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Multi-locus genetic evidence for rapid ecologically based speciation in *Daphnia*

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Abstract

The process of speciation involves the divergence of two or more subpopulations of a parent species into independent evolutionary trajectories. To study this process in natural populations requires a detailed knowledge of the genetic and ecological characteristics of the parent species and an understanding of how its populations can lose evolutionary cohesion. The cosmopolitan and speciose genus *Daphnia* provides many of these features by existing in multiple freshwater habitat types, particularly permanent lakes and temporary ponds, each of which presents distinct ecological challenges. We assayed the genetic composition of 20 temporary pond populations of members of the *Daphnia pulex* species complex in north-western Oregon and compared them to published data on related lake and pond populations. We collected molecular genetic data from 13 allozyme loci, from six microsatellite loci, and from the control region of the mitochondrial DNA. By assaying over 400 individual *Daphnia* for these data, we were able to compile composite genotypes not only of individual *Daphnia* but of each pond population as a whole. In these ponds, we discovered two distinct genotypic constellations, one which bears resemblance to the lake-dwelling taxon *D. pulicaria*, and one which bears resemblance to the pond-dwelling taxon, *D. pulex*. Using published genetic data from these and other species as a frame of reference, we characterized 13 of these ponds as being 'pond-like', three as being 'lake-like', and four as being 'mixed'. Unlike studies performed elsewhere, however, these ponds do not exhibit high probabilities of interspecific hybridization. Over 95% of all individuals have either a lake-like or a pond-like genotype at all three genetic systems, suggesting the two forms do not represent hybridized vs. nonhybridized genotypes. Because both types can be found in the same ponds at the same time in gametic disequilibrium, we also discount the possibility that they are two extremes of a single species that is highly genetically subdivided. With these genetic data, and with supporting life-history and ecological data previously gathered on these pond populations, we conclude that the most likely description of this system is of a taxon caught in the act of speciating, with new pond-adapted populations periodically stemming from lake-adapted sources during river flooding events.

Keywords: allozymes, *Daphnia*, ephemeral ponds, microsatellites, mtDNA, speciation

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Introduction

Speciation is the process by which one species splits into two. To study this process in natural populations requires a discrete definition of what a species is, so that divergences over time between populations can be measured quantitatively. In spite of a tendency of treating species as a fundamental unit of evolution, the definition of a species has remained elusive and typically is applied case-by-case by some operational criteria. The Biological Species Concept (BSC), the Phylogenetic Species Concept (PSC), and others have the potential for providing extrinsic

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criteria for the delineation of species in all taxa, but biologists have been slow in uniformly adopting any such convention (Coyne 1992; Avise & Wollenberg 1997). In practice, new species are often declared simply on the basis of a few genetic, behavioural, morphological, or ecological traits that are found to differ among populations, using previously such-defined species as the frame of reference. Nevertheless, regardless of how species are defined, many would argue that a population or a group of populations that share a common evolutionary trajectory deserve species status (cf. Ayala 1976). That is to say, groups of individuals who can exchange genes and who are subject to common selection pressures from their environment are good candidates for species, and divergences among such groups are good systems in which to study speciation.

Yet a critical obstacle facing those seeking to detect speciation in natural systems is the elucidation of a reproductive isolating mechanism, or an adaptive peak shift, or both, that completes the transition from a genetically subdivided species into multiple evolutionary trajectories. Though it is possible that genetic drift alone can promote speciation, recent studies have focused on ecological factors that promote populational divergence at both the genotypic and phenotypic levels in an attempt to clarify the role of natural selection in this process. Templeton (1981) pointed out that many of the observed genotypic differences among species may have developed subsequent to speciation and thus contributed nothing to reproductive isolation. Bush (1994) reiterated this problem, and stressed that trying to explain speciation within the context of a preconceived species concept is inappropriate. The sympatric generation of distinct evolutionary trajectories of the haw fly *Rhagoletis* has been traced by Bush and coworkers to a distinct ecological variable, in this case the anthropomorphic introduction of a new habitat, and serves as a good example of the utility of examining the genetics of speciation as opposed to the genetics of species differences (Feder *et al*. 1990; Bush 1994).

The freshwater microcrustacean *Daphnia* (water fleas) is a ubiquitous component of aquatic biotas and has a long evolutionary history stretching back at least 100 million years (Benzie 1987; Kerfoot & Lynch 1987; Taylor *et al*. 1996). An abundance of speciation events, many of which have been associated with the colonization of new aquatic habitats, have produced clades that likely include very distantly related forms, intermediately related forms, and extremely closely related forms (Colbourne *et al*. 1997). Phylogenetic reconstruction in this genus has accordingly been contentious, and researchers are now increasingly turning to molecular data in hopes of resolving long-standing systematic disputes (Colbourne & Hebert 1996). One group of *Daphnia* receiving particular scrutiny is the *D. pulex* species complex, an assemblage of 14–15 named species that inhabits both lake and pond habitats in the Holarctic. Two widespread members of this group include a pond-dwelling form (nominally *D. pulex*) and a lake-dwelling form (nominally *D. pulicaria*). In this group lies several putative cases of recent speciation and probable cases of collapse of species-isolating mechanisms (Lynch & Spitze 1994). It has been often speculated that this group's evolutionary plasticity is a result of the ability of populations to rapidly alter their mode of reproduction (sexual, asexual, or an alteration of the two = cyclical parthenogenesis) to meet the ecological demands of their habitat (Lynch & Gabriel 1983). However, because of the high potential for disjunct forms to interbreed, leading to apparent interspecific hybridization, it becomes extremely difficult to tease apart the genetic traits that are the distinguishing features of a given species. To confound this problem, *Daphnia* exhibit a strong amount of morphological plasticity in which rapid acclimatization can occur to enhance the survival of individuals in particular habitats. For example, in the presence of the dipteran predator *Chaoborus*, *Daphnia* will undergo a developmental alteration that produces spikes on their carapaces as an anti-predator strategy (Krueger & Dodson 1981; Spitze 1992). The relative lack of morphological synapomorphies in this genus has contributed greatly to taxonomic and phylogenetic confusion. Hence, the combined influences of genetic and ontogenetic fluidity complicate studies of speciation in this genus, and compel researchers to bring several independent data sets together to reconstruct the evolutionary history of pulex-group populations (Colbourne *et al*. 1997).

Daphnia populations are abundant in standing freshwater habitats such as lakes and ponds but are less often found in lotic systems such as rivers, streams, and groundwater. Movement of *Daphnia* from waterbody to waterbody presumably occurs when desiccation resistant resting eggs, termed ephippia, are transported by wind, water, or an animal vector. In the state of Oregon, there exist numerous waterbodies and river systems. In the north-western portion of the state, the lowland valley draining into the Willamette River supports many temporary ponds that contain *Daphnia* populations during the winter, spring, and early summer when the ponds are well hydrated. Though these *Daphnia* were originally characterized as the common pond species *D. pulex*, recent morphological studies identified a synapomorphic character, ephippial spinescence, that set Willamette valley pond *Daphnia* apart from *D. pulex* (Hebert 1995). This observation, in conjunction with detection of a somewhat unique set of allozyme allele frequencies in these ponds, led to the reclassification of these *Daphnia* into a new species, *D. arenata* (Hebert 1995). While subsequent allozyme and mitochondrial DNA (mtDNA) surveys of some of these populations did support a monophyletic association of this clade of *Daphnia*, the arenata clade is always found at a very low

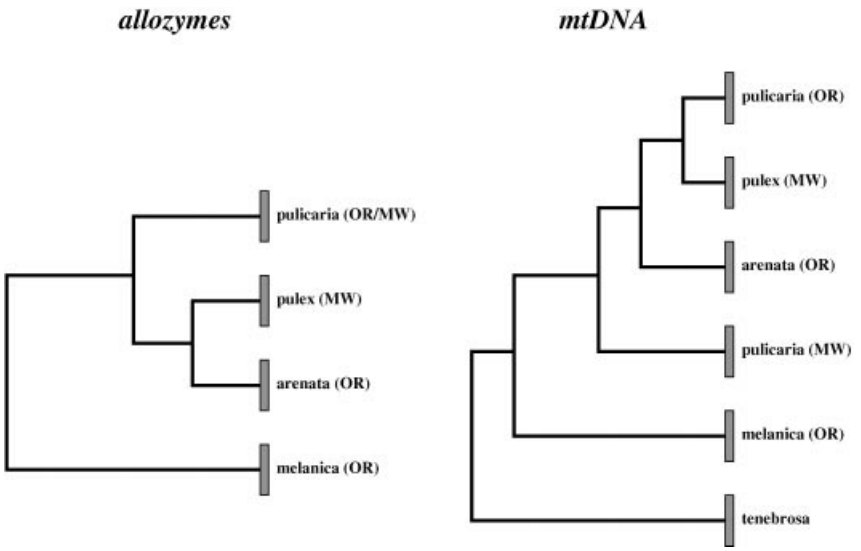


Fig. 1 Generalized distance-based phylogenetic relationships among taxa in or related to the *Daphnia pulex* species complex. Allozyme dendrogram represents the consensus relationships of four groups based on published analyses of 10–20 allozyme loci by Lynch & Spitze (1994) and Crease *et al*. (1997). Mitochondrial DNA dendrogram represents the consensus relationships of six groups based on the published control-region analysis of Lehman *et al*. (1995) and ND5 analysis of Colbourne *et al*. (1998). The branch lengths are not indicative of genetic distances, except that both analyses reveal a larger gap between the outgroup for the *D. pulex* species complex (*D. melanica*) and the ingroup taxa than among any ingroup taxa themselves. The current study focuses on populations putatively assigned to the *D. arenata* taxon.

genetic distance from other pulex clades (Lynch & Spitze 1994; Lehman *et al*. 1995; Crease *et al*. 1997; Colbourne *et al*. 1998) and in fact is almost always found imbedded in a larger grouping that includes *D. pulex* (Fig. 1).

