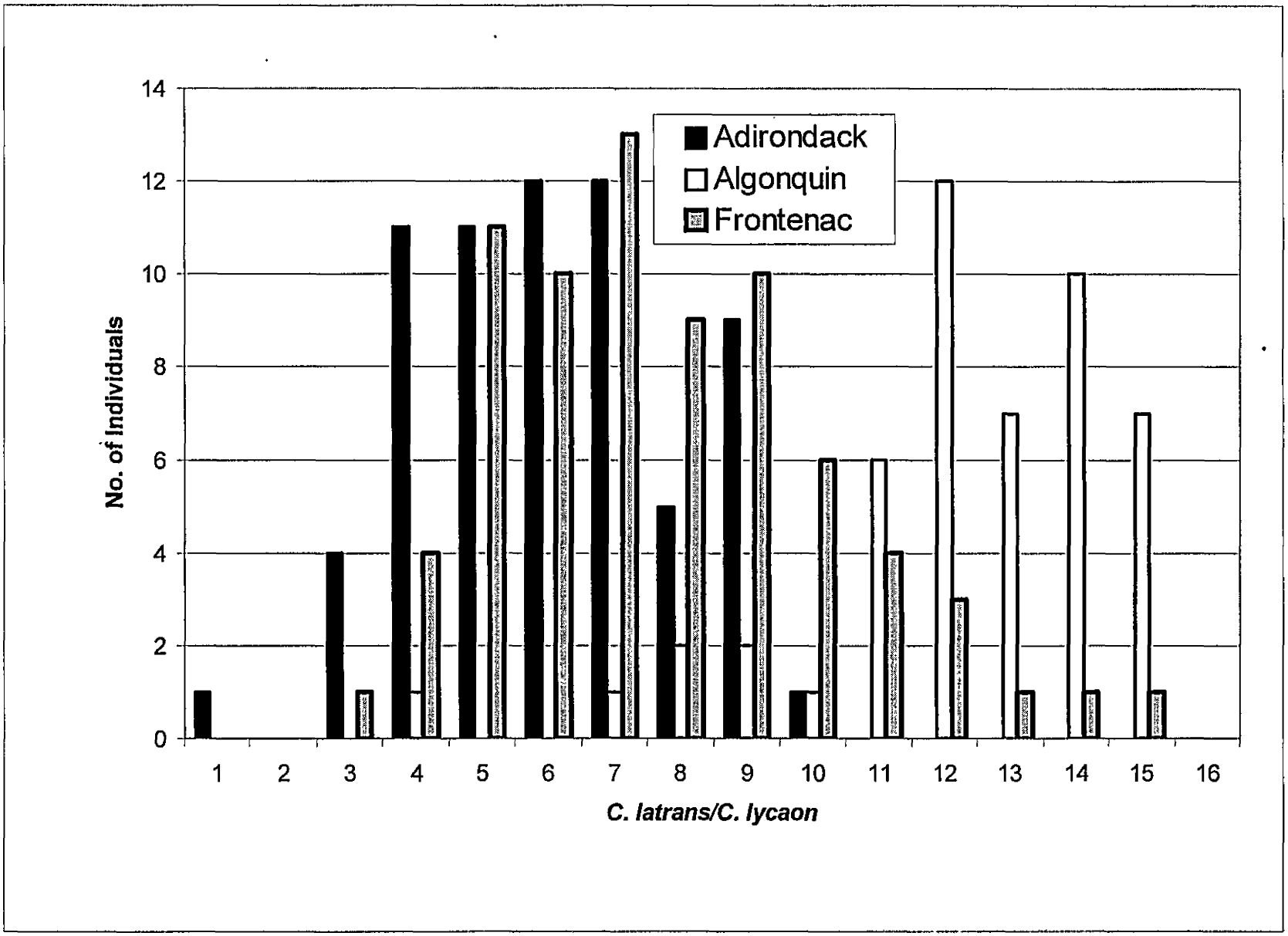

Adirondack
Algonquin
Frontenac
No. of Individuals
0
2
4
6
8
10
12
14
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
C. latrans/C. lycaon

Figure 5. Distribution of Individual Indices based on allele frequencies from representative *C. lycaon* and eastern coyotes (*C. latrans*) samples. The log likelihood of a genotype originating from Algonquin Provincial Park compared to the Adirondacks in northern New York State was determined for animals in these areas and the Magnetewan region.

