

A Preliminary Study of Genetic Variation Within and Among Populations of
Diaptomus leptopus (Copepoda: Calanoida): A DNA Sequence Analysis of the
Mitochondrial Cytochrome Oxidase I Gene

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Biology

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ABSTRACT

A Preliminary Study of Genetic Variation Within and Among Populations of *Diaptomus leptopus* (Copepoda: Calanoida): A DNA Sequence Analysis of the Mitochondrial Cytochrome Oxidase I Gene

Maria Guarnieri

Previous studies examining the population structure of aquatic organisms have been based generally on behavioral traits, morphological traits and allozyme studies. DNA sequence analysis of the mitochondrial cytochrome oxidase I gene may also be of use to study population structure. Thus, a study investigating the genetic variation within and among seven populations, distributed from Quebec to New York, of the freshwater copepod *Diaptomus leptopus* was conducted.

Variation among populations was shown in both the nucleotide sequence and the observed haplotype frequencies. These results indicate that the populations may differ. The phylogenetic tree obtained reveals that most of the individuals studied are most similar genetically within their population and that there are varying degrees of differentiation among populations. Most of the nucleotide substitutions are synonymous and a majority of the base changes occur at the third codon position. The amino acids that do change in the calanoid copepod occur at sites which are variable for other organisms sequenced. Hence, these sites are not thought to be important for the maintenance of the protein structure and function. Therefore, these results suggest that the gene product is not altered and the differences seen, in the mtCOI gene, among the populations, studied here, may be selectively neutral. The results obtained need to be supported by more individuals and genes studied, examination of historical aspects of population distribution and further analyses.

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INTRODUCTION

The population genetic structure of a species, which is the genetic differentiation of conspecific populations (Bucklin 1995), has been studied for several decades (see review articles in Ehlich & Raven 1969; Avise & Felley 1979; Hairston & Olds 1984; Randi et al., 1993) in organisms ranging from mammals (Randi et al., 1993; Ruiz-Garcia 1993) to insects (Chapco et al., 1992) to many aquatic organisms (Avise & Felley 1979; Morales et al., 1993), such as copepods (Hairston & Olds 1984, 1986; Neill 1992; Byrom et al., 1993). Population structure studies aid in better understanding the mechanisms which enable species to cope with their environment, either by adaptation or escape, and in determining the factors involved in genetic divergence, which leads to speciation.

Factors Influencing Genetic Variation

The degree of genetic divergence among natural populations is affected by three basic evolutionary forces: migration, genetic drift and natural selection (Allendorf & Phelps 1981). The interactions among these forces are complicated by the influence of other factors. For instance, the presence or absence of physical barriers affects the amount of migration and thus gene flow. If migration between populations is rendered difficult by the presence of physical barriers, gene flow will be low and unique gene pools in local populations may result (Burton 1987). In some marine copepods spatial separation is thought to be a possible barrier preventing the

panmixis of populations (see reviewed articles in Bucklin 1986). Sévigny et al. (1989) found allozyme variation at the GPI locus within the species *Pseudocalanus newmani* between samples that are from the Atlantic coast and Puget Sound (west coast), but not among samples close to Halifax. Some copepod populations are subdivided by small stretches of land. Tidal pool populations of the harpacticoid copepod *Tigriopus californicus* inhabit the rocky intertidal zone of the Pacific coast of North America. These habitats are irregularly interrupted by stretches of beach ranging from a few meters to tens of kilometres (Burton & Feldman 1981). Copepod populations from neighboring rock outcrops show strong differentiation at the loci studied and a high frequency of unique alleles. This strong differentiation among nearby populations is thought to occur due to restricted gene flow among the populations. The restriction of gene flow by distance may, with time, lead to gene flow restriction by biological barriers. In the genus *Tisbe*, 4 of 5 species studied were able to breed and produce fertile F₁ hybrids, from crosses between European and American populations. These F₁ produced viable offspring (Battaglia & Volkmann-Rocco 1973). The fifth species, *T. clodiensis*, produced no viable offspring or hybrids that died before the adult stage. They concluded that the reproductive barrier between these transatlantic populations of *T. clodiensis* is complete. Thus, isolation by land barriers and distance may lead to genetic variation among populations and may lead to reproductive barriers, thus increasing differences between populations of a species, ultimately resulting in speciation.

Bottlenecks, the reduction of population numbers, and founder effects influence the rate of genetic drift, which in turn influences genetic variation. Boileau & Hebert (1991) found that individuals from eastern populations of the copepod *Heterocope septentrionalis* have less genetic variation than their western counterparts that survived the last glaciation in a refugium in Alaska. In addition, the eastern populations contain alleles that are mainly a subset of those from the western populations. Both of these findings suggest that founder effects are responsible for these differences in the genetic variability. In addition, despite genetic distances suggesting divergence more than 100 thousand years ago, the populations could not have existed 20 000 years ago. Canada was completely covered by ice at least 18 000 years ago (CLIMAP 1976), thus reinforcing the suggestion that bottlenecks were responsible for increasing variation among populations.

Genetic differences can occur as a result of natural selection as well. Allan (1984) found life history trait differences between Florida and Michigan populations of the freshwater copepod *Mesocyclops edax*. Wyngaard (1986) found considerable additive genetic variance (genetic variance attributed to the additive effects of alleles [Futuyma, 1986]) in the body size of the Florida populations. The Michigan populations were found to have additive genetic variance in maturation times. Both authors interpreted their results as reflecting differential responses to selection in widely separate geographic localities. Thus, selection for local conditions could be responsible for sustaining variance among populations of a species. In addition, time

influences genetic divergence and eventually speciation. The longer populations endure in a particular habitat, the greater their adaptation to that environment and the more genetically distinct the populations will become, if the environments differ, and if selection occurs.

In summary, there are many factors affecting drift, selection and migration that need to be considered when investigating population genetic diversity. Nonetheless, population studies that examine variation do provide some insight into the evolutionary forces behind the numerous situations found in nature.

Molecular Techniques

Until recently, zooplankton genetic diversity has been assessed by morphological and behavioural traits (Hairston & Olds 1984, 1986; Neill 1992; Byrom et al., 1993), as well as with the use of allozymes (Boileau & Hebert 1988, Boileau 1991). However, more and more studies have used molecular techniques such as restriction fragment length polymorphism (RFLP) (Crease et al., 1989, Lynch et al., 1989), and the sequence analysis of various nuclear (nDNA) and mitochondrial (mtDNA) genes (Bucklin et al., 1992, 1995). Studies utilizing molecular techniques are more informative than those utilizing morphological or behavioural traits because the genetic material can be studied directly (nucleotide sequence) or indirectly (restriction fragment length polymorphisms).

The quality and quantity of genetic information obtained from different

molecular techniques varies. Sequence data, allozymes and RFLP all yield qualitative data, which can be converted to quantitative data. Sequencing offers advantages over allozymes and RFLP, but does have some disadvantages as well. Sequencing has a better resolution; changes both in the coding and noncoding regions can be found, whereas allozymes can be used to examine coding regions only. As for RFLP, changes could be found in both regions, so long as there is an enzyme restriction (RE) site within the regions. RFLP does uncover base substitutions, additions and deletions, but within RE sites only. Allozyme analysis cannot distinguish among any of these alterations, whereas sequencing does. These changes can be used as informative genetic markers (Awise 1994) to distinguish between species that are morphologically similar. Once a sequence is obtained, an amino acid sequence may be deduced, thus allowing synonymous and nonsynonymous (amino acid substitutions) substitutions to be noted. This information allows some speculation of whether the substitutions seen are selectively neutral or not. An amino acid sequence cannot be inferred from either allozyme or RFLP data. Sequencing offers the advantage of finding "application at virtually any taxonomic level" (Awise 1994). On the other hand, allozymes and RFLP analyses are considered as efficient for demonstrating genetic isolation of populations and being informative phylogenetically at the conspecific population and closely related species levels (Awise 1994), but not beyond. In addition, "the evolutionary relationships among alleles are not reflected by similarity in mobility" (Burton 1994), thereby rendering it difficult to determine

which alleles were introduced by immigrants and those present due to mutation. Therefore, it is difficult to assess which factors, migration, genetic drift or selection, are involved in the population structure of a species. Allozyme data may also underestimate population differences because the allozymes may have the same mobility, but different genetic structure, thus creating hidden genetic variance.

However, sequencing can be costly in both the supplies and manpower required. Allozyme analysis, unlike sequencing, is cheap and easy, thus can be used to look at several loci, many individuals and populations at one time. Sequencing is restrictive in both the number of loci and individuals studied. It also takes longer to sequence a few individuals than to obtain allozyme or RFLP data of more than one locus from several individuals. In sequencing, time can be spent by first inserting the desired DNA fragment into a plasmid and then cloning the plasmid in order to obtain enough DNA. However, enough DNA could be acquired for sequencing using the Polymerase Chain Reaction (PCR), which can amplify the target DNA directly from the organism without the use of plasmids and cloning. Still, DNA needs to be extracted from some source from the organism, like the eggs or sperm, and can sometimes be difficult. This difficulty is especially true for very small organisms, like *Diaptomus minutus*, a calanoid copepod only a few millimeters in length. However, direct amplification from the organism can be done without extraction (Bucklin et al., 1995; Bucklin & Lajeunesse 1994). Time spent sequencing can be reduced with the use of automatic sequencers, where 24 samples (once prepared for

sequencing, which may require a day's work) can be sequenced at once. Unfortunately, automatic sequencers are more expensive than the materials needed for allozyme or RFLP analyses. Finally, some DNA sequencing studies have confirmed what has been already found in allozyme studies, while other such studies have found opposing results (Avice 1994). Thus, certain criteria, such as the question being asked, the organism(s) studied and the available resources, equipment and cost, must be weighed before choosing a particular molecular technique.