Recent detailed quantitative genetic studies on 17 putative *D. arenata* populations from the Willamette valley and the central Oregon coast found little justification for distinguishing these populations from other *D. pulex* populations on the basis of life-history trait measurements (Lynch *et al*. 1999). However, this study did conclude that there was a significant amount of genetic subdivision among these pond populations, both at the quantitative genetic and molecular genetic levels, although the two did not appear correlated. In particular, ponds from the northern portion of the valley appeared to differentiate significantly from those from the southern portion of the valley and the coast. Of note was the fact that there were three additional ponds originally to be included in the quantitative genetic analyses, but these were removed from the study when it was realized that they did not consist primarily of genotypes characteristic of *D. pulex*. Yet from an micro-ecological point of view, the ponds from which these three populations were collected did not possess any features distinct from the other 17 populations. Here, we present a detailed molecular genetic analysis of all 20 pond populations and compare the results with data previously collected from other *Daphnia* species. By integrating distinct and independent data from allozyme allele frequencies, microsatellite allele frequencies, and mtDNA genotypes, in conjunction with published life-history trait data (Lynch *et al*. 1999), we are in a position to establish a frame of reference for interpopulational differentiation. We will use the combination of genetic and ecological information to test the hypothesis that sympatric speciation has recently occurred on a regional scale in *Daphnia*. In particular, we are interested in the potential for *D. pulex* complex members to exhibit gene flow between permanent lake populations and temporary pond populations, for these are the two most discrete ecological settings for the genus. Because speciation is often thought to involve the creation or the colonization of new habitats, this group of *Daphnia* and its dichotomous habitats may in fact, when studied at high enough resolution, provide an opportunity to observe the speciation process *in situ*.

Materials and methods

Sample collection

Twenty shallow (<1 m deep) temporary ponds from western Oregon, USA were sampled in January and February of 1996 and 1997 as originally described in Lynch *et al*. (1999). In addition to the 17 ponds investigated in that study, three additional ponds (GI, MP, and PW) were included in our population genetic study. At least 75 individuals in each population were isolated in separate beakers and allowed to reproduce parthenogenetically. Clonal derivatives of original isolates were concentrated in 95% ethanol and frozen at −20 °C for subsequent genetic analyses. These ponds were primarily located in the Willamette valley (Fig. 2) but one pond (KC) from the coastal mountain range and three ponds (BT, SW, and LO) from the central Oregon coast were included. 'Northern' and 'southern' groups of 10 ponds each were delineated on the basis of geography (Fig. 2) and the quantitative genetic analyses of Lynch *et al*. (1999) that supported such a distinction. The three ponds not included in the Lynch *et al*. (1999) study, but included here, were all in the northern

Fig. 2 Map of north-western Oregon depicting sampling localities in this study. Filled circles represent the 20 ephemeral (nondune) ponds in which pulex-group *Daphnia* were detected. The diamond denotes the location of ephemeral dune ponds containing *D. melanica* and the open squares denote the location of some of the permanent lakes referred to in this study. The Willamette River runs south to north, from sources in the Cascade mountain range and empties into the Columbia River. The middle fork of this river (shown) is now dammed at several locations, including Hills Creek Reservoir (HCR), in part to control flooding downstream. The dashed line separates the northern from southern groups of ponds as described in Lynch *et al*. (1999).

group. In addition to these samples, we utilized collections of pulex-group members from 42 Oregon lakes (Straughan & Lehman, 2000) and the unique melanic dune-pond species from the Oregon Dunes National Recreation Area, *Daphnia melanica* (Hebert 1995; Lehman *et al*. 1995). The latter has been shown to be a close outgroup to the pulex clade (Lehman *et al*. 1995; Colbourne *et al*. 1998) and is used as such in this paper. Quantitative genetic analyses of 18 life-history traits, such as clutch size at first reproductive event (C1) and body size at first adult instar (Sm), were performed previously on all 20 ponds (Pfrender 1998) including the three new ponds not reported in Lynch *et al*. (1999).

Allozyme electrophoresis

Because species in the *D. pulex* complex have been defined in the past two decades on the basis of allozyme genotypes at selected loci, we characterized the allele frequencies in all 20 pond populations using 13 potentially diagnostic loci. Allozyme alleles at the following loci: *AAT* (formerly *GOT*), *AMY*, *APK*, *FUM*, *G3PDH*, *HK* (formerly *HEX*), *LDH*, *MDH*, *ME*, *MPI*, *PEP*-1, *PGI*, and *PGM* were scored for their relative mobilities toward the '+' pole (anode) on cellulose acetate plates (Hebert & Beaton 1993). Allele designations F, M, S, etc. refer to 'fast', 'medium', and 'slow', etc. migrating alleles, respectively, and correspond to published allele designations (e.g. Spitze 1993; Lehman *et al*. 1995; Crease *et al*. 1997). Enzyme designations and substrates correspond to those published previously (Lynch 1983; Spitze 1993), except that the *PEP* locus in this study uses a Leu-Gly substrate and is not the same locus reported in Crease *et al*. (1997). Sample sizes varied with each pond, but at least 20 individuals per pond were targeted for typing at all 13 loci. Additional individuals were typed at polymorphic loci.

DNA extraction and amplification

Genomic DNA was liberated from live or preserved *Daphnia* using the Chelex method (Walsh *et al*. 1991; Morin *et al*. 1993). Single individuals were placed in 600 µL microcentrifuge tubes containing 300 µL 10% Chelex 100 resin (BioRad Laboratories) in ultra-pure water. Lid locks were secured to the microcentrifuge tubes and the tubes were autoclaved at 121 °C at 10 psi for exactly 20 min. Upon completion of the sterilization cycle, the autoclave chamber was fast exhausted and the microcentrifuge tubes were placed on wet ice. These samples were stored indefinitely (up to one year) at 4 °C before DNA amplification.

Microsatellite analysis

Twenty individuals from each pond population were chosen for microsatellite typing at six unlinked microsatellite loci as described previously (Pfrender 1998; Lynch *et al*. 1999). Briefly, microsatellite primers isolated from Midwestern *D. pulex* (Pfrender 1998; J. Colbourne, unpublished data) were 5′-end labelled using [γ-^{32}P]-ATP, and the products of 12.5 µL polymerase chain reactions (PCR) using these primers were separated on 6% polyacrylamide gels and visualized by autoradiography. Alleles were scored against a sequencing ladder to determine exact sizes, and size standards were chosen to run on all gels to assure scoring uniformity. The nucleotide sequences of selected homozygous alleles (see Results) were determined bidirectionally using the *Taq* FS dye-terminator cycle-sequencing method (Perkin-Elmer) on an ABI 373 automated sequencer, and spot checked using dideoxy sequencing with [α-^{35}S]-dATP, Sequenase 2.0 DNA polymerase (USB), and autoradiography.

mtDNA restriction fragment length polymorphism analysis

Between 8 and 15 individuals from each pond population were chosen for restriction site variation within the mtDNA control region as described previously (Lynch *et al*. 1999; Straughan & Lehman 2000). Briefly, the PCR product from the entire control region (approximately 770 bp) was digested with a panel of seven restriction enzymes (*Bam*HI, *Dra*I, *Hha*I, *Mse*I, *Pal*I, *Rsa*I, and *Sau*96I), which survey for variation at roughly 30 restriction sites, or roughly 100 nucleotide positions within the control region. Restriction fragments were separated on 2% or 3% agarose gels and visualized by ethidium bromide staining. Composite mtDNA genotypes were compiled by combining fragment profiles from all seven restriction enzymes. Restriction sites were confirmed and mapped using complete nucleotide sequences obtained from some of the individuals. A UPGMA dendrogram reconstruction of all pond similarities based on mtDNA genotypes was computed using these restriction sites by the PHYLIP software package (Felsenstein 1993). The extreme mtDNA sequence similarity of all control region genotypes under consideration (see Results) indicated that mutational saturation has not been reached in this locus among these populations, thus no correction for multiple hits was used in phylogenetic reconstruction. Lastly, the restriction fragment length polymorphism (RFLP) patterns were compared with published genotypes discovered in Midwestern pulex-group members (Van Raay & Crease 1994; Crease *et al*. 1997) and with genotypes discovered in Oregon lakes (Straughan & Lehman 2000).