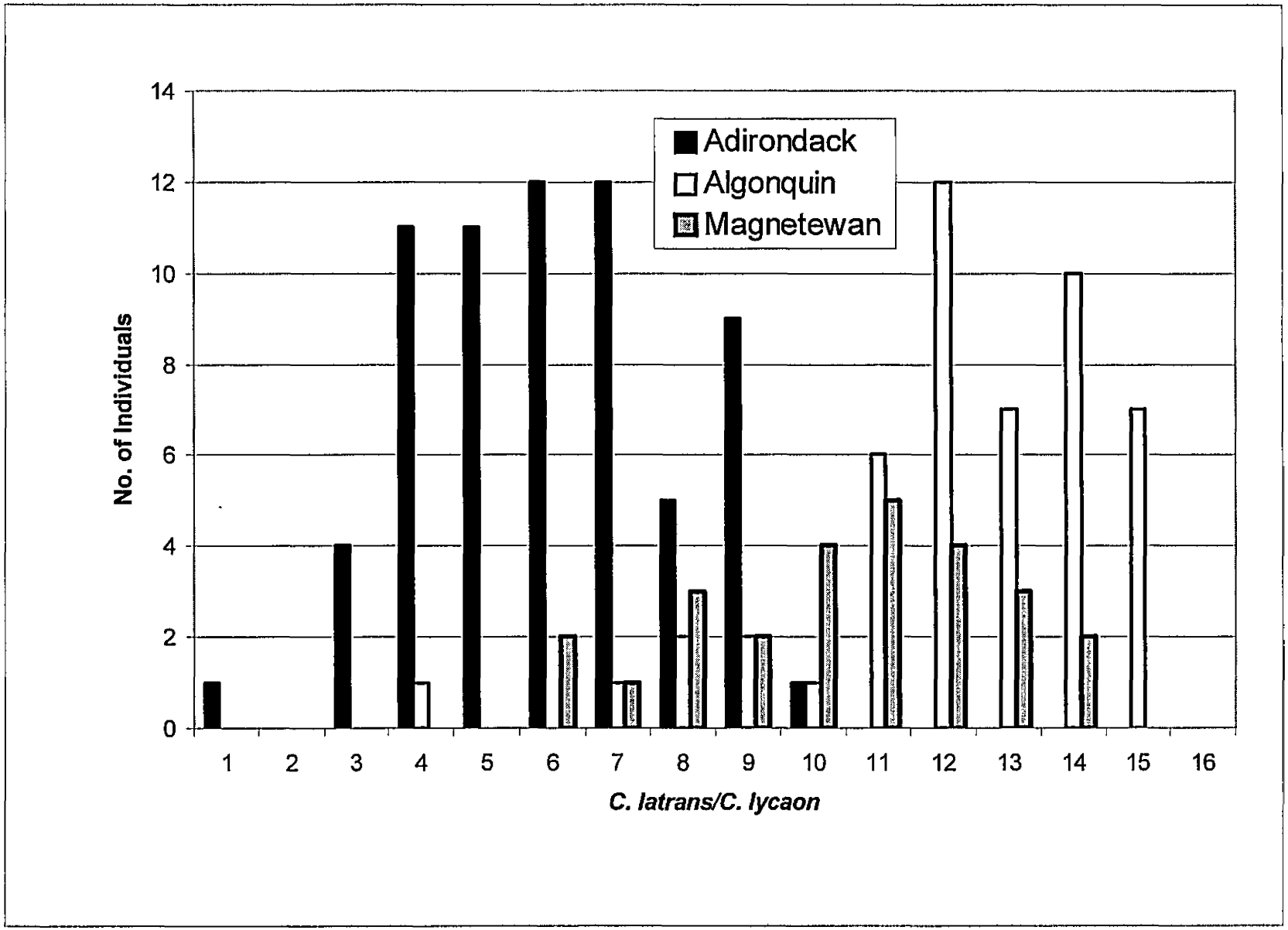

14
12
10
8
6
4
2
0
No. of Individuals
Adirondack
Algonquin
Magnetewan
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
C. latrans/C. lycaon

Figure 6: Distribution of Individual Indices based on allele frequencies from representative *C. lycaon* and gray wolves (*C. lupus*) samples. The log likelihood of a genotype originating from Algonquin Provincial Park compared to the Northwest Territories was determined in these areas and Pukaskwa National Park.