The type of DNA to be studied must be decided upon as well. In the past decade, more and more phylogenetic and population studies (Bucklin & Kann 1991; Bucklin & Lajeunesse 1994; Burton 1994) recognized mitochondrial DNA as being informative for the following two reasons. First, mtDNA has a high substitution rate. Mitochondrial DNA consists mainly of coding sequence, no introns, and very small or absent intergenic sequence, leading some to believe mtDNA to be quite conservative in its rate of nucleotide substitution. However, mtDNA has a high mutation rate in vertebrates (Brown et al. 1979; Barton & Jones 1983; Vawter & Brown 1986; Rand 1994), and invertebrates, like *Drosophila* (Moritz et al., 1987). Thus, the high evolution rate at the nucleotide level is useful for studying organisms at the intraspecific (within species) and interspecific (between species) levels. Second, mtDNA is inherited maternally and is not subjected to recombination due to mating as is nuclear DNA (Rand 1994), and thus mtDNA has the potential of passing from generation to generation without change, except changes caused by mutation.

Therefore, mitochondrial traits may "provide conserved markers for large-scale and/or long-term studies of dispersal" (Bucklin 1995). In addition, population differentiation may be revealed more with mtDNA than with nDNA. Burton (1994) used both allozyme (nDNA) and DNA sequence data (mtDNA) to elucidate the genetic structure of natural populations of a marine copepod, *Tigriopus californicus*. Mitochondrial DNA sequences reveal strong population differentiation between central and southern California populations, that is not apparent in the allozyme frequencies. Mitochondrial DNA may reveal differentiation more so than nDNA due to the lack of gene exchange by males, that may lead to mitochondrial genes being fixed more quickly than nuclear ones.

Bucklin & Lajeunesse (1994) studied several subspecies of the calanoid copepod, *Calanus pacificus*, to determine the geographic and systematic patterns of molecular variation within the species. Based on sequence comparisons of a portion of the mitochondrial 16S ribosomal RNA (rRNA) gene, these subspecies were found to be genetically distinct. Moreover, one subspecies shows differentiation within the sample. Therefore, there is subdivision within this particular species of *Calanus*. This study follows a similar course to the above study in that the mitochondrial sequence of a freshwater copepod, *Diaptomus leptopus*, will be examined.

Ecology of *Diaptomus leptopus*

Despite studies showing "that some [zooplankton] groups such as calanoid

copepods show more endemism than others, such as the cyclopoid copepods or cladocerans" (Hebert & Wilson 1994), some calanoid copepods have wide distributions and wide habitat ranges. For instance, *Diaptomus leptopus* Forbes, the calanoid copepod under study here, has been found in clusters all across Canada. Anderson (1974), found *D. leptopus* in 46 of 340 lakes and ponds examined in southern Alberta and British Columbia. However, Carter et al. (1980) found this species to occur in only nine of 696 glaciated eastern North American lakes studied - 8 lakes of Quebec, north of the St. Lawrence and Ottawa Rivers and 1 lake in southern New Brunswick. Despite this low occurrence, the authors still considered *D. leptopus* as a crustacean that can be expected to be found in almost any region. Sampling by E.J. Maly and associates (personal communication) has determined that *D. leptopus* is found in widely scattered locations between the Adirondack Mountains of New York State and Mont-Tremblant Park of Quebec. Thus, this calanoid copepod has quite a wide distribution.

The reasons for the wide distribution of many zooplankton have been and still are being debated. Maly & Bayly (1991) have attributed the current widespread distribution of the Australian centropagid copepod, *Boeckella triarticulata*, to continental drift, with some dispersal occurring at the local level. However, Maly (1991) found two species of Australian centropagid copepods, *Calamoecia lucasi* and *Boeckella fluvialis*, to have dispersed south in the time period from 1970 to 1989, in Victoria. Thus, its present day distribution is a result of recent dispersal or attributable

to vicariant events?

Diaptomus leptopus has been found locally (in Quebec, around Montreal) to inhabit both permanent and temporary bodies of water (Nishikawa, 1995). Various traits have been found to vary among these populations studied, with no particular association with habitat type. Size dimorphism (De Frenza et al., 1986) and diapausing egg production (Piercey, personal communication) have been found to vary among the populations of *D. leptopus*. A preliminary study done by Shimon & Maly (unpublished manuscript) suggests that mating success is higher between individuals among populations within a sampling area (town or regional park within which populations are situated) than among sampling areas. Also, development times have been found to vary among populations (Nishikawa, 1995). What has brought forth the variation in size dimorphism (DeFrenza et al., 1986) or diapause egg production (Piercey, personal communication) among these populations? Is it selection or genetic drift? What role has migration played in this variation?

In this study, seven populations of *Diaptomus leptopus*, from Quebec and New York State, are examined to determine the degree of genetic variation among and within populations. The genetic variation is examined by studying the sequence divergence of a region of the cytochrome oxidase I (COI) gene of mtDNA. At the intraspecific level, conservative genes and slowly evolving DNA would show no variation. Therefore, due to close relations between the individuals in this study,

mtDNA was an appropriate choice. Since COI was found to be three times more variable than 16S ribosomal RNA (rRNA) in the euphausiid, *Meganyctiphanes norvegica*, (Bucklin et al., 1995; personal communication) and it amplified most successfully (bright, sharp and consistently), COI was the logical choice as an indicator of population genetic structure.

Objectives

The objectives are to determine whether any genetic variation exists among populations and whether the variation is sufficient for studying the populations structure of *Diaptomus leptopus*. If there is enough variation among the mitochondrial COI gene sequences, then this genetic marker has the potential to answer some of the questions raised above about the zooplankton's distribution and its population structure. In addition to the analyses performed here, other statistical analyses are briefly discussed. These methods may help to answer questions such as: which factor is influential in any genetic variation seen? Is it genetic drift or selection or migration, or a combination of factors? The steps required to further this preliminary study to a population structure study will be discussed as well.

MATERIAL & METHODS

Plankton samples were collected by net tow. In Quebec, a total of six populations from Mont-Tremblant, Lachute and Morgan Arboretum were sampled, along with one population from the Adirondack Mountains of New York State (Table 1). Once collected, water samples from these sampling sites were brought back to the lab, where the zooplankton were sorted. Adult copepods were preserved in 1.5 ml microcentrifuge tubes with 95% ethyl alcohol. These alcohol-preserved copepods were stored at room temperature until the DNA was amplified and sequenced.

DNA Amplification

A primer party (pilot study which attempts to amplify various regions of DNA with four or five different primer sets, refer to Table 3) was carried out in order to find primers that could yield an amplification product that showed consistency in sharpness, brightness and with no artifacts. The primers chosen to amplify a 670 base pair (bp) segment of the cytochrome oxidase I (COI) gene of mtDNA were LCO1490 and HCO2198, based on the *Drosophila yakuba* sequence (Folmer et al., 1994). The primer sequences are as follows:

LCO1490: 5' GGTCAACAAATCATAAAAGATATTGG 3'

HCO2198: 5' TAAACTTCAGGGTGACCAAAAAATCA 3'

The L and H indicate the light and heavy strands (referring to the buoyant densities in a Cesium chloride gradient) respectively, CO denotes cytochrome oxidase and the

numbers represent the positions of the primers in the *Drosophila* sequence (Folmer et al., 1994).

DNA from individual copepods from each of the seven populations was amplified directly from the organism, i.e. without prior extraction. Three to six copepods were surveyed from each population. Preserved copepods were individually placed in 1.5 ml microfuge tubes containing 400 μ l of distilled water approximately four hours prior to amplification to remove enough ethanol from the copepods to allow proper amplification of the DNA. Specimens were then crushed in 0.5 ml microfuge tubes containing 35 μ l of distilled water, 5 μ l of 10X Taq Polymerase (FMC BioProducts, Rockland, ME) buffer and 4 μ l of 25mM MgCl₂. To this mixture, 5 μ l of 2mM dNTP, 0.5 μ l of 10mM of each primer and 0.25 μ l of Taq polymerase were added, to yield an overall reaction volume of 50 μ l. Lastly, two drops of oil were added to prevent evaporation. Tubes were then centrifuged for about 20 seconds to ensure that all reagents were at the bottom of the tube. Amplification was carried out in a Perkin Elmer Thermal Cycler, model 480, with the following protocol conditions:

1. DNA was denatured at 94°C for 1 minute (min)
2. Primers annealed at 37°C for 2 min
3. Extension reaction occurred at 72°C for 3 min (DNA synthesis)

These conditions were executed for 40 cycles, after which, samples were held at 4°C. Amplified products were then verified for appropriate size with a standard molecular

weight marker (used to estimate size of the amplified products) on a 1.4% agarose gel. In addition, a negative control, sample with all reagents except DNA, was always run with the other amplification reactions to ensure that no contamination of the amplification products occurred. The gel already contained 5 μ l ethidium bromide, so staining after electrophoresis was not necessary. Only samples which showed single, bright, sharp bands of the correct molecular weight, about 670 bp, were chosen to be purified (Figure 1 - lanes 2, 4 & 5 (a) and 4 & 5 (b)).