Population genetic analyses

Populations were first defined to include all members from the same pond; groupings of ponds then could be made to create larger populations. Among-population genetic differentiation was quantified by the use of analogs to Wright's *F*-statistics.

For the microsatellite data, observed genotype and allele frequencies at each of six loci were used to calculate estimates of genetic diversity for each pond and for average diversity estimates for several ponds grouped together. The effective number of alleles, n_e, is a measure of diversity that describes the equivalent number of equifrequent alleles that are segregating in a pond, and was calculated for each locus by $n_e = [\Sigma(x_i)^2]^{-1}$ (Nei 1987; p. 187) and average values across all ponds or across all loci can be made as simple unweighted arithmetic averages because the same number of individuals (20) were typed with all six loci in all 20 ponds. Gene diversity, h, is the expected fraction of heterozygotes in a population under the assumption of random mating and was calculated for each locus by $h = 1 - \Sigma(x_i)^2$ where x_i is the frequency of microsatellite allele i in a pond and the summation is performed over all alleles observed at the locus (Nei 1987; p. 177). Among-population genetic differentiation was estimated in two ways. First, G_{ST} values were obtained by considering the weighted deviations of average h values across all loci from the average squared allele frequencies (Nei 1973). Similarly, excess homozygosities in each pond were calculated as F_{IS} using the method of Weir & Cockerham (1984). Second, the R_{ST} statistic (Slatkin 1995) was calculated using the RST-CALC program of Goodman (1997, 1998) to correct for significant mutation rates inherent in microsatellite loci. R_{ST} values were computed for all 20 ponds, for the 10 northern and 10 southern ponds grouped together as aggregate populations, for the southern ponds and seven northern ponds (CP, FC, OA, PG, SH, SL, and WM) and three northern ponds (GI, MP, and PW) ponds grouped together as aggregate populations, and for all pairwise comparisons between ponds. Linkage disequilibria among nuclear loci was assessed by considering the

agreement of composite genotype and allele frequencies to Hardy–Weinberg equilibrium (HWEQ) expectations using χ^2 goodness-of-fit tests (Weir 1996; pp. 112–135). Lastly, we assessed the genotypic associations of specific multilocus genotypes in various populations by estimating the relatedness *R* (Queller & Goodnight 1989) of these genotypes to two reference populations. The reference populations comprised 20 randomly chosen genotypes from both northern (GI and PW) and southern (AM, BT, and MV) populations for a total of 40 genotypes. The relatedness of 34 randomly chosen genotypes from certain critical populations to these reference populations was calculated with the program RELATEDNESS 5.0.5 (Queller & Goodnight 1989).

For the mtDNA data, the genotype of each individual was considered as the composite presence-absence matrix of the 30 restriction sites surveyed in the seven-enzyme RFLP screen. Deletions and insertions large enough to be visible on agarose gels were typically scored as the loss or gain of a single restriction site. mtDNA genotypes and genotype frequencies in valley and coastal ponds were compared to those found in dune ponds, in Oregon lakes, and in non-Oregon pulex-group populations (Lehman *et al*. 1995; Crease *et al*. 1997; Straughan & Lehman 2000).

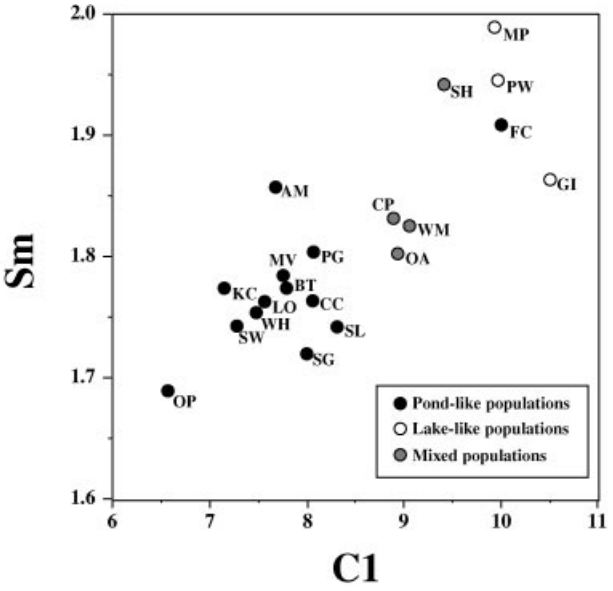


Fig. 3 Sample bivariate plot of average life-history characteristics in pond populations (Pfrender 1998). In this comparison, average clutch size at first reproductive event (C1) is plotted against average size at maturity in mm (Sm) for all 20 populations in this study.

Results

All 20 ponds sampled for this study are less than 25 m in diameter and have the potential to dry up in the late summer or fall; their locations are depicted in Fig. 2. Preliminary quantitative genetic and population genetic analyses of these ponds indicated that 17 of them were approximately in HWEQ and were thus likely to contain cyclically parthenogenetically reproducing populations (Lynch *et al*. 1999), a characteristic of *Daphnia pulex*. Thus, they reproduce asexually for several generations before undergoing a bout of sexual reproduction that is often triggered by an environmental cue such as pond desiccation. However, one pond (MP) did not appear to be in HWEQ, presenting the indication that it may be composed primarily of asexual clones, as is often the case with populations inhabiting more permanent waterbodies such as lakes. Two other ponds (GI and PW) were in HWEQ but appeared to have genetic characteristics of lake-dwelling individuals. Lake populations of the pulex complex are often described as a distinct species, *D. pulicaria*, with the potential to hybridize with pond-dwelling species such as *D. pulex*. Because lake- and pond-dwelling forms may experience distinct ecological forces to which adaptation is geared, we first examined the placement of these three peculiar populations (GI, MP, and PW) within bivariate plots of average life-history characteristics (Pfrender 1998). One such plot is shown in Fig. 3, in which the GI, MP, and PW populations exhibit the most extreme values. These populations, and to a lesser extent SH, FC, WM, CP, and OA, in that order, possess the largest individuals, who generate the largest first clutches at reproduction. This pattern, with GI, MP, and PW as outliers to the other populations, is mirrored in nearly all other bivariate analyses (Pfrender 1998). From these data it can be inferred that these three populations may have recently experienced a different set of selective forces than the other pond populations.

Allozymes

Analysis of allozyme allele frequencies allows an independent establishment of traits that may be characteristic of independent evolutionary trajectories. Previous research on the genetics of the *D. pulex* species complex in North America has produced the convention that the fast allele of the lactate dehydrogenase locus (*LDH*-F) is a distinguishing feature of the predominantly lake-dwelling form (i.e. *D. pulicaria*), while the *LDH*-S allele is a distinguishing feature of the predominantly pond-dwelling form (i.e. *D. pulex*) (Hebert *et al*. 1989; 1993). The hexokinase locus is also potentially diagnostic, with *HK*-S being found in high frequencies in lakes, while *HK*-M is found in high frequencies in ponds (Hebert *et al*. 1989). These trends are very strong among Oregon lakes, where *LDH*-F is present at a frequency of 0.95 and *HK*-S is present at a frequency of 0.97 (Crease *et al*. 1997).