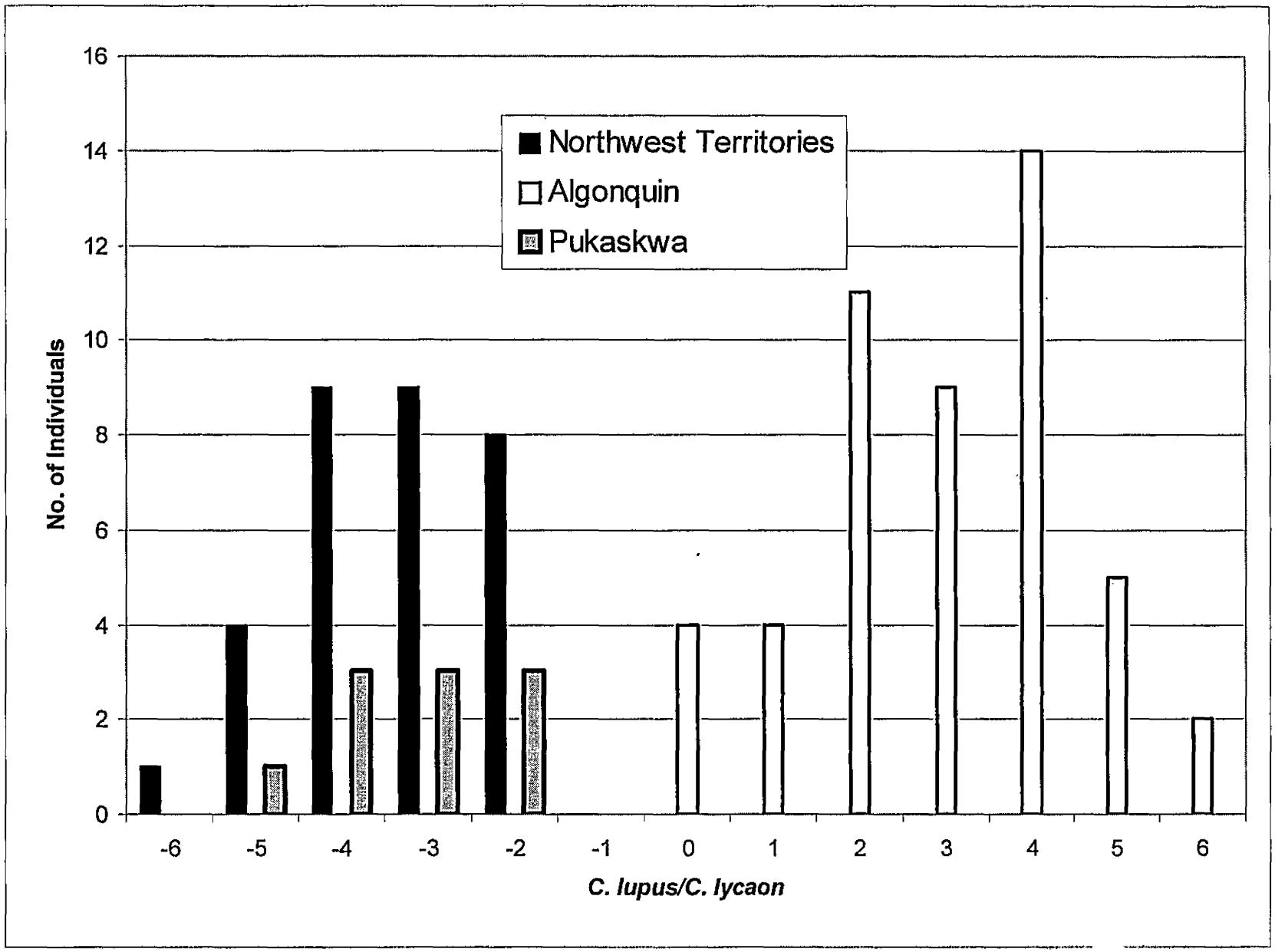

16
14
12
10
8
6
4
2
0
No. of Individuals
Northwest Territories
Algonquin
Pukaskwa
-6
-5
-4
-3
-2
-1
0
1
2
3
4
5
6
C. lupus/C. lycaon

Figure 7. Distribution of Individual Indices based on allele frequencies from representative *C. lycaon* and gray wolves (*C. lupus*) samples. The log likelihood of a genotype originating from Algonquin Provincial Park compared to the Northwest Territories was determined in these areas and the northeastern Ontario region.