Purification was achieved with the following steps: 40 μ l of the amplified product was loaded onto a 1% Nusieve (FMC BioProducts, Rockland, ME) gel containing ethidium bromide and electrophoresed at 65 volts for 30-40 min. The gel was then viewed by U.V. radiation, and the bands were cut out with a clean razor blade. The cut gel fragments were melted by heating to 65°C in 1.5 ml microfuge tubes. The temperature was lowered to 37°C, at which point 2-3 units of beta-agarase enzyme (Sigma Chemical Cop., Chicago, Il.) were added. The samples were incubated overnight at 37°C to ensure complete digestion of the agarose.

Sequencing Reactions

The sequencing reaction was carried out in a Perkin Elmer Thermal Cycler, model 480, using a Cycle-Sequencing Kit (Applied Biosystems, Inc., Foster City, CA). An asymmetrical amplification using the LCO1490 primer was performed to incorporate fluorescently labelled dideoxynucleotides. This primer was chosen over

HCO2198 for data analysis due to its consistency in the production of good sequence data.

The nucleotide sequencing was carried out in an Applied Biosystems, Inc., Automated DNA Sequencer (Figure 2). The sequencer used 6% acrylamide gels, which were electrophoresed for 11 hours. The automatic sequencer read the fluorescent emission from each base, recorded the emissions in a gel file, which was then read as a four-colour sequence chromatogram (Figure3) by the computer software SeqEd (version 2.0). These chromatograms were then verified visually for accurate machine reading.

Data Analysis

The Genetics Computer Group (GCG) Sequence Analysis software package (Smithies et al., 1981) was used for sequence alignment, preliminary analysis and to avoid human bias. Sequences were aligned by the multiple sequence alignment program Pileup (Devereaux et al., 1984), which was used to determine the haplotypes and their observed frequencies for all populations. In order to establish whether the observed haplotype frequencies found were different, a chi-square test by Monte Carlo simulations (Roff and Bentzen 1989) from the REAP analysis software package was carried out.

A phylogenetic tree was constructed using a neighbor-joining method (Saitou & Nei 1987) in the software package MEGA (Molecular Evolutionary Genetics

Analysis; Kumar et al., 1993) to show the molecular relationships among the various haplotypes, i.e. to determine which haplotypes were more similar genetically, thus more closely related phylogenetically. The neighbor-joining method was chosen because it produces a unique final tree under the principle of minimum evolution, which is a principle based on acquiring the smallest number of evolutionary changes required to explain the observed differences among operational taxonomic units (Avice 1994). The neighbor-joining method is also quite efficient in obtaining the correct tree topology, in comparison with other tree-making methods like the UPGMA cluster analysis method (Saitou & Nei 1987). The tree building protocol required a distance matrix to be generated before the dendrogram was built. Thus, a distance matrix, based on Tamura-Nei distances with pairwise deletions was produced. Tamura-Nei distances are genetic distances based on the nucleotide divergence between pairs of haplotypes. These genetic distances are sensitive to the varying types of substitutions, i.e. transitions versus transversions, and the varying rates of nucleotide substitution from site to site (Kumar et al., 1993). One thousand bootstrap replications were performed to carry out the statistical analysis of the tree.

RESULTS

HAPLOTYPE AND POPULATION VARIATION

The nucleotide differences show genetic variation among individuals, within and among populations (Appendix 1). The observed haplotype frequency matrix (Table 2) shows the presence of unique haplotypes in some populations (TR, HI, QU & IN - refer to Table 1 for initials) and common ones among other populations (MA, MB & RT - refer to Table 1). The unique haplotypes are those which are present in only one population, but the haplotypes' observed frequency may not necessarily be 1. The common haplotypes have observed frequencies that can vary among the populations within which it is present. Haplotype B, occurs in 1 out of 6 individuals (i.e. 1/6) in one of the two ponds in which it is found (Malloy 1 Pond) and 5/5 in the other (Rats Lake). The other haplotype found in more than one population is haplotype A, which is present in both Lachute ponds. Despite these two common haplotypes, all other observed haplotypes have been found to be thus far unique to a population, thereby indicating that these populations may differ. The chi square with Monte Carlo simulations confirms the haplotype frequencies among populations to be significantly heterogeneous ($\chi^2 = 156.98$, $P \leq 0.0005$). Therefore, based on these data, not only do the haplotypes differ, but the uniqueness of the haplotypes observed here could suggest that populations may differ as well.

However, are there varying degrees of differentiation among haplotypes, i.e. are some haplotypes more similar genetically to each other than to others? The

neighbor-joining tree (Figure 4) illustrates that most haplotypes within their populations are more similar genetically to one another in comparison to others. Individual #6 from Malloy 1 (MB6), however, clusters with the Rats haplotype (RT 1-5), inferring that it is more closely related genetically to the Rats haplotype than to the other haplotype present in the same pond. In addition, certain populations do seem to be more similar genetically to other populations not too distant geographically. The clustering of Malloy 0 & 1 indicates that individuals are very similar, and more so than to any other population. In fact, all individuals, except MB6, from the Malloy ponds share a common haplotype. Quarry and Hill ponds also cluster before being joined to the other ponds and lakes in the dendogram. Yet, some populations that are further apart cluster (in the tree - Figure 4) before clustering to populations geographically closer. Hill and Quarry Ponds are geographically closer to the other sites studied than is Indian Lake. Yet the population from Indian Lake is genetically closer to these populations than are Hill or Quarry populations.

SUBSTITUTIONS

From a total 297 nucleotide base sequence, 21% (62) of the bases were substituted, some with double substitutions occurring (Appendix 1). Another 2% (6) of all nucleotide bases were indistinguishable and indicated by the letter N (Appendix 1). Of the 62 base substitutions, 9.7% (six bases) were nonsynonymous substitutions (amino acid altering substitutions), of which two occurred at second codon position

and four occurred at the first codon base position. The synonymous (non-amino acid altering) substitutions were mainly at the third base position of the codon with a few occurring at the first codon base. These first codon substitutions were changes such as, TTA/G to a CTN, where both of these codons code for a leucine residue. Four of the six indistinguishable bases occurred at second codon positions, making it difficult to determine the amino acid at that residue (Appendix 2). The other two indistinguishable bases occurred at the third codon position, thereby not affecting the amino acid outcome at that site. Overall, 77.4% of all substitutions (not including the indistinguishable ones) occurred at the third codon position, 19.4% at the first codon position and only 3.2% at the second position. There was a strong transition bias at the first codon position, but not at the second or third codon positions. Overall, there was a transition bias; 61.3% of the substitutions were transitions (purine substituted for purine and pyrimidine for a pyrimidine), as opposed to 38.7% transversions (purine substituted for pyrimidine and vice versa).

AMINO ACID SEQUENCE

From all of the substitutions that occurred, only five out of the 99 residues were different (5.1%), with one of the five having a double amino acid replacement (Appendix 2). The amino acid sequence (inferred from the nucleotide sequence) was then compared to the mtCOI amino acid sequence of forty other organisms (sequences not shown), that were supplied by J.A. Kornblatt (personal communication). There

were nine residues that were identical in amino acid in all forty species, of which *D. leptopus* was in accordance, and one residue in 38 of the forty organisms, that the copepod was not. In *Diaptomus leptopus*, this particular residue was occupied by a neutral amino acid, as it was in the other two species (from the total 40), which were not in consensus. This neutral amino acid within the copepod was replaced by two other neutral amino acids (site of the double amino acid replacement as mentioned earlier). Among the remaining 89 residues, 37 occurred in 75% or more of the organisms and the remaining 52 were at variable sites, where there were always four or more amino acids recurring.

DISCUSSION

Genetic Variation

The existence of genetic variation within the species *Diaptomus leptopus* is manifested in both the sequence data by nucleotide diversity (Appendix 1) and the observed haplotype frequencies (Table 2). The nucleotide diversity demonstrates that some individuals within and most individuals among populations differ. The haplotypes from different populations, as indicated by the Monte Carlo analysis, are significantly heterogeneous, hence the populations are considered to differ as well. However, this analysis does not indicate which populations differ and by how much they differ.

Degree of Variation

The neighbor-joining tree (Figure 4) shows that there is a range of variation among the populations and that a majority of the individuals are most related genetically to other individuals within their respective populations. However, there are exceptions to these trends.

Individual 6 from Malloy 1 (MB) differs from all others in its pond and is similar to all other individuals in Rats Lake (RT). Thus, it is possible that there is more than one common haplotype present in both the MB and RT populations. This pattern would indicate that there may be more similarity between these populations than indicated by the Monte Carlo analysis. On the other hand, there may have been

a contamination of Rats' haplotypes into Malloy 1 Pond by Maly and associates, who have sampled both populations in the past few years. However, these two populations have rarely, if ever, been sampled on the same day, due to the distance and travelling time between them. If they were sampled in the same sampling trip, and any copepods were trapped in the sampling nets, it is unlikely that the copepod would survive the long trip between the sites. In addition, the sampling nets were thoroughly rinsed in the pond or lake that had just been sampled before going on to the next one. Only individuals from one pond were alcohol-preserved at one time, and when handling either the copepod or its DNA before the sequencing, the microcentrifuge tubes were always marked beforehand to know which samples should be placed in which tubes, thus avoiding confusion and reducing error. Therefore, cross contamination of individuals seems unlikely.