The allele frequencies from 13 allozyme loci in all pond populations are diagrammed in Fig. 4; 10 of these loci

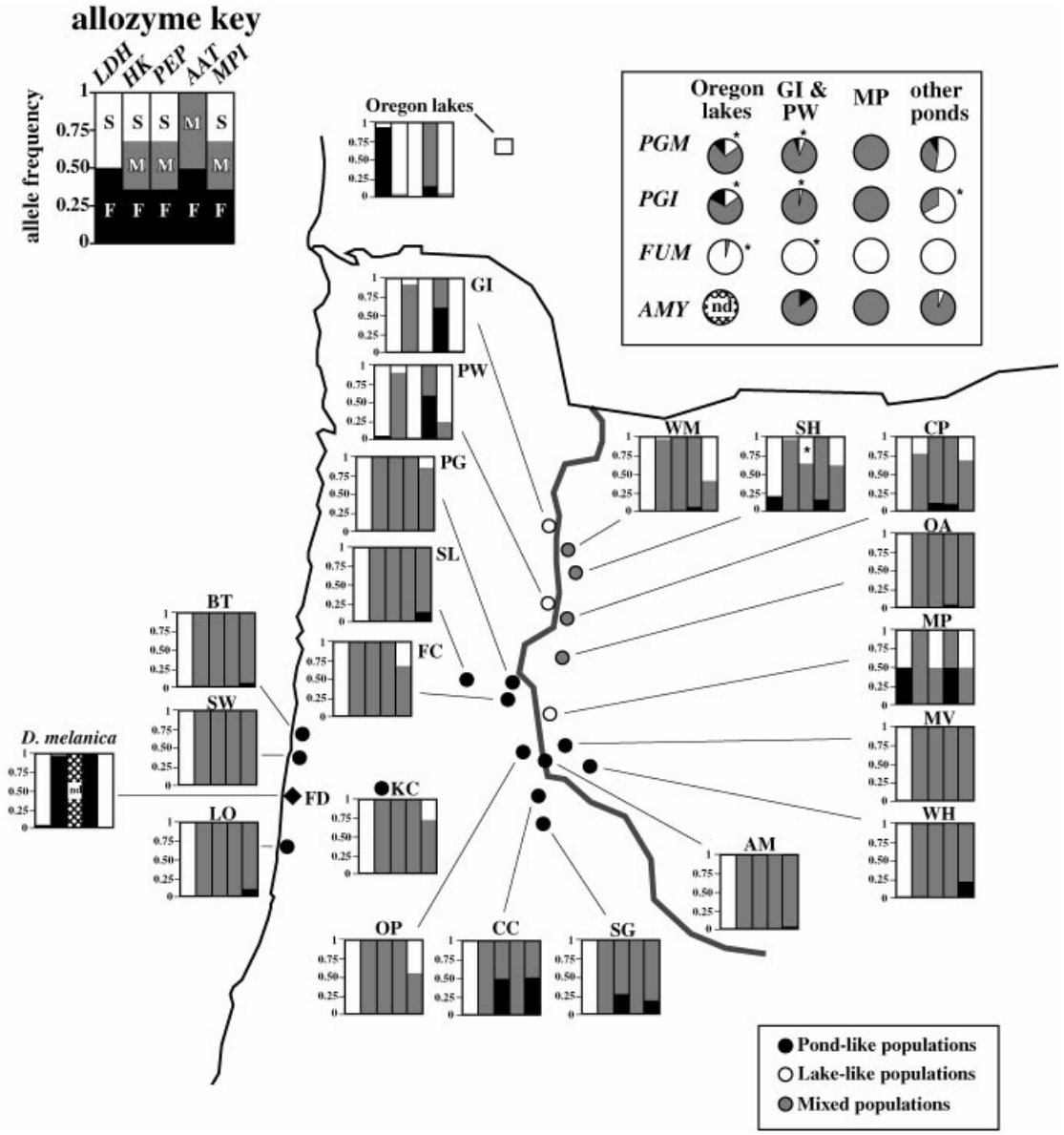


Fig. 4 Distribution of allozyme allele frequencies among Oregon *Daphnia* populations. For the five most diagnostic loci (*LDH*, *HK*, *PEP*, *AAT*, and *MPI*), the frequencies within each pond population is given by bar graphs. For four additional polymorphic loci (*PGM*, *PGI*, *FUM*, and *AMY*), populations are pooled such that pie graphs depict composite allele frequencies among one or more populations, and all nondune populations were fixed for M alleles at three loci (*G3PDH*, *ME*, and *APK*). For some loci, S and S-alleles were pooled to give the frequencies denoted in the white portions of the graphs indicated with asterisks. In these cases the frequency of S-alleles in the population ranged from 1 to 6%. Data from Oregon lakes and from the dune-pond species *D. melanica* are from Crease *et al*. 1997. Notation 'nd' = not determined.

were polymorphic among ponds. An average of 37.2 individuals per pond were partially typed at the polymorphic loci, and 17 or more individuals per pond were typed at all 13 loci. Among these samples, the *LDH*-F allele is very rare, being only detected in pond MP, at a frequency of 0.500, and in two other ponds at low frequencies: 0.047 in PW and 0.211 in SH. All other individuals possess the *LDH*-S allele. The *HK*-S allele is a little more common, being found in low frequencies in the CP, GI, OA, PW, SH, and WM ponds; the *HK*-M allele predominates otherwise. However, a marked pattern is seen at a third locus, *PEP-1*. The M allele is the most common, being fixed in 13 of the ponds. But the S allele is fixed in GI, nearly so in PW, and has a frequency of 0.500 in MP and 0.286 in SH. Among Oregon lakes, the S allele at this locus is nearly fixed (frequency >99%, K. Spitze, unpublished data). The

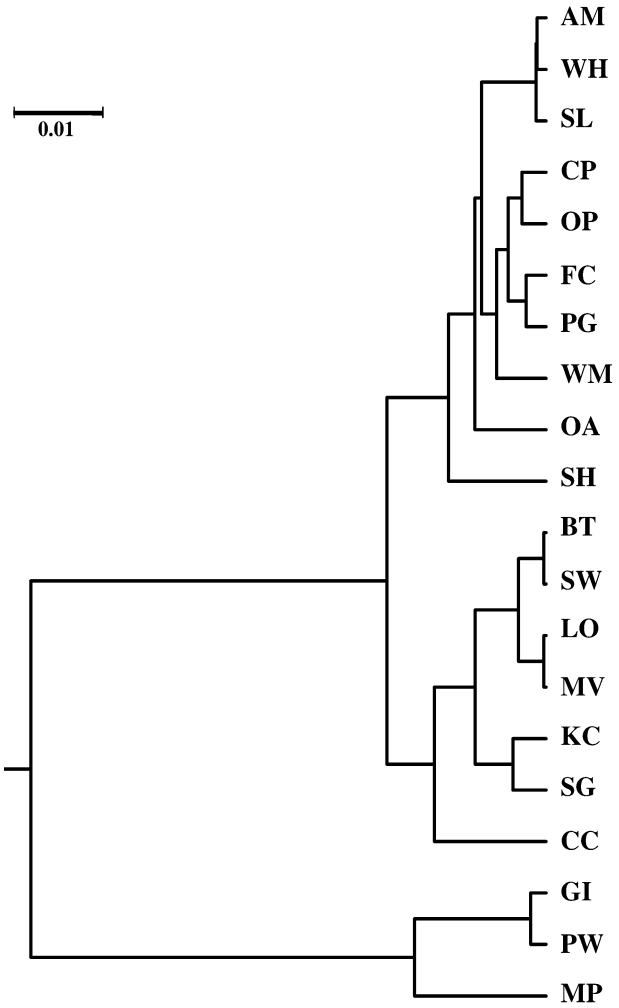


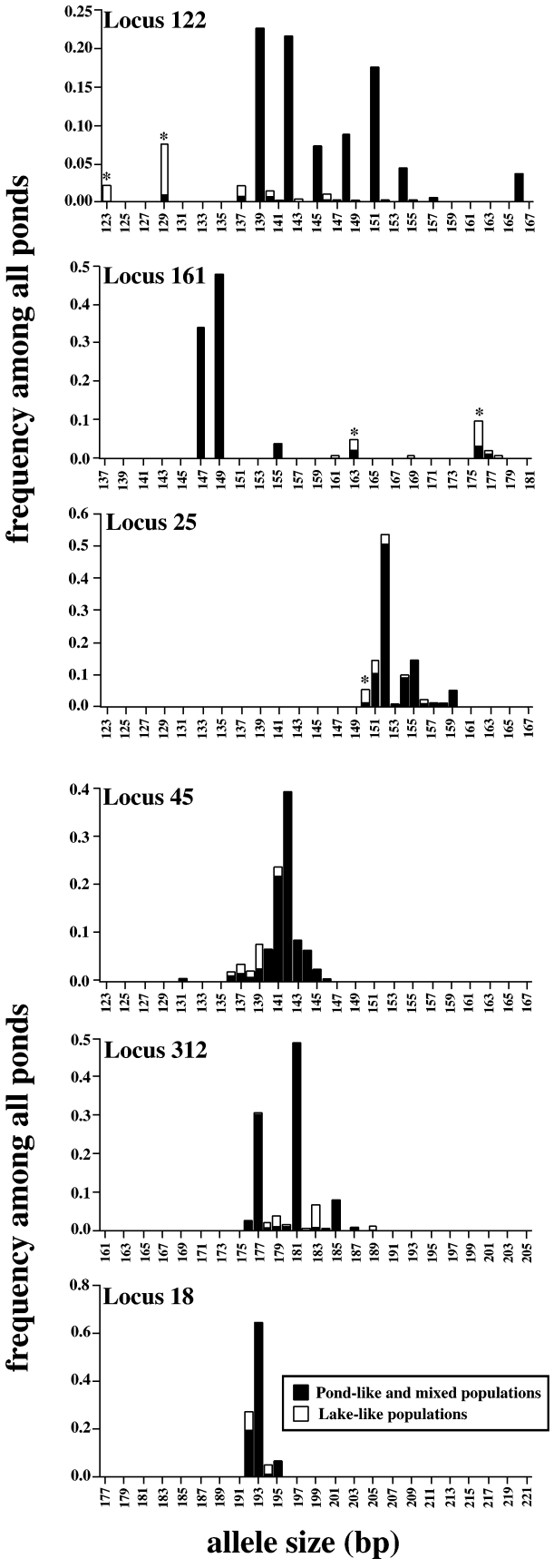
Fig. 5 UPGMA dendrogram for all 20 pond populations of Oregon *Daphnia*, based on the among-population estimates of Nei's (1987) genetic distance from allele frequencies at all 13 allozyme loci. To construct the tree, all loci were assumed to be evolving in the absence of selection; however, there is some indication that certain alleles of a subset of these loci may be selectively correlated with habitat type: lake or pond (see text).