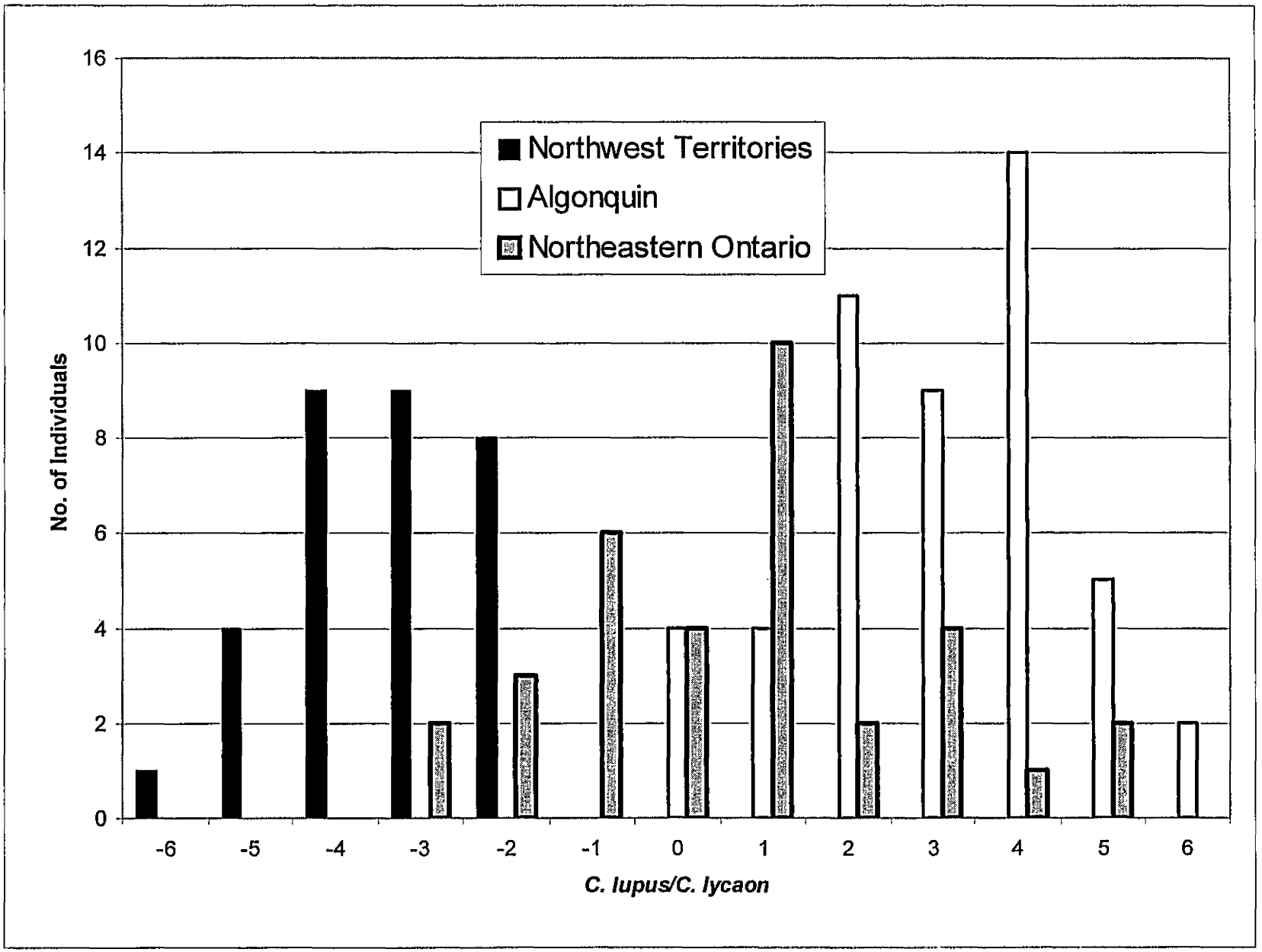
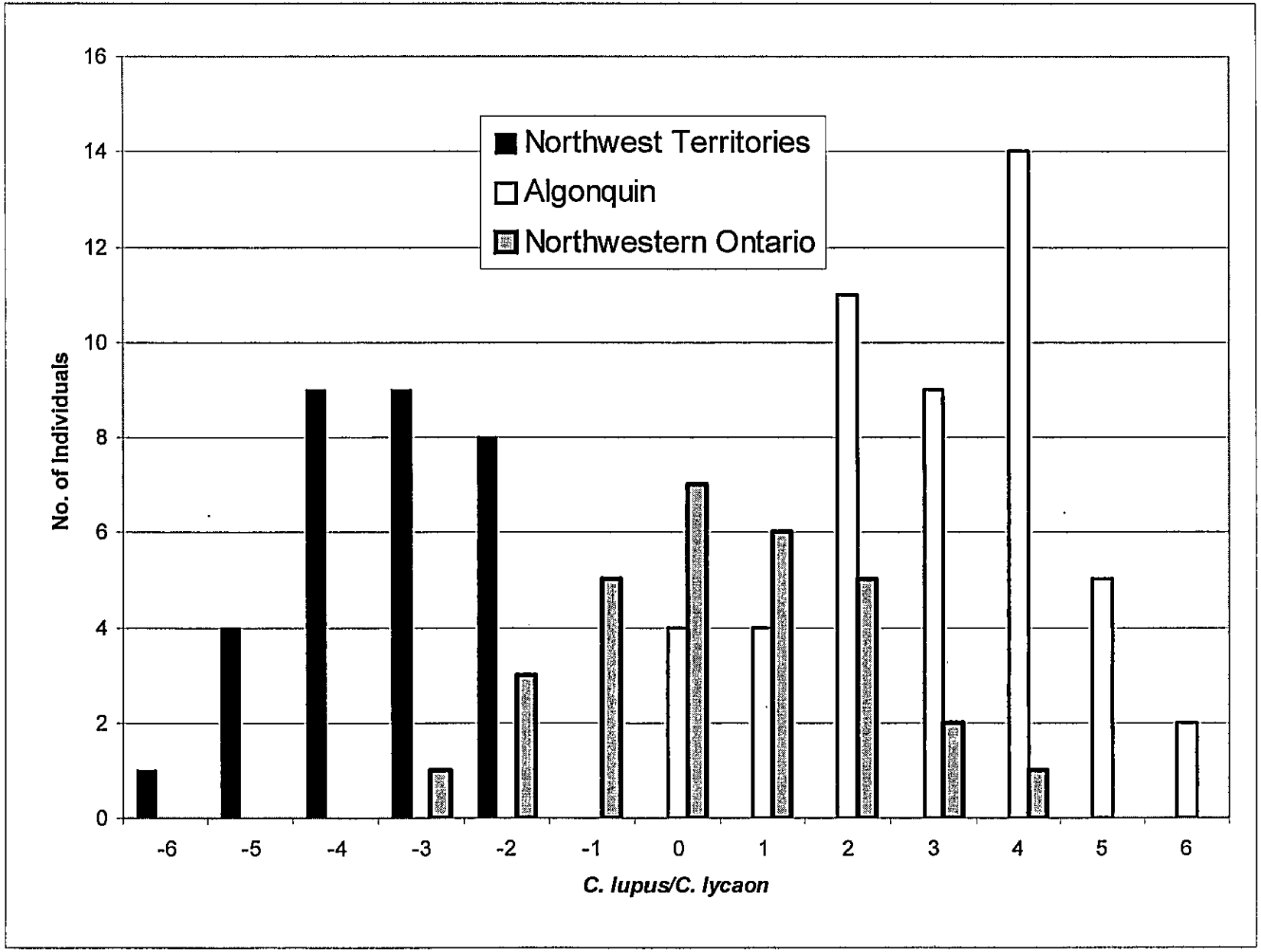

Northwest Territories
Algonquin
Northeastern Ontario
No. of Individuals
0
2
4
6
8
10
12
14
16
-6
-5
-4
-3
-2
-1
0
1
2
3
4
5
6
C. lupus/C. lycaon

Figure 8. Distribution of Individual Indices based on allele frequencies from representative *C. lycaon* and gray wolves (*C. lupus*) samples. The log likelihood of a genotype originating from Algonquin Provincial Park compared to the Northwest Territories was determined in these areas and the northwestern Ontario region.


Northwest Territories
Algonquin
Northwestern Ontario
No. of Individuals
0
2
4
6
8
10
12
14
16
-6
-5
-4
-3
-2
-1
0
1
2
3
4
5
6
C. lupus/C. lycaon

Chapter 9

General Discussion

This thesis set out to test two hypotheses: 1) Neutral DNA markers can be used to accurately reconstruct the evolutionary history of populations; 2) Neutral DNA markers are concordant with adaptive distinctiveness in reconstructing the adaptive history of populations. An additional objective was to assess the criteria for identifying conservation units and assigning conservation priorities using specific criteria such as isolation, amount of divergence, adaptive history or adaptive potential.

Conservation Genetics as an Applied Science

Since its beginning, conservation genetics has been described as an applied science (O'Brien 1994a, Carvalho 1998). The conceptual view of this applied science was the integration of multiple subdisciplines of biology (genetics, morphometrics, physiology, behaviour and ecology) to make realistic approximations of the 'history of natural populations, the present status and future prognosis of populations'within a conservation framework (O'Brien 1994a). Despite this foundation, the field of conservation genetics has recently entered into a time of reflection (Carvalho 1998, Bowen 1999, King and Burke 1999, Taylor and Dizon 1999, Crandall et al. 2000) as to whether molecular genetic techniques are accurately representing the adaptive significance of species and populations. These recent reviews have identified that few studies have adopted a multidisciplinary approach to reconstruct the evolutionary and adaptive history of populations for the purpose of identifying conservation units and establishing conservation priorities.