If this common haplotype is not an error, then there could be a similar situation to that present among the Lachute ponds (Malloy 0 & Malloy 1). The Lachute populations have a common haplotype, and is not thought to be an error because there are five individuals in one pond (MB) and three in the other (MA) of the same haplotype. Thus, getting more samples from both the RT & MB populations would determine whether the occurrence of the common haplotype is a real result or the consequence of an error. If no more common haplotypes between the two populations are found, then the common haplotype between RT & MB probably resulted from an error. However, if there are more common haplotypes, it would

explain why the RT is clustered with MB & MA (Figure 4), before being clustered with the other Mont-Tremblant Park population, Truite Rouge Lake.

The occurrence of both unique and common haplotypes presents an interesting situation. One can theorize that as individuals were transported, perhaps by glaciers, into new habitats, some individuals of the similar haplotype entered the same habitat, while other individuals and other haplotypes entered other habitats, thereby explaining how there could be both some common and unique haplotypes among and within some of the ponds. Selection on the other hand can explain the presence of unique haplotypes but it is difficult to explain the presence of common ones. Since there are traits, which are thought to be under genetic control, that differ among these populations (development time, size dimorphism, diapause egg production), it is assumed that adaptation will result in the haplotypes becoming different among the populations, thus explaining the presence of unique haplotypes by individual adaptation to a particular habitat. However, it is difficult to explain the presence of common ones. Nevertheless, there may be a combination of factors or other phenomena occurring, thus rendering it difficult to differentiate between selection and genetic drift, and requiring further analyses, but see discussion below. In addition, it is possible that some common haplotypes have come about by migration, or dispersal of resting eggs, yet adding another factor to distinguish from the others. However, despite the dispersal of diapausing eggs being unknown, migration among these populations does not seem too likely since none of these populations have any

direct water connections to allow adults to migrate. Nonetheless, this does not rule out egg dispersal.

If migration is occurring, then populations which are physically closer to one another should be genetically more similar. It would be easier for a copepod to migrate 10 meters, as opposed to 100 m. Therefore, a correlation analysis such as the Mantel Correlation Statistical Analysis (Manly 1992) among the geographic and genetic distances can be performed. A positive correlation would indicate that geographically closer populations are more similar and that migration is occurring. However, it is difficult to assess which factor is influential based on these speculations, and without more data and further analyses, to be discussed later. Nevertheless, based on the results obtained here, it would seem that the variation, in mtCOI, as seen here, among populations is selectively neutral. If so, then genetic drift may be considered as more influential than either selection or migration.

Substitutions

Most of the base substitutions in this study do not lead to an amino acid change, thus residues may be considered as conserved. A comparison of the *D. leptopus* amino acid sequence with that of forty other organisms indicates that certain amino acid sites that are not altered in all or almost all organisms, including the copepod's, are conserved amino acids. In addition, there are some variable sites within *D. leptopus* which are variable among all species compared as well.

Therefore, the conserved amino acids that are maintained in *D. leptopus* are assumed to be important in the structure, and thus functioning of the protein. The amino acids that do change in *D. leptopus* are at variable sites, and are perhaps not important in the maintenance of the protein structure and function. These results suggest that the gene product is not altered. In addition, most of the substitutions occur at the third codon position, presumably because the third codon position is less subject to functional constraints than the first or second positions, due to the degeneracy of codons. Therefore, as predicted by the neutral theory, nucleotide positions, which are functionally less constrained will exhibit the highest rate of change (i.e. number of nucleotide substitutions) and should harbour most of the substitutions (Avice 1994). Therefore, since there are more codons with changes at the third codon position, than at the first or second position, that do not alter the one amino acid, then the rate of change should be higher at the third codon base than at either the first or second codon base (Nei 1987). In fact, out of the 62 nucleotide base substitutions, 48 occurred at the third codon base. In addition, there were 12 nucleotide substitutions at the first codon position and only two at the second codon. Since a majority of the substitutions at the first codon position occurred at a leucine site (i.e. the changes at the first codon mainly coded for leucine - refer to the results), then it can be inferred that the first codon position is less functionally constrained than the second and the rate of change, as seen in this mitochondrial gene, is lowest at the second position, than at either the first or third position (Nei 1987). Therefore, the data seems to

support the possibility that the substitutions seen, in the mitochondrial gene studied here, among the populations, within *D. leptopus*, are selectively neutral. If this is true, it is likely that genetic drift may be more influential than either selection or migration in shaping the observed variation at this gene, within *D. leptopus*. Nevertheless, the number of individuals sampled are few and only one gene was examined. Therefore, there are certain steps that should be taken in future studies.

FUTURE STEPS

All of the above is based on the few individuals sampled. Nevertheless, the above speculation demonstrates that more than one aspect of the organism must be considered in order to make a reasonable conclusion about the current variation present in mtCOI among *D. leptopus* populations studied here, and how it came to be. Thus, in order to make a more accurate conclusion several steps should be taken, including further analyses.

Sample Sizes

In order to establish whether the presence of MB6 in both Rats Lake and Malloy 1 Pond resulted from an error or natural causes more samples need to be studied. Some studies used pools of individuals (Bucklin & Kann 1991; Bucklin et al., 1992; Burton 1994), but pools do not show the true amount of variation since all the individuals that are being pooled may not be identical. Thus, individual variation

is hidden and underestimated. Other studies range in sample sizes from 27 individuals of one species (Bucklin & Lajeunesse 1992) to sample sizes ranging from 24 per species to 162 per species, with more than two species studied (Bucklin et al., 1995). Yet, other studies have samples of 2 and 4 per species of several species (Brown et al., 1979). Authors using such small samples when examining more than one species have justified their small sample sizes by stating that the interspecific variation was found to be much greater than the intraspecific variation. Thus, one individual from each species can provide genetic distance estimates provided that the genetic distance between the species is sufficiently large (Nei 1978). Sample sizes should therefore be decided upon by the researchers and one may refer to Nei (1987) for guidelines. If small samples sizes are used, then justifications are recommended. Many statistical analyses like Monte Carlo (Roff & Bentzen 1989), Amova (Excoffier et al., 1992), and the Mantel test (correlation analysis; Manly 1992) involve permutations, which create a distribution with the original data, since the distribution of the data is unknown. These permutations thereby allow the significance of the data, i.e. the probability of obtaining the data by chance alone to be tested (Roff & Bentzen 1989) with the data itself. However, a large sample size will not only indicate whether the haplotype present in both Rats Lake and Malloy 1 Pond populations is real, but offers better probability of obtaining rare alleles (Bucklin 1995) and more confidence in the results, thus is a more attractive option. Therefore, more individuals, which were preserved at the same time as the individuals

studied here, were examined to increase the sample sizes. The additional data is in the process of being compiled and analysed, thus could not be presented here.

Gene Trees

The results analysed are based on one gene. Since all functional mitochondrial genes are transmitted as a nonrecombining unit through female lines, from a phylogenetic perspective, the entire 16kb mtDNA molecule can be considered to represent only a single "gene" (Avise 1994). However, due to variation in substitution rates among the mitochondrial genes, the results from one gene cannot be applied generally to the entire mitochondrial genome. Therefore, one gene tree reflects only that gene and no other. For this reason, gene trees may not represent species trees accurately (Moritz et al., 1987). Since genes mutate at different rates, nucleotide differentiation based on the number of base substitutions will differ among genes and relationships between haplotypes may differ. Hence, obtaining trees from several genes is best for obtaining a more general view of what is contributing to the variation of these populations of this species. Thus, future studies, wishing to build upon this one, could try to sequence the 16S rRNA gene, which has shown some consistency in sharpness, brightness and no amplification artifacts. The rRNA genes are not thought to be very variable in comparison to other mitochondrial genes (Moritz et al., 1987), but some have found the gene to be variable for some of the organisms that they studied and not for others (Ann Bucklin, personal

communication). Also, more variable genes like those of the D-loop of mtDNA, which has been found to be the most variable region in mtDNA (Moritz et al., 1987) are worth studying as well. Obtaining results from a more conserved and more variable gene will provide a more complete picture of the variation present in *D. leptopus*.

Sequence Data

To rely on data from molecular techniques requires having faith that what is being sequenced is the target DNA and that errors during synthesis will be as few as possible. However, Taq polymerase, the enzyme used during DNA synthesis, is not error free. Taq can replace one nucleotide base for another which does not correspond to the template sequence. If this error occurs early in the cycle stages, then it is difficult to detect. If it occurs later on, then reverse sequencing (sequencing the complementary strand) the target DNA may help in detecting the error. In addition, sometimes it is difficult to decide which base is present if two bases seem to occur at one site. Two bases at one nucleotide site can arise when artificial termination in more than one of the four nucleotide sequencing reactions occurs (McArdle et al., 1991). Therefore, reverse sequencing will help to decide which base is truly present because the complementary strand will contain the nucleotide's "binding partner". Overall, reverse sequencing increases the confidence in the data by clarifying such ambiguous results as two bases at one site. With more confidence

in the data, and more individuals and genes studied, further analyses may be conducted.