AAT locus also shows a similar pattern, with the M allele being fixed in 13 ponds and the F allele persisting in high levels in GI, MP and PW, and low levels in CP, OA, SH, and WM. The F allele of *AAT* is generally very rare among North American *D. pulex* populations (Hebert *et al*. 1989; Crease *et al*. 1997) but is present among Oregon lakes at a frequency of 13% (Crease *et al*. 1997). These data indicate a uniqueness for the GI, MP and PW ponds, with the CP, OA, SH, and WM ponds having some of the same characteristics. A distance phenogram constructed from these allozyme data depicts the relative levels of separation the three outstanding ponds (GI, MP, and PW) have from the other 17 (Fig. 5). The allozyme distinctiveness exhibited in these ponds has some 'lake-like' features as deduced from the *PEP*, *AAT*, and *MPI* allele frequencies, but the lack of *LDH*-F alleles among at least two of these ponds would fail to characterize them as *D. pulicaria* populations by the traditional convention. The combination of the life-history data (Fig. 3) and the allozyme data (Figs 4 and 5) provides strong support for a marked genetic distinction of at least three populations (GI, MP, and PW) despite a lack of obvious ecological differentiation.

Microsatellites

The microsatellite data from six loci reinforce the above trend. With the assumption that the GI, MP, and PW ponds are harbouring alleles and genotypes that may be distinct from the other ponds, we examined the microsatellite allele frequencies to see if there were alleles found disproportionately in these ponds. When allele frequency histograms are plotted for each locus, it can be seen that these ponds do harbour unique alleles, especially at the *Dpu122*, *Dpu161*, and *Dpu25* loci (Fig. 6). For example, the 123 and 129 alleles at locus *Dpu122* comprise 100%, 10%, and 60% of the GI, MP, and PW populations, respectively, but no more than 7.5% of any other population. Similarly, we identified 11 microsatellite alleles that are present only in, or predominantly in, the 'lake-like' ponds GI, MP, and PW (Table 1). In fact, the three other ponds that possess low frequencies of 'lake-like' allozyme alleles (CP, SH, and WM), along with a fourth pond (OA), also contain low frequencies of these alleles. In these four 'mixed' populations, individuals with lake-like alleles at one locus tend to have lake-like alleles at all other loci. Among the 20 individuals typed from each of the CP, OA, SH, and WM populations, 1, 1, 3, and 1 individuals, respectively, have diagnostic lake-like alleles at all of the *Dpu122*, *Dpu161*, and *Dpu25* loci.

Of note are the relative sizes of the lake-like microsatellite alleles; most of them lie within the tails of the allele frequency distributions (Fig. 6). This observation is most striking at locus *Dpu122*, and thus we chose several homozygous individuals, both from the ponds in this study and from two Oregon lakes, for sequence analysis at this locus (Fig. 7). The individuals from the AM, WH, LO, and SH ponds, none of which possessed a lake-like genotype, all showed trimeric $(TTC)_n$ repeat variation in the microsatellite, with n ranging from 7 to 12. The individuals from the actual lake populations showed n ranging from 8 to 9, but with a 2-bp deletion in the 5′ flanking sequence relative to the pond sequences. The same 2 bp deletion was observed in the *D. melanica* outgroup individual from the dune ponds. The individuals from the lake-like ponds PW and GI however, showed n ranging from 4 to 6, but with a 7-bp deletion in the 5′ flanking sequence relative to the pond sequences. Thus, the lake and lake-like sequences



both show a small deletion but not of the same size. All of these types vary in the number of TTC repeat units they possess, but each group is out of register with the others by consequence of block deletions in the 5′ flanking region.

We then used microsatellite allele-frequency data to assess geographical structure. Previously it was determined that the ponds could be grouped into northern and southern clades (Lynch *et al*. 1999) with more genetic diversity being detected in the northern clade. Here, the three additional ponds (GI, MP, and PW) are all in the north (Fig. 2). With these additional ponds, diversity is still greater in the north; for all ponds north of Junction City, the average effective number of microsatellite alleles is 2.69, while south of this point they average 1.63 alleles, and this difference is significant (*t*-test, $P < 0.05$). This increase in diversity can be traced in large part to the distinctiveness of the lake-like ponds in the north. Excess homozygosity (F_{IS}) at microsatellite loci averages 0.126 per pond in all 17 ponds not characterized as lake-like, while it averages −0.238 in GI, MP, and PW. The mixed ponds (CP, OA, SH, and WM) exhibit the highest positive F_{IS} values of any group, averaging 0.156. Although these deviations from HWEQ were significant, there was no evidence for asexual reproduction in any pond except MP (Lynch *et al*. 1999). As discussed below, the high F_{IS} values are likely a result of within-pond inbreeding.

Population subdivision in the ponds is significant by any measure. G_{ST} for microsatellite data when each pond is considered a separate population is 0.365 (SE = 0.023), a value that is not significantly different from a value of 0.325 (0.068) calculated from the allozyme data (Monte Carlo resampling test, $P < 0.05$). For the microsatellite data, $R_{ST} = 0.431$ for all 20 ponds averaging over variance components at all six loci. When the ponds are grouped into northern and southern groups, the R_{ST} value drops to 0.170, but this is still significantly non-zero ($P < 0.001$). Again, much of this subdivision can be traced to the lake-like ponds. When GI, MP, and PW are removed from the northern group and placed into their own, third population, the overall R_{ST} drops, compared to the 20 population value, to 0.406. However, pairwise R_{ST} values are 0.485 when the lake-like ponds are compared to the other northern

Fig. 6 Microsatellite allele sizes and frequencies at six loci. The ordinate refers to the composite frequency of the indicated allele in the total (20 pond) population. The fraction of each bar that is white refers to the fraction of all alleles of the indicated size that are found in the lake-like populations (GI, MP, and PW). The asterisks denote alleles chosen as highly diagnostic for the lake-like genotype, being found almost exclusively in ponds GI, MP, or PW, or in mixed ponds but associated with lake-like allozyme alleles and lake-like mtDNA genotypes. The lake-like alleles at the trimeric repeat locus *Dpu122* are out of register with the pond-like alleles as a consequence of a 7-bp insertion/deletion region (Fig. 7).

Table 1 Frequencies of microsatellite alleles that are potentially diagnostic for the lake-like nuclear genotype. Alleles indicated in boldface type are considered the most diagnostic, being found in high frequencies in lake-like populations (n = 60 individuals) and nearly absent from pond-like populations (n = 260 individuals). Values for 'all others' are average frequencies per pond

	Dpu122		*Dpu161*						*Dpu25*	*Dpu45*	*Dpu312*
	123	**129**	161	**163**	169	**176**	177	178	**150**	146	189
GI	0.225	0.775	0	0.450	0.050	0.450	0.050	0.025	0.275	0	0.025
MP	0.100	0	0	0	0	1.000	0	0	0.625	0	0
PW	0.150	0.450	0.05	0.075	0	0.825	0	0.05	0.375	0	0.05
CP	0	0.025	0	0	0	0	0.050	0	0.025	0	0
OA	0.025	0	0	0	0	0.050	0	0	0.050	0	0
SH	0	0.075	0	0.025	0	0.200	0	0	0	0	0
WM	0	0	0	0	0	0	0.050	0	0.025	0.025	0
all others	0.002	0.006	0	0.025	0	0.019	0.002	0	0.004	0	0