For the purpose of this thesis, three biological systems were selected to reconstruct the adaptive and evolutionary history based on their potential to integrate molecular data with other data, e.g. ecology, morphology, physiology and behaviour. These studies also reflected differential impacts from anthropogenic and natural factors such as severe natural environmental conditions, altered landscapes, habitat fragmentation, disease and hunting pressure.

Limitations of Neutral DNA Markers in Reconstructing Evolutionary History

An understanding of evolutionary history is important for assessing populations in relation to the degree of isolation, levels of gene flow, population demographic events such as bottlenecks and expansions, divergence times and hybridisation. These evolutionary processes are often considered in a background of anthropogenic factors influencing the current distributions and genetic variation of populations (Lande 1998). Among the three groups of species in this thesis, the tilapia and the wolf raised significant issues that affected the accuracy of reconstructing evolutionary history while the moose study gave consistent results at neutral loci with the proposed population histories.

The Lake Magadi tilapia study (Chapters 2 and 3) examined minisatellite DNA loci and the variable control region of the mtDNA. The detection of significant population differentiation with DNA fingerprinting did not support the hypothesis of gene flow among lagoon populations. This data supported the proposal of allopatric lagoon populations within Lake Magadi. Among geographically separated lagoons, the maintenance of common Magadi haplotypes suggested a common heterogeneity in the absence of gene flow resulting from balancing selection on the mitochondrial DNA. This violates the previously held assumption that mtDNA variation is selectively neutral. Additional neutral DNA markers will be required to further assess the isolation and to confirm the proposed selection on mtDNA sequences. Additional evidence of selection acting on the mtDNA is present in the recent literature (Castro et al. 1999, Garcia-Martinez 1998, Peek et al. 2000).

The study examining the history of eastern North American wolves, specifically the eastern timber wolf (*C. lycaon*) and the red wolf (*C. rufus*) (Chapters 5, 6), demonstrated a significant conflict in the interpretation of mtDNA and microsatellite data in the taxonomic classification. The conflicting interpretation of these data was not within the studies in this thesis but was identified when compared to previous genetic studies examining wolf taxonomy (Wayne and Jenks 1991, Roy et al. 1994, Roy et al. 1996). Our data are consistent with the hypothesis of a North American-evolved wolf independent of the gray wolf (*C. lupus*). This suggests that coyote-like mtDNA in eastern timber wolves and red wolves is not of recent coyote origin but in fact represents divergent sequences of a closely related species. The

difference in interpretation can be attributed to some extent to the *a priori* consideration of the taxonomy of each sample. The earlier genetic studies classified samples as wolves or coyotes and pooled them in the "appropriate" population based on the assignment given in the field (Wayne and Jenks 1991, Roy et al. 1994, Roy et al. 1996). As a result samples not of red wolf origin, particularly from museums (Roy et al. 1996) may not have been representative of the species under investigation due to misclassification (Gardner 1998, Nowak personal communication) or sampling from heavily impacted hybridisation zones (Nowak and Federoff 1998). The two historic samples from wolves killed well before the arrival of coyotes to the region allowed a direct test of our proposed model (Chapter 6). Furthermore, our use of individual assignment tests does not rely on the *a priori* taxonomic classification of the samples under investigation (Chapter 5).

Assignment tests are becoming prevalent in molecular genetic studies to examine migration and gene flow (Bossart and Prowell 1998, Waser and Strobeck 1998, Cornuet et al. 1999, Davies et al. 1999, Pritchard et al. 2000). Within the new model of North American *Canis* evolution, assignment tests, e.g. individual index, were used to characterise eastern coyotes and Ontario canid "types" (Chapters 7 and 8). A similar problem of pooling eastern coyotes for analysis based on field assignments was intrinsic to the original conclusion of the lack of wolf gene introgression in *C. latrans* (Roy et al. 1994). This was further compounded by the assumption that the gray wolf (Lehman et al. 1991, Wayne and Lehman 1992) and not the eastern timber wolf was the contributing parental species within these hybrid crosses, thereby misidentifying the origin of a number of the mtDNA haplotypes within eastern coyotes (Chapter 7). This study demonstrates the problem of the *a priori* consideration given to the origin of samples in both hybridising species and sympatric cryptic species.

The moose study showed population structure and genetic variation at the neutral DNA markers consistent with the proposed histories of the populations we surveyed (Chapter 4). An extension of this study would be to increase the number of microsatellite loci and assess finer-scale population structuring and indirect estimates of and migration using assignment tests (Bossart and Prowell 1998, Waser and Strobeck 1998, Cornuet et al. 1999, Davies et al. 1999, Pritchard et al. 2000).

One important observation from the moose study was that heterozygosity at neutral DNA loci was not a good measure of genetic variation, particularly low number of alleles. Theoretical (Lacy 1987, Leberg 1992) and experimental evidence (Spencer et al. 2000) support this finding. While heterozygosity may define genetic variation as accurately as allelic diversity, particularly following population bottlenecks, the relationship between heterozygote excess and allelic diversity have been utilised to identify recent bottleneck events (Luikart et al. 1998, Luikart and Cornuet 1998). Furthermore, heterozygosity remains an important measure for detecting events such as a Wahlund effect in the reconstruction of the evolutionary processes influencing populations. The importance of the relationship between heterozygosity and allelic diversity in assessing adaptive variation with neutral DNA markers is discussed in more detail in the next section.