Analysis of Molecular Variance

Questions about which evolutionary factors (i.e. migration, natural selection and genetic drift) influence the pattern of variation present can be addressed with the aid of statistical analyses, like the analysis of variance for molecular data (AMOVA). This analysis is similar to an analysis of variance (ANOVA), but is used for molecular data. Amova yields analogous F-statistics, called phi-statistics (σ^2) which are haplotypic correlation measures at each hierarchial level (Excoffier et al., 1992). These hierarchial levels refer to the ranked division of an organism's population structure, i.e. within populations, among populations within a region and among populations among regions. Each one of these phi statistics acts like an F_{ST} at each level, i.e. defining the amount of gene flow at a particular hierarchial level. Just like an F_{ST} , a phi value of 1 indicates that there is no gene flow among populations and that the populations are very different, thus migration would not be occurring in such a case. This molecular analysis also yields variance components for each hierarchial level, which are extracted by equating the mean sum of squares to its expected mean sum of squares deviation (Excoffier et al., 1992). These variance components indicate where most of the variation lies, i.e. of the total variation found, which of the levels has the highest percent of variation. Therefore, if the percent variation is

higher among the individuals within a population, in comparison to the percent variation among populations, then this is an indication that migration could be occurring. However, if percent variation is higher among populations than within populations, then genetic drift is occurring, assuming that the variation is neutral. As for selection, there are two possible outcomes depending on the nature of the habitats. If habitats are similar, then the percent variation within populations and among populations would be similar. However, if habitats differ, then percent variation within populations would be lower than that among populations, rendering it difficult to distinguish between genetic drift and selection. If this is the case, i.e. both selection and drift are possible influential factors, then data, such as third codon substitution, will be required to resolve the influence of each. Geographical or historical situations may also serve as a resolution, or partial resolution, to such a predicament mentioned above.

History

Along with more individuals sampled, genes studied and further analyses, perhaps historical events could support findings implied from statistical analyses. For instance, if an Amova analysis would be performed, the percent variation at the different levels may help to determine the relative roles of selection and genetic drift versus the role of dispersal or migration in the genetic variation found. Therefore, examining the glacier retreats and water drainage paths may help explain how and if

genetic drift could be involved in the variation present and either confirm or refute the possibility.

Historical occurrences may also shed some light on the common haplotype present between RT and MB, in a similar manner. Since water basins were in the process of being formed during glacier retreat, water basins were not defined physically and water runoffs had no distinct path. Therefore, water runoff heading mainly south as ice sheets headed north (Dadswell 1974; Lacasse & Magnan 1994) may have had several paths. In addition, retreating glaciers caused shifts in the earth's crust to lift the west portion of Quebec (Lacasse & Magnan 1994), thereby possibly causing water run off to head southwest, where MA & MB are situated in relation to RT, thus bringing haplotypes from RT to MB. This pattern would explain why Malloy 1 has more genetic relatedness to Rats than does Truite Rouge, as indicated from the phylogenetic tree obtained here. However, this is one possibility of many others, and without more data and a more detailed study of the distribution and movements of this copepod, this possibility is a theory requiring further study. Nevertheless, historical events should be considered as well, to better understand the current geographic and genetic findings, even though exact water drainage patterns are difficult to assess.

In conclusion, the presence of genetic variation among these populations of *Diaptomus leptopus* has been established with the use of molecular data. The data

obtained so far from these populations of *D. leptopus* indicates that there is very little variation occurring within populations and much more variation among populations. To properly assess which evolutionary forces are more influential in the population structure of this calanoid, historical factors, the number of substitutions at the codon positions and the nonsynonymous vs the synonymous substitutions should be taken into consideration. A correlation analysis between the genetic and geographic distances and the Amova analysis may shed some light into the debate of which of the evolutionary factors (i.e. migration vs natural selection or genetic drift) is influential. Lastly, certain steps should be taken to ensure confidence of the data, such as obtaining adequate sample sizes, reverse sequencing and studying more than one gene. Mitochondrial studies have been breaking new grounds and I believe will continue to do so in the near future.

Figure 1: Amplification products from both the COI and 16S regions, as they appear on 1.4% agarose gels.

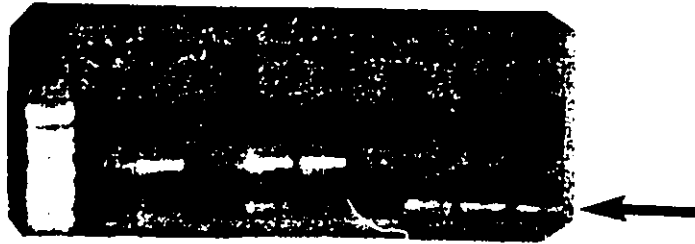
a: Lane M represents the lane with the pgem marker (Promega Corporation, Madison, W.I.); lanes 1-5 have amplification products using the COI primer set (670 base pairs [bp]); lane 6 contains the negative control and lanes 7-9 have the 16S primer amplification products (280bp). The arrow indicates the position of the 16S products that are too difficult to see due to the lightness of the products. The COI amplification products in lanes 2, 4 & 5 were chosen to be sequenced.

b: Lane M represents the lane with the pgem marker (Promega Corporation, Madison, W.I.); lanes 1-5 have amplification products using the COI primer set (670 base pairs [bp]); lane 6 contains the negative control. The amplification products in lanes 1, 2 & 3 were not chosen to be sequenced, because there were amplification artifacts present (difficult to see in lane 2). Lanes 4 & 5 had no artifacts, were sharp and bright, thus were chosen to be sequenced.

c: Lane M represents the lane with a 1 Kb DNA ladder; lanes 1-14 have amplification products using the 16S primers (280bp) and lane 15 contains the negative control. Only the amplification products in lanes 2, 3, 4, 9, 12 & 14 were chosen to be sequenced because they had no artifacts, such as in lanes 1, 5, 7 & 8, nor were they smeared like those in lanes 5, 10, 11 & 13.

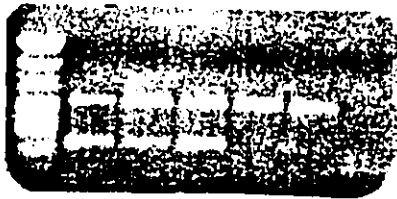
M 1 2 3 4 5 6 7 8 9

a)



M 1 2 3 4 5 6

b)



M 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15

c)



Figure 2: The automatic sequencer. The arrow indicates the position of the gel, behind which is the laser which reads the fluorescent emissions. The computer on the left side of the sequencer records the gel files, which can then be transferred to another connected computer for analysis.

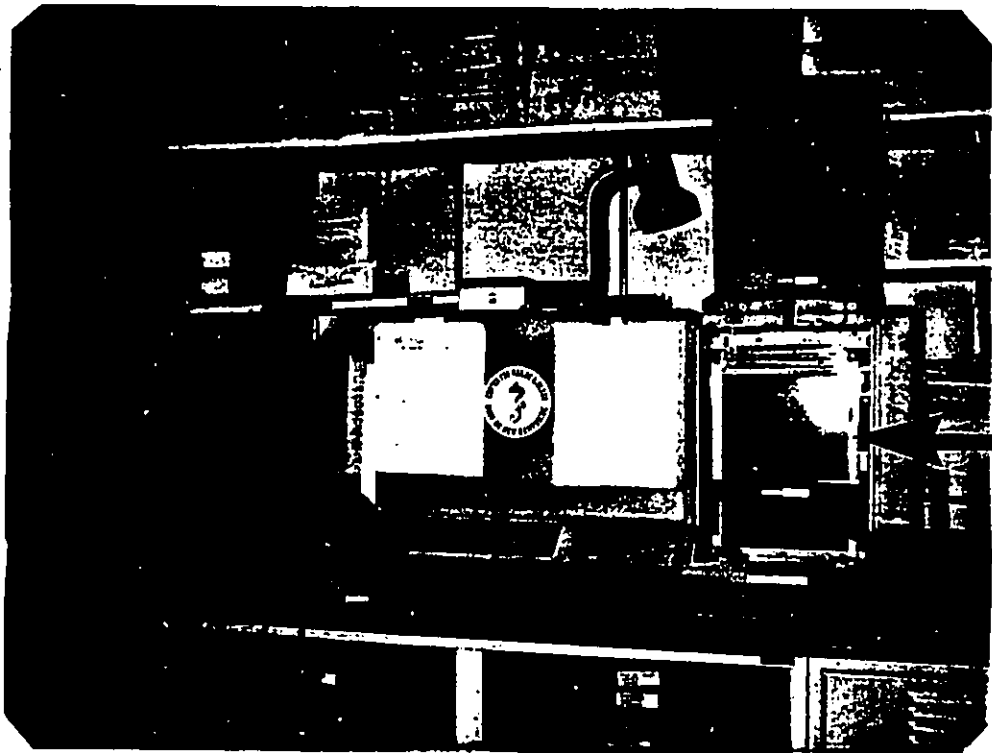


Figure 3: The four-colour chromatogram as read by the SeqEd computer program (version 2). The darkened region (indicated by the top arrow) in the sequence, shown above the chromatogram (indicated by the bottom arrow), is the portion of the sequence for which the chromatogram is displayed. Each peak has a different colour representing a different base, with their respective nucleotide base written above them. Therefore, the sequence is verified visually to be sure that the computer did not make any errors in reading the sequence.