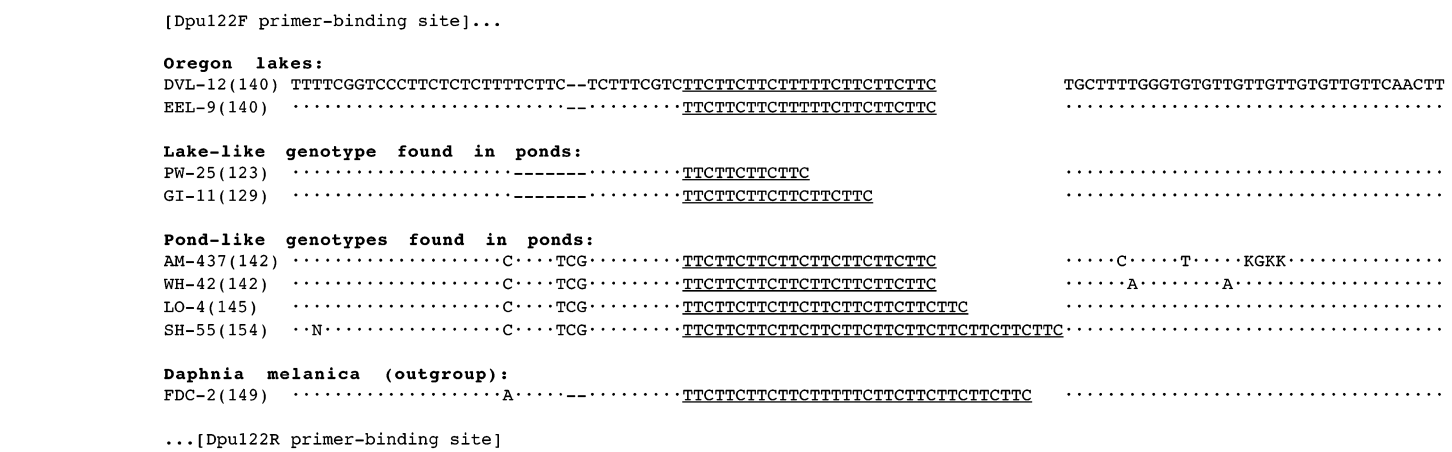


Fig. 7 Nucleotide sequences of the *Dpu122* microsatellite locus and flanking regions in selected homozygous individuals. Sequences have been deposited in GenBank with accession nos AF142086–AF142093. Individual source (two-letter codes = ponds, three-letter codes = lakes) and individual number are indicated, along with allele sizes of the PCR products detected in these individuals (in parentheses). Underlined regions indicate the primary trimeric repeat region $(TTC)_n$, while dashes indicate small block deletion in lake- and lake-like genotypes with respect to the pond-like genotypes. Dots denote nucleotides that are the same as in the DVL-12 sequence. N = undetermined nucleotide; K = G or T.

ponds, 0.617 when the lake-like ponds are compared to the southern ponds, but only 0.088 when the northern and southern nonlake-like ponds are compared. All of these values are significantly non-zero ($P < 0.01$). The microsatellite data as a whole, taken together with the other genetic data, describe a set of populations under a variety of genetic influences, not the least of which is the presence of at least two essentially noninterbreeding forms, sometimes coexisting in the same pond.

mtDNA

In total, we typed 178 of the 400 pond individuals for their mtDNA control region genotype by the seven enzyme RFLP screen. We typed between 8 and 15 individuals per pond (average = 8.9) and detected a total of 22 distinct genotypes (Table 2). Three of these genotypes were apparently derivatives of others by single moderate-sized insertions or deletions (i.e. 20–30 bp); these are indicated in Table 2 in the 'ind' column, and these mutations were confirmed by nucleotide sequence analysis (data not shown). Of particular interest is the observation that the genotypes #5–8 were also detected in high frequency (>67%) in a survey of over 400 pulex-group *Daphnia* from 42 Oregon lakes (Straughan & Lehman 2000). Notably, none of the lake specimens in that study were found to contain any of the other genotypes in the present study (genotypes #18–33). These 'lake' mtDNA genotypes (#5–8) are found in four ponds, GI, MP, PW, and SH at frequencies of 100%, 100%, 78%, and 25%, respectively (Fig. 8). Thus the three ponds (GI, MP, and PW) that have strong 'lake-like' characteristics by other genetic analyses are fixed or nearly

Table 2 Mitochondrial DNA control-region RFLP genotypes, presented in the format recommended by Leberg & Neigel (1999). The presence (1) or absence (0) of restriction sites are indicated. Restriction enzyme recognition sequences are as follows: *Bam*HI = GGATCC, *Dra*I = TTTAAA, *Hha*I = GCGC, *Mse*I = TTAA, *Pal*I = GGCC, *Rsa*I = GTAC, *Sau*96I = GGNCC; 'ind' refers to large (i.e. >10 bp) insertions or deletions. Composite RFLP genotypes given as in Straughan and Lehman (2000) with indels excluded. Putatively all Oregon *pulicaria* (lake-like) genotypes lack *Hha*I restriction site #1 (indicated with asterisk) and all *pulex* (pond-like) genotypes possess this site. Amana is a pond found in Iowa, in the Midwestern United States, and represents published *Daphnia pulex* sequence information (Van Raay & Crease 1994). Three-letter codes refer to Oregon lakes and two-letter codes refer to Oregon ponds (see Fig. 2). Total number of genotypes detected in this study = *n*; the relative proportions of this total among 'pond-like' ponds, 'mixed' ponds, and 'lake-like' ponds are given as *f*(P), *f*(M), and *f*(L), respectively. Lake genotypes #5–7 have been found in the ponds in this study (G1, MP, and PW) as well as among Oregon lakes including LAV, DOL, HCR, PHR, EEL, and DVL (Straughan & Lehman 2000). Lake genotype #8 was not detected in the ponds in this study, and is included only for comparison. Genotype #24 was by far the most frequent, being found in 49% of all individuals outside of ponds GI, MP, and PW

#	Group	Type specimen	*Bam*HI	*Dra*I	*Hha*I *	*Mse*I	*Pal*I	*Rsa*I	*Sau*96I	ind	Composite	*n*	*f*(P)	*f*(M)	*f*(L)
1	(pond)	Amana	1	00111	10	1101001100101011	00	00	1010	1011	ABCAFFC	0	0	0	0
5	lake	DOR-109	1	00111	11	1101001100101011	00	00	1010	1011	ABAEFFC	4	0	0	1
6	lake	LAV-6	1	00111	11	1101000100101011	00	10	1010	1011	ABAEFBC	2	0	1	0
7	lake	HCR-A1	1	00111	11	1101000100101011	00	00	1010	1011	ABAAFFC	20	0	0	1
8	lake	PHR-A2	1	00111	11	1101000100101011	00	10	1010	1011	ABAAFBC	0	0	0	0
18	lake	PW-11	1	00111	11	1101001100101011	01	00	1010	1011	ABAEAFC	2	0	0	1
19	lake	OA-43	1	00111	11	1101000100101011	10	00	1110	1011	ABAABFB	2	0	1	0
20	lake	SH-29	0	00111	11	1101000100101011	00	10	1110	1011	FBAAFBB	1	0	1	0
21	lake	WM-27	1	00111	11	0101001100011111	10	00	1110	1011	ABAGBFB	2	0	1	0
22	lake	WM-31	0	00111	11	1100000100011111	00	10	1010	1011	FBACFBC	1	0	1	0
23	pond	MV-10	1	00111	01	1110000110101011	01	00	1010	1011	ABBDAFC	5	1	0	0
24	pond	WH-3	1	00111	01	1101000100101011	01	00	1010	1011	ABBAAFC	75	0.87	0.13	0
25–	pond	SL-5	1	00111	01	1101000100111011	01	00	1010	0011	ABBBAFC	6	1	0	0
25	pond	WM-10	1	00111	01	1101000100111011	01	00	1010	1011	ABBBAFC	7	0.43	0.57	0
25+	pond	FC-3	1	00111	01	1101000100111011	01	00	1010	1111	ABBBAFC	14	1	0	0
26	pond	LO-17	1	00111	01	1101000100101011	00	00	1010	1011	ABBAFFC	3	1	0	0
27–	pond	FC-4	1	00111	01	1101000100101011	01	00	1000	1001	ABBAAFD	1	1	0	0
27	pond	CP-14	1	00111	01	1101000100101011	01	00	1000	1011	ABBAAFD	7	0	1	0
28	pond	PG-26	1	00111	01	1101000100101011	00	00	1110	1011	ABBAFFD	3	1	0	0
29	pond	SL-22	1	00111	01	1101000100101011	01	00	1110	1011	ABBAAFB	2	1	0	0
30	pond	WM-29	0	00111	01	1101000100111011	01	00	1110	1011	FBBBAFB	3	0	1	0
31	pond	SH-27	0	00111	01	1101000100101011	01	00	0111	1011	FBBAAFE	8	0.88	0.12	0
32+	pond	KC-31	0	00111	01	1101000100101011	01	00	1110	1011	FBBAAFB	4	1	0	0
33+	pond	OP-101	0	00111	01	1101000100101011	00	00	1110	1011	FBBAFFB	6	1	0	0
35–	*D. melanica*	FD-K8.93	0	00111	11	1100000100011111	00	01	1011	1011	FBACFAA	0	0	0	0

Locations of restriction sites, given in bp from the 5′ end of the DPUDL-1 PCR primer used to amplify the *Daphnia* mtDNA control region (Lehman *et al*. 1995): *Bam*HI: 150; *Dra*I: 115, 200, 208, 544, 715; *Hha*I: 92, 618; *Mse*I: 41, 57, 88, 101, 113, 135, 194, 205, 294, 333, 353, 490, 506, 514, 538, 705; *Pal*I: 400, 521; *Rsa*I: 64, 98; *Sau*96I: 55, 152, 523, 570.