Discordance between neutral DNA markers and adaptive distinctiveness

Evolution of adaptive distinctiveness can be considered at the species level (adaptive radiation) or population level (locally adapted genomes) (Givnish 1997). The studies in this thesis involved both microevolutionary and macroevolutionary processes to assess both adaptive radiation and local adaptations. The Magadi tilapia study (Chapters 2 and 3) considered morphological, physiological and behavioural characterisation as representative of adaptive distinctiveness as a result of macroevolutionary processes. The genetic assessment of moose populations (Chapter 4) used a functional DNA marker, the *Mhc* gene DRB, as representative of adaptive variation in an intra-specific comparison, i.e. microevolutionary level. Morphological data of wolves and coyotes, representative of adaptive differentiation, formed the basis for evaluating the *Canis* types within eastern North American (Chapters 5, 6, 7 and 8). Morphological differentiation among *Canis* types likely reflects both microevolutionary selection pressures and macroevolutionary adaptive radiation.

The Magadi tilapia showed a pronounced difference between the apparent "neutral" mtDNA marker and morphological, physiological and behavioural adaptations, while the differentiation observed at the neutral minisatellite data was consistent with the adaptive divergence observed with phenotypic

characters (Chapters 2 and 3). As described in the previous section, the evidence from the tilapia analysis suggests the mtDNA genome should be considered a potential adaptive marker. The suggestion of selection acting on the mtDNA genome to maintain haplotypes will require confirmation by examining larger regions of the molecule, specifically functional genes potentially under selection, e.g. COI (Peek et al. 2000).

A limited number of studies have identified discordance between mtDNA data and phenotypic characters. Finding informative DNA markers has been a constant challenge in assessing phylogenetic relationships concordant with adaptive characters among African cichlids (Kornfield and Parker 1997) due to the rapid divergence rates and high incidence of parallel evolution (Ruber et al. 1999). Examples of discordance between mtDNA phylogenetic relationships and ecological/morphological characters have been seen in sturgeon (Doukakis et al. 1999) and the madtom catfish (Bennets et al 1999).

There was discordance between the neutral mini- and microsatellite loci and the functional DRB *Mhc* locus in moose (Chapter 4). Lower allelic diversity was observed in Newfoundland moose compared to its source population but a number of the other populations showed discordance between neutral and functional DNA markers. Isle Royale showed higher *Mhc* allelic diversity than predicted by neutral DNA markers, conversely, mainland Ontario moose showed the opposite pattern. The overall low level of genetic variation within moose does have limitations as to the amount of genetic change that can be assessed. It is reasonable and conservative from a conservation genetics approach, to predict that a loss of neutral genetic variation will reflect loss at functional loci. Other studies have observed the low allelic diversity at *Mhc* loci due to population bottlenecks and/or isolation (Seddon and Baverstock 1999). The opposite trend may not be so obvious, where high neutral genetic variation accurately reflects functional loci that are influenced by local selection pressures.

In inferring adaptive variation, allelic diversity is more sensitive than heterozygosity in identifying disparate gene pools and this is particularly important for gene loci under selection, e.g. *Mhc* (Hughes 1991) as the loss of such alleles are far more crucial a loss to populations. Despite this, the majority of studies examining genetic variation utilise heterozyosity (Hedrick 1999). Recently, alternative measures of allelic diversity and allele frequency distribution have been recommended (Petit et al. 1998). While allelic

Isolation & Phylogenetic Divergence

The ESU concept as a means of identifying conservation priorities has shifted to deep-rooted phylogenetic separations based on shared derived characters (Vogel and DeSalle 1994) or reciprocal monophyly of mtDNA (Moritz 1994a). A number of studies have applied the Moritz criterion for ESU status, however, the majority of them have resulted in modifying the criteria to suit morphological or ecological characters in the absence of reciprocal monophyly (Legge et al. 1996, Duvernall and Turner, Barratt et al. 1999, Bennetts et al. 1999, McLean et al. 1999, Sherwin et al. 2000). Ultimately, adaptive characteristics have been considered overriding when the appropriate mtDNA sequence resolution was unavailable. Furthermore, a number of studies have identified the limitations of the Moritz ESU approach (Duvernell and Turner 1998), some so far as to recommend it be discarded and replaced with a ranking criterion based on genetic and ecological exchangeability (Crandall et al. 2000).

The ESU concept may be effective under certain circumstances, but this definition does not handle several conservation scenarios including populations with paraphyletic histories (Crandall et al. 2000), or rapid adaptive radiation and hybridisation. While phylogenetic divergence may prove useful it should not be at the exclusion of other criteria with respect to identifying conservation units. One of the serious limitations of a systematic or phylogenetic approach is that it excludes important genetic and adaptive variation below the species or subspecies level. While this may be appropriate for regions with high levels of endemism, temperate regions that have been subject to evolutionarily more recent Pleistocene events may represent evolutionary significance or at least adaptive significance at the population level as representative of a species "evolutionary legacy" (Waples 1991, Dizon et al. 1992). This relates to the original objective of identifying adaptive intra-specific units, locally adapted genomes, and assigning ESU status (Ryder 1986). The theory of molecular evolution and adaptive radiation has undergone a similar transition in the progression from speciation as a necessary outcome of adaptive radiation (Skelton 1993, Schluter 1996) to ecological and adaptive divergence without speciation (Givnish 1997).