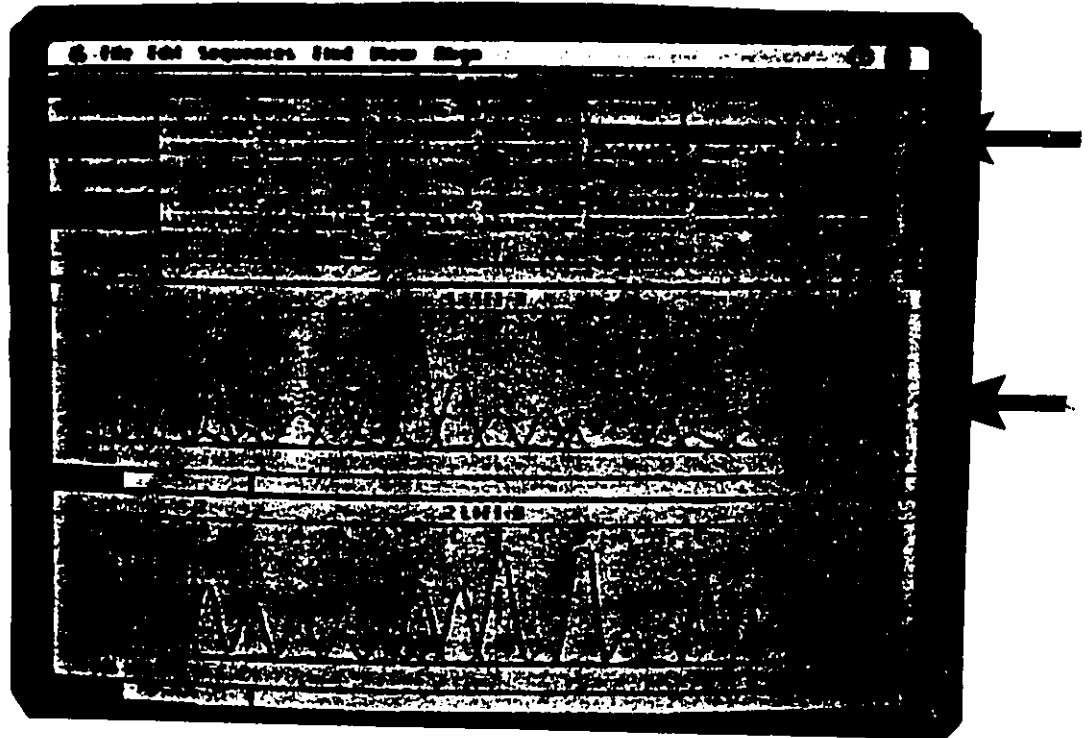


Figure 4: Neighbor-joining tree, based on Tamura-Nei distance ($\alpha=0.5$), showing the phylogenetic relationship between all haplotypes studied. Bootstrap confidence limits (1000X) are given at branch points in italics and bold, and genetic distances are given along branches. All numbered abbreviations are different individuals. Refer to table 1 for corresponding names of populations. Scale: each centimeter is approximately equal to the genetic distance of 0.008585.

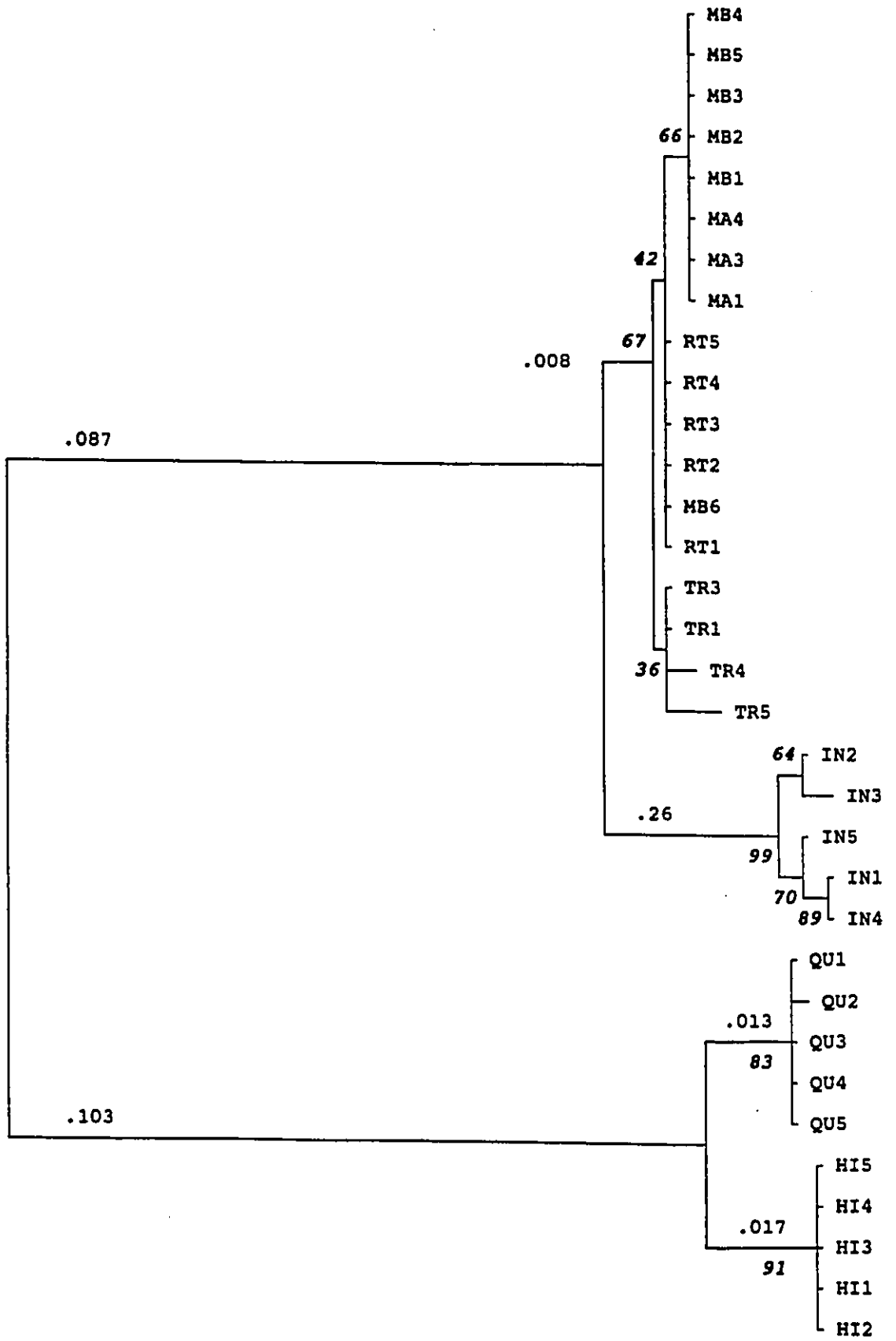


Table 1: Some characteristics of all *Diatomus leptopus* populations used in this genetic analysis of the COI gene in mtDNA. Populations are arranged according to the alphabetic order of the regional park or town (SAMPLING AREA) within which they are found. All populations were sampled during the summer of 1994. The parentheses contain the abbreviations for each pond and lake used in all other tables. POP TYPE refers to the temporary (T) or permanent (P) state of the habitat.

SAMPLING AREA	POND/LAKE	LOCATION	MAX KNOWN DEPTH (m)	MAX LENGTH (m)	MAX WIDTH (m)	POP TYPE
Adirondacks	Indian Lake (IN)	43°37'24" N 74°45'44" W	10.7	12 276	9 042	P
Lachute	Malloy 0 Pond (MA)	45°43'16" N 74°28'55" W	2.1	50	55	T
	Malloy 1 Pond (MB)	45°43'12" N 74°29'02" W	1.8	200	100	T
Mont-Tremblant	Rats Lake (RT)	46°26' N 74°19' W	9.1	2 100	700	P
	Truite Rouge Lake (TR)	46°27'30" N 74°15'30" W	7.6	2 100	780	P
Morgan Arboretum	Hill Pond (HI)	45°25'50" N 73°56'45" W	2.4	30	10	T*
	Quarry Pond (QU)	45°25'45" N 73°52'30" W	0.6	110	50	T

* indicates that the pond, although does not dry up in the summer, freeze to the bottom in the winter.

Table 2: Results from the chi-square analysis using Monte Carlo simulations. Each haplotype is represented by letters A-K. Values are the occurrences of a particular haplotype found in each population. Original X^2 (in text below) refers to the calculated chi square from the matrix below.

	A	B	C	D	E	F	G	H	I	J	K
Malloy 0	3	0	0	0	0	0	0	0	0	0	0
Malloy 1	5	1	0	0	0	0	0	0	0	0	0
Rats Lake	0	5	0	0	0	0	0	0	0	0	0
Truite Rouge	0	0	2	1	1	0	0	0	0	0	0
Hill	0	0	0	0	0	5	0	0	0	0	0
Quarry	0	0	0	0	0	0	5	0	0	0	0
Indian	0	0	0	0	0	0	0	2	1	1	0

Calculated X^2 value obtained from above matrix	= 156.98
Average X^2 from 1000 Monte Carlo simulations	= 61.69
Number of replicates which exceeded original	= 0.00
Probability of exceeding original X^2 by chance	= 0.00

Table 3: Different primer sets, from various regions of the mitochondrial DNA, used in the primer party. SOURCE refers either to the person from whom the primer was obtained or where the primer sequences can be found. Refer to Clary & Wolstenholme (1985) for locations of primers in the mitochondrial genome.