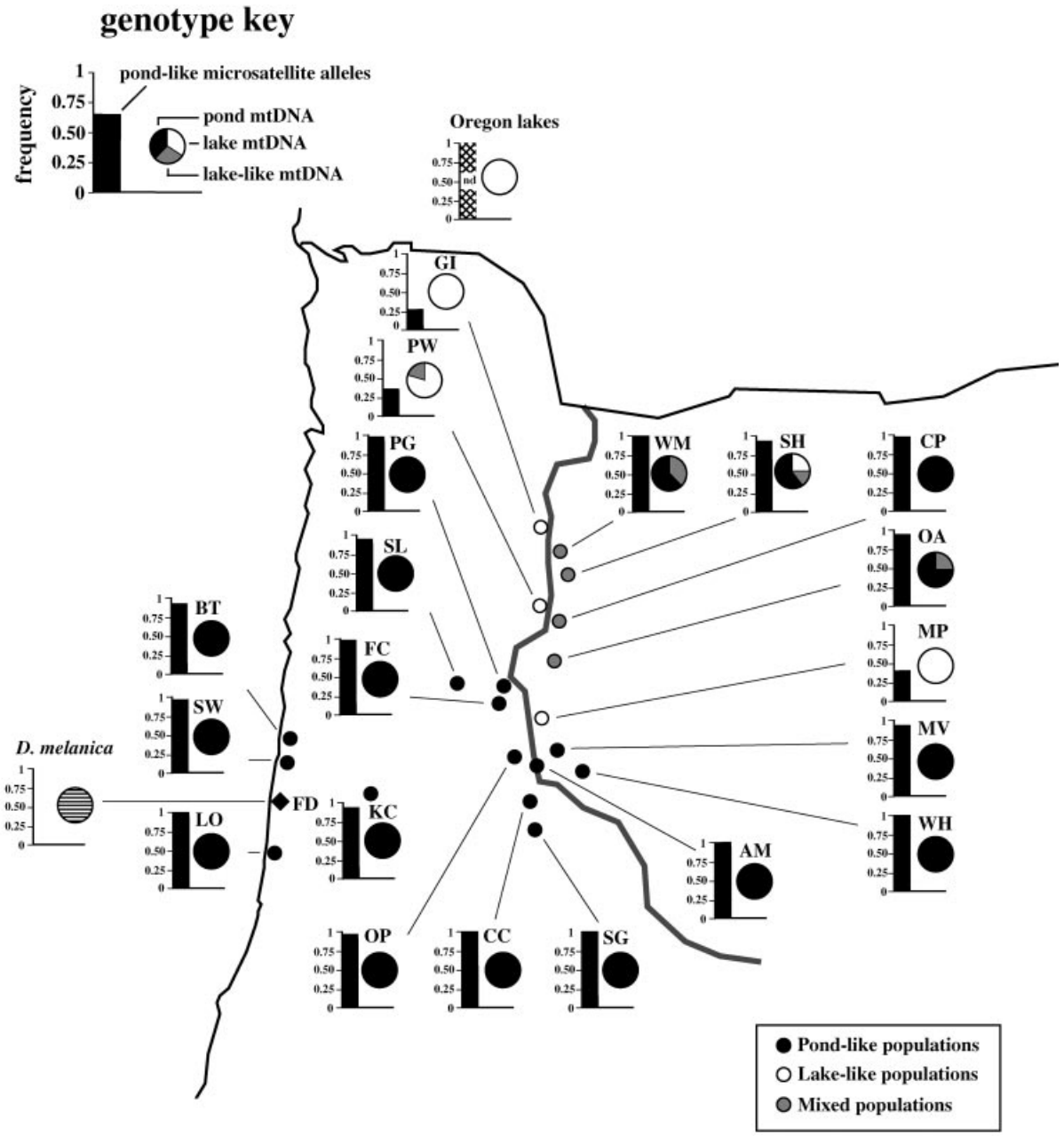


Fig. 8 Summary of microsatellite allele frequencies and mtDNA control-region genotype frequencies among Oregon *Daphnia* populations. Bar graphs depict the populational frequencies of all 'pond-like' microsatellite alleles (i.e. those not designated as lake-like in Table 3). Twenty individuals per pond were typed at six microsatellite loci (see text). Pie graphs depict the frequencies of mtDNA genotypes as defined in Table 2. Between 8 and 15 individuals per pond were typed with seven restriction enzymes for their mtDNA genotype. Lake mtDNA genotypes are #5–8, and have been found at high frequencies in a survey of Oregon lakes (Straughan & Lehman, 2000). Lake-like mtDNA genotypes are #18–22 and those not detected among Oregon lakes but which contain a *Hha*I restriction site putatively diagnostic of lake-derived mtDNA. Pond-like genotypes are #23–33 and have only been found in Oregon nondune ponds. The dune species *D. melanica* contains unique mtDNA genotypes not found elsewhere (Lehman *et al*. 1995). Of the 178 individuals from nondune ponds typed for mtDNA genotype, only five (two in pond OA and three in pond WM) possess a lake-like mtDNA genotype and a pond-like nuclear genetic constellation.

so for a putative lake-specific mtDNA genotype. Examination of Table 2 and of nucleotide sequence data (not shown) reveals that all of these mtDNA genotypes are closely related, having estimated uncorrected pairwise nucleotide sequence similarities ranging from 90.5% to 99.3%. Moreover a parsimony analysis of all 22 genotypes fails to produce any most-parsimonious cladogram that has significant phylogenetic signal by the g_1 statistic of Hillis

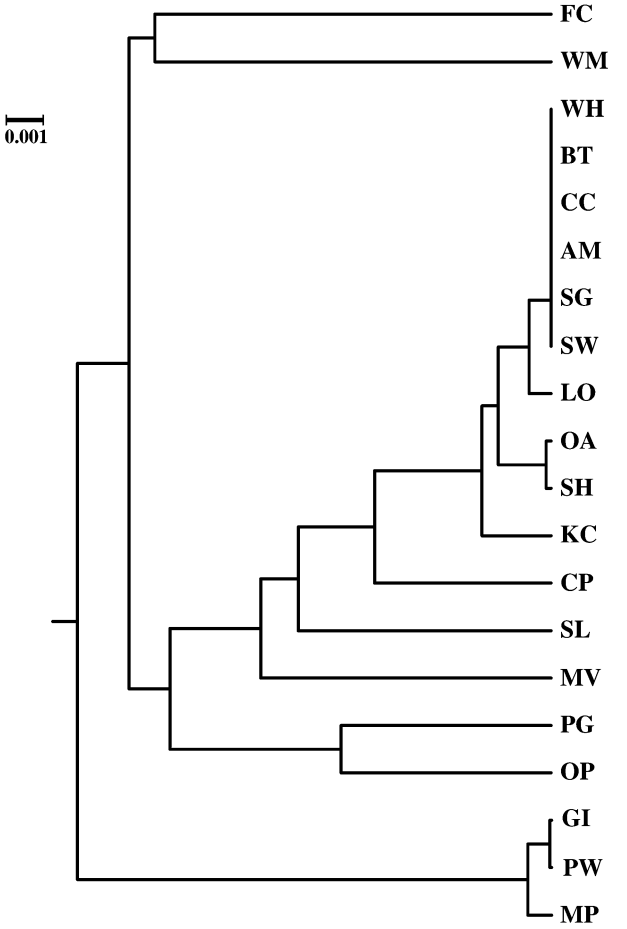


Fig. 9 UPGMA dendrogram for all 20 pond populations of Oregon *Daphnia*, based on the among-population estimates of nucleotide diversity obtained from a mtDNA control-region RFLP analysis.

& Huelsenbeck (1992). However, a UPGMA cluster analysis of these genotypes in populations (Fig. 9) and examination of restriction site patterns (Table 2) does reveal the existence of at least one restriction site, for *Hha*I, that is diagnostic of the lake-type genotypes. Classifying all genotypes with this *Hha*I site as being lake-like, yields five additional genotypes (#18–22) as belonging to this group. These five genotypes are rare among the pond populations surveyed in this study; being only found in between 1 and 3 individuals in the OA, PW, SH, and WM ponds (Fig. 8). With this expanded definition, the PW population becomes 100% lake-like in mtDNA and the OA, SH, and WM populations exhibit a low frequency of lake-like genetic characteristics, as they did with the previous analyses.