In addition to the recent application of phylogenetic divergence and a cladistic systematic approach to establishing conservation units (Vogel and DeSalle 1994, Moritz 1994a), isolation has been a

critical criterion for inferring adaptive significance (Waples 1991, Dizon et al. 1992). This was extended to the lower classification of Management Units (MU) when mtDNA and nuclear DNA markers were significantly differentiated but were not reciprocally monophyletic (Mortiz 1994). Isolated populations have been considered a priority in most conservation genetic considerations due to the proposed loss of genetic variation and increased risk of extinction (O'Brien 1994a, O'Brien 1994b, Frankham 1995). This is a viable criterion under certain circumstances but again should not be an overriding consideration (Crandall et al. 2000). Studies on metapopulations have identified higher risks of extinction in "fringe" sink populations despite low levels of gene flow (Saccheri et al. 1998,Westemeier et al. 1998). The overall increase in merging genetics with metapopulation theory (Harrison and Hastings 1986) offers the potential for identifying important local adaptations among subpopulations with the end result being a proactive approach in preventing isolation among key regions of the larger metapopulation conservation unit.

At present the taxa studied in this thesis would not be considered evolutionary significant units under the most common criterion of reciprocal monophyly. *Acolapia* sp. from Lakes Magadi and Natron (Chapters 2 and 3), despite demonstrating significant and unique adaptations within different lakes and among different lagoons in some of the most severe environmental conditions would not warrant ESU status under the most common classification of Moritz's (1994a). Monophyly was not considered in the re-classification of *Oreochromis alcalicus alcalicus* and *O. a. grahami* to *Alcolapia alcalicus* and *A. grahami* (plus two additiona species), respectively (Seegers et al.1999). They proposed a model of balancing selection on the mtDNA (Chapter 3) suggests a common selection pressure of heterogeneous environments common to all the isolated Magadi lagoon systems with isolation supported by differential selection pressues among lagoons. Ultimately, adaptive distinctivness is the important consideration for the Magadi *Alcolapia* and not reciprocal monophyly of mtDNA sequences.

Moose, representing an intra-specific comparison among different regions and populations, would unlikely warrant ESU status under any criteria but this example shows the importance of identifying adaptive genetic diversity within a metapopulation and what local selection pressures may be influencing adaptive selection (Chapter 4). The conservation unit for wildlife species such as moose should be the metapopulation unit with subpopulations being considered within the management strategy of this unit.

Neutral DNA markers can reconstruct the metapopulation structure by assessing gene flow and *Mhc* genes can be used to identify differential selection pressures and locally adapted genomes within the conservation unit.

The eastern timber wolf and red wolf (Chapters 5 and 6) would also not be considered as ESUs under the reciprocal monophyly criterion or even an isolation criterion for two reasons. Firstly, the phylogenetic patterns of the mtDNA for *C. lycaon* appear paraphyletic in relationship to *C. latrans* (Chapter 5). Secondly, the extensive hybridisation between *C. lycaon* and *C. latrans* (Chapter 7 and 8) and to a lesser degree *C. lycaon* and *C. lupus* are not consistent with isolated entities (Chapter 8). There is no basis to eliminate conservation consideration of eastern wolves as a result of failure to meet the specific criterion of an ESU concept or other ranking approach. Hybridisation alone would eliminate populations from the existing criteria for identifying conservation units, despite the fact this is often a significant risk facing one parental species and it relates directly to adaptive potential (see below). This is reflected in the eastern coyote which is a hybrid that contains important genetic material of the New World evolved wolf (Chapter 7).

Adaptive History & Adaptive Potential

Adaptive history reflects the response of species or populations to selective ecological variables through their evolutionary history. Adaptive potential represents the genetic diversity allowing organisms to respond to environmental change (Lande and Shannon 1996, Bowen 1999). Recent studies of island populations examining parallel adaptations on independent islands and translocation experiments suggests that ecological selection pressures are more causal in determining geographic differentiation in small island populations (Thorpe and Malhortra 1996). Furthermore, morphological differentiation appeared within islands and appears independent of molecular phylogenetic history.

Lake Magadi tilapia demonstrate significant distinctiveness as a result of their adaptive history within the severe environmental conditions of the lagoons of the Soda lake (Chapters 2 and 3). African cichlids in general represent an important speciose taxonomic group that is the common example of the use of adaptive potential in assigning conservation priorities (e.g. Bowen 1999). Similar to other conservation

criteria, the use of adaptive potential has focused on phylogenetic species lineages (Bowen 1999, Soltis and Gotzenfdanner 1999). The comparison of neutral DNA markers to the functional *DRB* gene in moose (Chapter 4) shows the adaptive potential of maintaining the genetic diversity of important adaptive loci in different subpopulations under differential local selection pressures. The partitioning of alleles at functional loci within locally adapted populations of a metapopulation provides the potential to adapt to respond to environmental change along its geographic range.

Wolf evolution demonstrates important aspects of both adaptive history and adaptive potential. The convergence of two lineages to similar wolf-like characteristics on two different continents shows the importance of accurately reconstructing the history of *Canis* species. An inaccurate assessment of this history is the difference between intensely hybridised, "swamped", population of gray wolves in eastern North America *versus* a distinct New World wolf lineage hybridising with a closely related sister-species. The recommendations for conservation would be very different under these two conflicting models.

Furthermore, the adaptive potential of the existing eastern wolf-like canids with a history of hybridisation must be factored into conservation considerations. The functional role of the eastern wolves and coyotes within ecosystems is a question faced by conservation efforts surrounding the Florida panther, as to whether the "pure" must be recovered or whether a functional predator is more important (Bowen 1999). Conservation efforts favored the second option. Adaptive potential allows a reasonable assessment of conservation units with the increasingly common scenario of hybridisation. The adaptive potential of hybrid canids is important as it contains the genetic material of parental species, e.g. *C. lycaon* x *C. latrans* or *C. lupus*. This further raises the possibility that the most well adapted wolf-like canid for a human altered landscape may be the introgressed form.