REGION/GENE	PRIMER SET	SOURCE
16S rRNA	16sar	Palumbi <i>et al.</i> , 1991
	16sbr	Palumbi <i>et al.</i> , 1991
Cytochrome b	cyb1	Palumbi <i>et al.</i> , 1991
	cyb2	Palumbi <i>et al.</i> , 1991
D-loop	Iso A	Bucklin (UNH)
12S RNA	12sbi	Palumbi <i>et al.</i> , 1991
12S RNA	12se	Palumbi <i>et al.</i> , 1991
	12sbi	Palumbi <i>et al.</i> , 1991
Cytochrome Oxidase I	COI f	Palumbi <i>et al.</i> , 1991
	COI 609	Bucklin (UNH)
	COI a	Palumbi <i>et al.</i> , 1991
	COI f	Palumbi <i>et al.</i> , 1991
	COI N	Bucklin (UNH)
	COI K	Bucklin (UNH)
Cytochrome Oxidase II	COII	Palumbi <i>et al.</i> , 1991
Cytochrome Oxidase III	COIII	Bucklin (UNH)

Literature Cited

- Allan, J.D. 1984. Life history variation in a freshwater copepod: Evidence from population crosses. *Evolution* 38(2): 280-291.
- Allendorf, F.W. and S.R. Phelps. 1981. Use of allelic frequencies to describe population structure. *Can. J. Fish. Aquat. Sci.* 38: 1507-1514.
- Anderson, R.S. 1974. Crustacean plankton communities of 340 lakes and ponds in and near the National Parks of the Canadian Rocky Mountains. *J. Fish. Res. Board Can.* 31: 855-869.
- Avise, J.C. Molecular Marker, Natural History & Evolution. Chapman & Hall Publishers; New York, New York, 1994.
- Avise, J.C. and J. Felley. 1979. Population structure of freshwater fishes. I. Genetic variation of bluegill (*Lepomis macrochirus*) population in man-made reservoirs. *Evolution* 33(1): 15-26.
- Barton, N. & J.S. Jones. 1983. Mitochondrial DNA: New clues about evolution. *Nature* 306: 317-318.
- Battaglia, B. & B. Volkmann-Rocco. 1973. Geographic and reproduction isolation in marine harpacticoid copepod *Tisbe*. *Marine Biology* 19: 156-160.
- Boileau, M.G. 1991. A genetic determination of cryptic species (Copepoda: Calanoida) and their postglacial biogeography in North America. *Zool. J. Linn. Soc.* 102:375-396.
- Boileau, M.C. and P.D.N. Hebert. 1988. Genetic differentiation of freshwater pond copepods at arctic sites. *Hydrobiologia* 167/168: 393-400.
- Boileau, M.G. and P.D.N. Hebert. 1991. Genetic consequences of passive dispersal in pond-dwelling copepods. *Evolution* 45(3): 721-733.
- Brown, W.M., M. George Jr. & A.C. Wilson. 1979. Rapid evolution of animal mitochondrial DNA. *Proc. Natl. Acad. Sci. USA* 76(4): 1967-1971.

- Bucklin, A. 1986. The genetic structure of zooplankton populations. In *Pelagic biogeography - Proceedings of an international conference*. Edited by A.C. Pierrot-Bults, S. van der Spoel, B.J. Zahuranec & R.K. Johnson. Netherlands: Unesco Publ, p 35-41.
- Bucklin, A. 1995. Molecular markers of zooplankton dispersal in the ocean. *U.S. Nat.Rept. Internat. Union Geodesy Gaffes. Supply July 1995*: 1165-1175.
- Bucklin, A. and L. Kann. 1991. Mitochondrial DNA variation of copepods: Markers of species identity and population differentiation in *Calanus*. *The Biol. Bull.* 181(2):357.
- Bucklin, A., B.W. Frost and T.D. Kocher. 1992. Mitochondrial 16S rRNA sequence variation of the *Calanus* (Copepoda: Calanoida): Intraspecific and interspecific variation. *Mol. Marine Biol. Biotech.* 1(6): 397-407.
- Bucklin, A. and T.C. Lajeunesse. 1994. Molecular genetic variation of *Calanus pacificus* (Copepoda: Calanoida): Preliminary evaluation of genetic structure and subspecific differentiation based on mtDNA sequences. *CalCOFI Reports* 35: 45-51.
- Bucklin, A., B.W. Frost and T.D. Kocher. 1995. Molecular systematics of *Calanus* (Calanoida: Copepoda). *Mar. Biol.* 121: 655-664.
- Burton, R.S. 1987. Differentiation & Integration of the genome in population of the marine copepod *Tigriopus californicus*. *Evolution* 41(3): 504-513.
- Burton, R.S. 1994. Inferring the genetic structure of marine populations: A case study comparing allozyme and DNA sequence data. *CalCOFI Reports* 35: 52
- Burton, R.S. & M.W. Feldman. 1981. Population genetics of *Tigriopus californicus*: II. Differentiation among neighboring populations. *Evolution* 35(6): 1192-1205.
- Byrom, A.E., C.W. Burns and G.P. Wallis. 1993. Experimental hybridization of alpine and lowland forms of *Boeckella dilatata*, a calanoid copepod. *Heredity* 71: 508-515.

- Carter, J.C.H., M.J. Dadswell, J.C. Roff and W.G. Sprules. 1980. Distribution and zoogeography of planktonic crustaceans and dipterans in glaciated eastern North America. *Can. J. Zool.* 58: 1355-1387.
- Chapco, W., N.W. Ashton, R.K.B. Martel, N. Antonishyn and W.L. Crosby. 1993. A feasibility study of the use of random amplified polymorphic DNA in the population genetics and systematics of grasshoppers. *Genome* 35: 569-574.
- Clary, D.O. and D.R. Wolstenholme 1985. The mitochondrial DNA molecule of *Drosophila yakuba*: Nucleotide sequence, gene organization, and genetic code. *J. Mol. Evol.* 22: 252-271.
- CLIMAP Project Members. 1976. The surface of the ice-age earth. *Science* 191: 1131-1137.
- Crease, T.J., D.J. Stanton and P.D.N. Hebert. 1989. Polyphyletic origins of asexuality in *Daphnia pulex*. II. Mitochondrial-DNA variation. *Evolution* 43(5): 1016-1026.
- Dadswell, M.J. 1974. Distribution, ecology and postglacial dispersal of certain crustaceans and fishes in eastern North America. *Natl. Mus. Can. Publ. Zoology* 11.
- DeFrenza, J., R.J. Kirner, E.J. Maly and H.C. Van Leeuwen. 1986. The relationships of sex size ratio and season to mating intensity in some calanoid copepods. *Limnol. Oceanogr.* 31: 491-496.
- Devereaux, J., P. Haerberli and O. Smithies. 1984. A comprehensive set of sequence analysis programs for the VAX. *Nuc. Acids Res.* 12: 387-395.
- Ehrlich, P.R. and P.H. Raven. 1969. Differentiation of populations. *Science* 165: 1228-1231.
- Excoffier, L., P.E. Smouse and J.M. Quattro. 1992. Analysis of molecular variance inferred from metric distances among DNA haplotypes: Application to human mitochondrial DNA restriction data. *Genetics* 131: 479-491.
- Folmer, O., M. Black, W. Hoeh, R. Lutz and R. Vrijenhoek. 1994. DNA primers for amplification of mitochondrial cytochrome c oxidase subunit I from diverse metazoan invertebrates. *Mol. Marine Biol. Biotech.* 3(5): 294-299.

- Futuyma, D.J. *Evolutionary Biology*. Second ed. Sinauer Associates, Inc Publishers: Sunderland, Massachusetts, 1986.
- Hairston, N.G.Jr. and E.J. Olds. 1984. Population differences in the timing of diapause: Adaptation in a spatially heterogeneous environment. *Oecologia* 61: 42-48.
- Hairston, N.G.Jr. and E.J. Olds. 1986. Partial photoperiodic control of diapause in three populations of the freshwater copepod *Diatomus sanguineus*. *Biol. Bull.* 171: 135-142.
- Hebert, P.D.N. and C.C. Wilson. 1994. Provincialism in plankton: Endemism and allopatric speciation in Australian *Daphnia*. *Evolution* 45(4): 1333-1349.
- Kimura, M. 1968. Evolutionary rate at the molecular level. *Nature* 217: 624-626.
- Kumar, S., K. Tamuar and M. Nei. 1993. MEGA: Molecular Evolutionary Genetics Analysis, version 1.0. Pennsylvania State University, University Park, PA 16802, USA.
- Lacasse, S. and P. Magnan. 1994. Distribution post-glaciaire de l'omble de fontaine dans le bassin hydrographique du fleuve Saint-Laurent: Impact des intervention humaines. Université du Québec à Trois-Rivières, pour le Ministère de l'Environnement et de la Faune du Québec. 83p.
- Lynch, M., K. Spitze and T. Crease. 1989. The distribution of life-history variation in the *Daphnia pulex* complex. *Evolution* 43(8): 1724-1736.
- Maly, E.J. 1991. Dispersal ability and its relation to incidence and geographic distribution of Australian centropagid copepods. *Verh. Internat. Verein. Limnol.* 24: 2828-2832.
- Maly, E.J. & I.A.E. Bayly. 1991. Factors influencing biogeographic patterns of Australian centropagid copepods. *Journal of Biogeography* 18: 455-461.
- Manly, B.F.J. 1992. RT - A Program for Randomization Testing, version 1.02. Center for Application of Statistics and Mathematics, University of Otago, P.O. Box 56, Dunaden, New Zealand.