Establishing Conservation Priorities

Systematics, ecology and evolutionary potential should not be ranked as to their level of importance as they are not mutually exclusive. In addition, no one criterion should be considered exclusively. It is difficult to assess the adaptive potential of taxa without an understanding of their adaptive history. For example, African cichlids would warrant consideration for conservation based on

their adaptive potential (Bowen 1999). However, the speciose nature and rapid divergence of African cichlids would not have been identified were it not for the extensive work in reconstructing the phylogenies and taxonomic history of these species flocks (Meyer 1993).

The underlying reasoning for developing criteria and assigning priorities has been the statement that with limited resources the most appropriate taxonomic groups requiring protection will have to be determined (e.g. O'Brien 1994a, Wayne 1992). This approach is not realistic. Although described as the underlying reason in specific studies, direct comparisons in the literature among taxa to assign conservation priority status have not been made. Ironically, the best example has been the attempt to de-list the red wolf as a conservation priority based on the proposed gray wolf/coyote hybrid history (Nowak et al. 1995).

Conservation genetics (O'Brien 1994) and molecular ecology (Carvalho 1998) have been classified as applied sciences and as such are rooted in a realistic framework. Genetic analyses are introduced into conservation studies for many reasons: 1) existing endangered status; 2) politics; and 3) economics, e,g. sustainable harvesting; and sometimes 4) biological distinctiveness (Soltis and Gotzenfdanner 1999). Conservation genetics provides information for making management decisions to implement conservation policy and a shift from a strictly academic consideration of conservation units to an applied or "academic plus policy" approach has been recommended on a case-by-case basis (Taylor and Dizon 1999). Many aspects of a conservation plan are subjective and may be better dealt with in this manner.

The examples in this thesis identified several different reasons for recommending conservation efforts supporting the case-by-case argument. The Lake Magadi tilapia populations represent distinct adaptive histories and although not directly threatened by anthropogenic factors, they represent an important example of "life-on-the-edge" and are therefore an important model biological system for future study (Chapters 2 and 3). Moose as a game species (Chapter 4) requires effective management for sustainable harvest and genetic data should be used to manage the metapopulation.

Eastern North American wolves and coyotes (Chapters 5, 6, 7 and 8) represent a highly impacted taxonomic group with significant political issues on economic impacts and recovery in Canada and the US.

Due to the high levels of disturbance, altered distributions and extensive hybridisation, any wolf recovery or management program must assess the historic context of North American wolves. What wolf existed in a specific geographic region; what wolf-like canid exists there at present; and what wolf should be re-introduced or recovered in this region are questions that must be addressed before any actions are taken. This is particularly significant in the northeastern US, where future wolf re-introductions are planned. Furthermore the above questions have direct impacts on the de-listing of wolves under the US Endangered Species Act (ESA), where assumptions of wolf numbers are based on the presence of one wolf species and not on the potential for sympatric wolf species inhabiting the Great Lakes region.

This thesis presents situations where specific criteria do not fit all conservation needs and that current genetic criteria based on exclusively "neutral" DNA markers are flawed. A fundamental objective of conservation genetics should be the identification of adaptive significance at different levels (genes, populations and species) in the context of evolutionary history for the purpose of identifying units of conservation and developing effective management strategies. Adaptive significance (distinctiveness and potential) may result in protection at the species level and/or in effective management at the population level. Conservation genetics should not be exclusively focused on protection or recovery of endangered or threatened species but also on sustainable and effective management of a wide-range of species that are considered renewable resources. The growing importance of adaptive characters, genotypic or phenotypic, in reconstructing adaptive history should continue with future directions focusing on defining these characteristics. Despite the importance of adaptive characters, neutral DNA markers should remain essential in reconstructing evolutionary history although the characteristics of specific DNA markers should be well-defined before interpreting their significance (Chapters 3 and 4, Hedrick 1999).

Developing a Strategy using Genetic Markers to Identify Conservation Units

Having identified the importance of incorporating functional genetic or phenotypic characters to assess adaptive significance a number of recommendations can be made to identify such characters or traits:

1) Examine genes potentially under selection, such as *Mhc* (Chapter 4), the transferrin gene (Ford et al. 2000) and LDH (Powers and Schulte 1996) to assess locally adapted genomes and local

selection pressures. The increasing amount of research in Comparative Genomics and commercially important species such as humans, cattle and dogs will provide valuable marker systems to assess genetic variation.

2) Examine population genetic structure using neutral and functional DNA markers to identify discordant patterns (Chapter 4, Mitton 1998).

3) Identify changes in genetic structure corresponding to landscape differences or ecological variables. Incorporating genetic parameters into Geographic Information Systems (GIS) for spatial statistical analyses will allow the development of the field of Landscape Genetics. An example of the influence of environmental conditions on genetic structure was described for the long-finned pilot whales that showed structure not by simple isolation-by-distance but between different ocean temperatures (Fullard et al. 2000).

Recent studies have applied molecular genetics to examine the underlying genetic basis for adaptively important characteristics (Turner et al. 1997), studies that have been recommended as a significant future direction for molecular ecology and conservation genetics (Carvalho 1998, Bowen 1999).

Conclusion

This thesis has addressed the use of DNA markers within natural populations and the limitations that exist in reconstructing evolutionary and adaptive history. Neutral DNA markers serve an important role in reconstructing population demographic history, although congruence among multiple marker systems should be examined. These same markers less accurately reflect adaptive variation, therefore actual phenotypic or genes underlying adaptively important characteristics should be used for this purpose. The integration of a wide range of data into conservation genetics requires additional examples and further requires a critical assessment of the interpretation of DNA markers in comparative studies when reconstructing evolutionary and adaptive histories.

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