- McArdle, B.F., M. Hane and C.W. Fuller. Using sequenase in automated DNA sequencing. Editorial COMMENTS Spring 1991 17(4): 1-8, supplied by the United States Biochemical Corporation, Cleveland, Ohio.
- Morales, J.C., B.G. Hanks, J. W. Bickham, J.N. Derr and B.J. Gallaway. 1993. Allozyme analysis of population structure in arctic cisco (*Coregonus autumnalis*) from the Beaufort Sea. *Copeia* 3: 863-867.
- Moritz, C., T.E. Dowling & W.M. Brown. 1987. Evolution of animal mitochondrial DNA: Relevance for population biology and systematics. *Ann. Rev. Ecol. Syst.* 18: 269-292.
- Nei, M. 1978. Estimation of average heterozygosity and genetic distance from a small number of individuals. *Genetics* 89: 583-590.
- Nei, M. 1987. Molecular Evolutionary Genetics. Columbia Univ. Press, New York.
- Neill, W.E. 1992. Population variation in the ontogeny of predator-induced vertical migration of copepods. *Nature* 356: 54-57.
- Nishikawa, T. 1995. Variation in sexual size dimorphism in the calanoid copepod *Diaptomus leptopus*: A test of 2 hypotheses. Master of Science Thesis, Concordia University, Montreal, Quebec.
- Palumbi, S., A. Martin, S. Romano, W.O. McMillan, L. Stice & G. Grabowski 1991. The Simple Fool's Guide to PCR (unpublished).
- Rand, D.M. 1994. Thermal habitat, metabolic rate and the evolution of mitochondrial DNA. *Tree* 9(4): 125-131.
- Randi, E., V. Lucchini and F. Francisci. 1993. Allozyme variability in the Italian wolf (*Canis lupus*) population. *Heredity* 71: 516-522.
- Roff, D.A. and P. Bentzen. 1989. The statistical analysis of mitochondrial DNA polymorphisms: X^2 and the problem of small samples. *Mol. Biol. Evol.* 6: 539-545.
- Ruiz-Garcia, M. 1993. Analysis of the evolution and genetic diversity within and between Balearic and Iberian cat populations. *J. of Heredity* 84: 173-180.

- Saitou, N. and M. Nei. 1987. The neighbour-joining method: A new method for reconstructing phylogenetic tree. *Mol. Biol. Evol.* 4: 406-425.
- Sévigny, J.M, I.A. McLaren & B.W. Frost. 1989. Discrimination among variation within species of *Pseudocalanus* based on GPI locus. *Marine Biology* 102: 321-327.
- Smithies, O., W.R. Engles, J.R. Devereaux, J.L. Slightom and S. Shen. 1981. Base substitutions, length differences and DNA strand asymmetries in the human G-gamma and A-gamma fetal globin gene region. *Cell* 26: 345-353.
- Vawter, L. & W.M. Brown. 1986. Nuclear and mitochondrial DNA comparisons reveal extreme rate variation in the molecular clock. *Science* 234: 194-196.
- Wolstenholme, D.R. & D.O. Clary. 1985. Sequence evolution of *Drosophila* mitochondrial DNA. *Genetics* 109: 725-744.
- Wyngaard, G.A. 1986. Heritable life history variation in widely separated populations of *Mesocyclops edax* (Crustacea: Copepoda). *Biol. Bull.* 170: 296-304.

Appendix I: Complete 297 nucleotide sequence for all haplotypes of *Diaptomus leptopus* studied. The dots represent the similar base as in the first haplotype (IN1) and any substitution is shown with the different base. The N represents an indistinguishable base.

	1																	54
IN1	TCT	GGG	ATG	GTG	GGG	ACT	GGG	CTA	AGT	GTA	ATC	ATT	CGG	ATA	GAG	TTA	GGC	CAA
IN5
IN4
IN2	A..
IN3	A..
MA1	G A..
MA3	G A..
MA4	G A..
MB1	G A..
MB2	G A..
MB3	G A..
MB4	G A..
MB5	G A..
MB6	G A..
RT1	G A..
RT2	G A..
RT3	G A..
RT4	G A..
RT5	G A..
TR1	T..	...	G A..
TR3	T..	...	G A..
TR4	T..	...	G A..
TR5	T..	...	G A..
QU1	A	C	...	T..	...	A A..	...	C	A
QU2	...	N	A	C	...	T..	...	A A..	...	C	A
QU3	A	C	...	T..	...	A A..	...	C	A
QU4	A	C	...	T..	...	A A..	...	C	A
QU5	A	C	...	T..	...	A A..	...	C	A
HI1	A	C	...	T..	...	A A..	...	C	A
HI3	A	C	...	T..	...	A A..	...	C	A
HI4	A	C	...	T..	...	A A..	...	C	A
HI5	A	C	...	T..	...	A A..	...	C	A
HI2	...	N	A	C	...	T..	...	A A..	...	C	A

	55																	109
IN1	GCC	GGC	TCG	TTA	ATT	GGG	GAC	GAC	CAA	ATT	TAC	AAT	GTA	GTT	GTC	ACA	GCT	CAT
IN5
IN4
IN2	A..
IN3	A..
MA1
MA3
MA4
MB1
MB2
MB3
MB4
MB5
MB6
RT1
RT2
RT3

RT4
RT5
TR1
TR3
TR4
TR5
QU1	.TTCTG	.AN	...	
QU2	.TT	.NCTG	.AN	...	
QU3	.TTCTG	.AN	...	
QU4	.TTCTG	.AN	...	
QU5	.TTCTG	.AN	...	
HI1	.TC	.ATG	.AN	...	
HI3	.TC	.ATA	
HI4	.TC	.ATA	
HI5	.TC	.ATA	
HI2	.TN	.C	.ATA	

109

162

IN1	GCT	TTT	ATT	ATG	ATT	TTT	TTT	ATG	GTT	ATA	CCT	ATT	TTG	ATT	GGC	GGG	TTT	GGC
IN5
IN4
IN2
IN3
MA1	.CAG
MA3	.CAG
MA4	.CAG
MB1	.CAG
MB2	.CAG
MB3	.CAG
MB4	.CAG
MB5	.CAG
MB6	.CAG
RT1	.CAG
RT2	.CAG
RT3	.CAG
RT4	.CAG
RT5	.CAG
TR1	.CAG
TR3	.CAG
TR4	.CAG
TR5	.CAG
QU1C	.AAGA
QU2C	.AAGA
QU3C	.AAGA
QU4C	.AAGA
QU5C	.AAGA
HI1C	.AAGA
HI3C	.AAGA
HI4C	.AAGA
HI5C	.AAGA
HI2C	.AAGA

163

216

IN1	AAT	TGA	TTA	GTG	CCT	TTA	ATA	CTA	GGC	GCA	GCG	GAC	ATA	GCT	TTC	CCC	CGT	ATA
IN5
IN4
IN2
IN3

HI4A ..CG ..TA ..T T.. ..A ... T.G T.AT
 HI5A ..CG ..TA ..T T.. ..A ... T.G T.AT
 HI2A ..CG ..TA ..T T.. ..A ... T.G T.AT

271

287

IN1 TCT CTT GTA GAA AGA GGT GCC GGA ACA
 IN5
 IN4
 IN2
 IN3G ..
 MA1
 MA3
 MA4
 MB1
 MB2
 MB3
 MB4
 MB5
 MB6
 RT1
 RT2
 RT3
 RT4
 RT5
 TR1
 TR3
 TR4
 TR5
 QU1A .A.AG ..
 QU2A .A.AG ..
 QU3A .A.AG ..
 QU4A .A.AG ..
 QU5A .A.AG ..
 HI1 C.. T.AAG ..
 HI3 C.. T.AAG ..
 HI4 C.. T.AAG ..
 HI5 C.. T.AAG ..
 HI2 C.. T.AAG ..

Appendix II: Amino acid sequence of all haplotypes from all ponds. Amino acids in all haplotypes that are similar to the first haplotype (IN1) are represented by a dot. Unknown amino acids (due to a base change at the second position of the codon) are represented by a ? and all other amino acids changes are shown by the different amino acid symbol at the each site.

	1				60
IN1	SGMVG	TGLSV	IIRME	LQAG	SLIGDDQIYN VVVTAHAFIM IFFMVMPILI GGFGNWLVEL
IN5
IN4
IN2MT.
IN3MT.
MA1M
MA3M
MA4M
MB1M
MB2M
MB3M
MB4M
MB5M
MB6M
RT1M
RT2M
RT3M
RT4M
RT5M
TR1M
TR3M
TR4M
TR5M
QU1MV.?
QU2MV.?
QU3MV.?
QU4MV.?
QU5MV.?
HI1M
HI3M
HI4M
HI5M
HI2M?
	61				99
IN1	MLGAADMAFP	RMNNMSFWFL	IPALV	MLLSS	SIVESGAGT
IN5
IN4
IN2
IN3
MA1
MA3
MA4
MB1
MB2
MB3
MB4
MB5
MB6
RT1

RT2
RT3
RT4
RT5
TR1
TR3
TR4
TR5
QU1
QU2?
QU3
QU4
QU5
HI1?
HI3?
HI4?
HI5?
HI2?