Multilocus analyses

To examine more closely whether lake- and pond-like genetic characteristics were consistent across genetic

Table 3 Genotypic compositions of ponds. Allozyme frequencies are defined by average frequency of *AAT*-F and *PEP*-S alleles within ponds. Microsatellite frequencies are defined by average frequency of individuals with *Dpu122*(123), *Dpu122*(129) or *Dpu25*(150) alleles. mtDNA frequencies are defined by tallying individuals with control-region *Hha*I restriction site #1 (Table 2)

		Frequencies of lake-like genotypes					
Pond	Pond type	allozymes	*n*	microsatellites	*n*	mtDNA	*n*
AM	pond-like	0	23	0	20	0	8
BT	pond-like	0	56	0	20	0	9
CC	pond-like	0	42	0	20	0	10
FC	pond-like	0	31	0.05	20	0	15
KC	pond-like	0	30	0.05	20	0	9
LO	pond-like	0	38	0	20	0	8
MV	pond-like	0	36	0.05	20	0	8
OP	pond-like	0	51	0.05	20	0	8
PG	pond-like	0	41	0	20	0	10
SG	pond-like	0	28	0	20	0	9
SL	pond-like	0	17	0.05	20	0	8
SW	pond-like	0	30	0	20	0	9
WH	pond-like	0	55	0	20	0	8
CP	mixed	0.052	33	0.05	20	0	8
OA	mixed	0.048	23	0.05	20	0.25	8
SH	mixed	0.23	17	0.15	20	0.38	8
WM	mixed	0.021	68	0.05	20	0.33	9
MP	lake-like	0.5	37	1	20	1	9
GI	lake-like	0.802	45	1	20	1	8
PW	lake-like	0.80	43	1	20	1	9

analyses within individuals, we compiled composite genetic characteristics of each pond population (Table 3). This assembly of data allowed us to define three discrete types of populations. In the lake-like populations (GI, MP, and PW) all or nearly all individuals possess genetic characteristics that cluster together at a disjunct genetic distance from all others. Many of these characteristics have independently been associated with lake populations (e.g. allozyme allele *HK*-S, a deletion in the 5′-flanking region of microsatellite locus *Dpu122*, and mtDNA genotypes #5–8), but it is clear that the lake-like genotypes in the ponds are not identical to the genotypes found exclusively in the lakes. In the 13 pure pond-like populations (AM, BT, CC, FC, KC, LO, MV, OP, PG, SG, SL, SW, and WH) the potentially distinguishing allozyme alleles (*LDH*-F or *HK*-S) are absent, the 11 lake-like microsatellite alleles (Table 1) are extremely rare (average frequency = 0.01 per locus per pond), and lake-like mtDNA genotypes (#5–22) are absent. In the 'mixed' populations (CP, OA, SH, and WM) pond-like genotypes are common (75–95% by any measure) but not fixed. In these mixed ponds however, the associations across genetic systems within individuals are very strong. A bivariate plot of multilocus nuclear genotypes reveals a strongly bimodal distribution

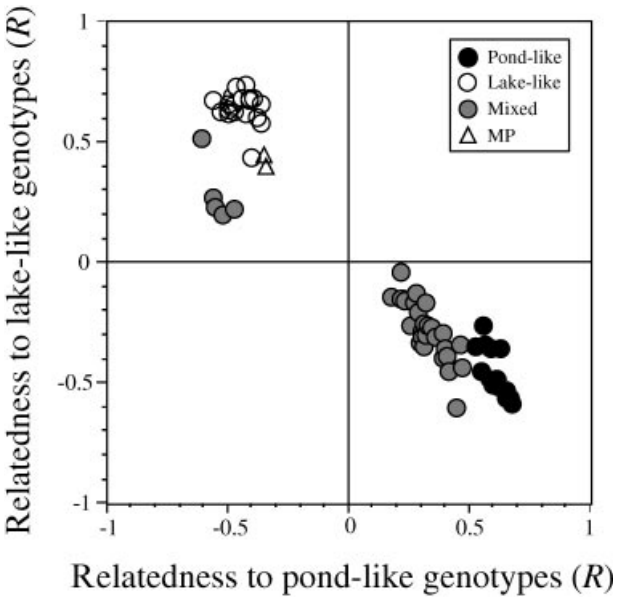


Fig. 10 Bivariate multilocus genotypic associations using Queller & Goodnight's (1989) relatedness statistic. Twenty randomly chosen individuals from the lake-like populations (GI and PW) were used to construct a 'lake' reference population, while 20 randomly chosen individuals from unambiguously pond-like populations (AM, BT, and MV) were used to construct a 'pond' reference population. Individuals from putative mixed populations were not included in the reference populations. The relatedness statistic *R* was then calculated using the program Relatedness 5.0.5 for 20 additional randomly chosen individuals from lake-like ponds (open circles), for 20 additional randomly chosen individuals from pond-like ponds (closed circles), for 34 randomly chosen individuals in the mixed ponds (CP, OA, SH, and WM; grey circles), and for the only two clones in the pond MP (open triangles) using allele frequency data from all six microsatellite loci and the 10 polymorphic allozyme loci. For each individual, relatedness to the lake reference population and relatedness to the pond reference population were each calculated and plotted on the bivariate graph.

within the mixed ponds; individual *Daphnia* in these ponds are either closely related to lake-like genotypes found in GI and PW, or to pond-like genotypes found in southern ponds, but none fall in between (Fig. 10). In pond SH for example, all five individuals who possess *LDH*-F, *AAT*-F, *PEP*-S, and *HK*-S alleles possess at least one such allele at all four loci, and all five of these individuals possess lake-like microsatellite alleles at the diagnostic loci (*Dpu122*, *Dpu161*, and *Dpu45*). None of these alleles, both of allozymes or of microsatellites, are found in any other typed individual in this pond. Similarly, in CP and OA, only one individual in each pond was found to have a lake-like genotype and this is consistent at all three genetic systems in both cases. Only in the mixed pond WM is there any evidence of intermixing of lake- and pond-like genotypes. Of the 68 individuals from this pond typed by the allozyme analysis, three have *LDH*-F, *AAT*-F, *PEP*-S, or *HK*-S alleles, but only one of these has lake-like microsatellite alleles, and this is the only individual out of 20 that has lake-like microsatellite alleles in this pond.

Thus, only two individuals out of 400 typed by both nuclear methods have discordant lake- and pond-like genotypes. Though this is dependent on our definitions of lake-like and pond-like, the allele distributions (Figs 4, 5, 6) are strongly bimodal, and χ^2-tests of independence (Weir 1996; p. 127) show a significant disequilibrium between these two constellations of alleles in the mixed ponds (e.g. $P < 0.0001$ for all pairwise allozyme microsatellite comparisons in pond SH) regardless of their source. When representative multilocus genotypes are compared among the various types of ponds (Fig. 10) it can be seen that the lake-like individuals are lake-like at all loci, except that the *Daphnia* in GI and PW have the pond-diagnostic alleles *LDH-S* and *HK-M*.

Moreover, in no case does an individual with a mtDNA genotype actually found in Oregon lakes (#5–8), fail to have any of the highly diagnostic microsatellite alleles (Table 1: 123 or 129 at locus *Dpu122*, 163 or 176 at locus *Dpu161*, or 150 at locus *Dpu25*; $n = 25$). If lake-like mtDNA genotypes are broadened to include all those with the diagnostic *Hha*I restriction site (#5–22), the association is still very strong with these microsatellite alleles (*G*-test of independence, $P < 0.001$). Conversely, only two of 28 individuals with one or more of these lake-diagnostic microsatellite alleles fail to have a lake-like mtDNA genotype. In total, only six individuals out of 400 show any mixing of pond-like nuclear genomes with lake-like mtDNA genomes or vice versa. Five of these appear in 'mixed' populations (OA, $n = 2$; WM, $n = 3$) and seem to have pond-like nuclear genomes with lake-like mtDNA genomes, at least in the broad sense of having the *Hha*I restriction site (Fig. 8). Curiously, all five of these individuals have unique lake-like mtDNA genotypes (#19, #21, and #22) not found elsewhere in any lake or pond (Table 5). The single other mixed-genome individual appears in a pond-like population (MV) and seems to have a lake-like nuclear genome with a pond-like mtDNA genome. To summarize, the vast majority of individuals assayed in this study appear to have their nuclear and mtDNA genomes derived from the same source, and cross-type introgression, if it occurs, is very rare (i.e. <2%).

Discussion

In the genetic composition of 20 ephemeral ponds, we have detected two disjunct forms of *Daphnia*. The more common form resembles that of the ubiquitous pond-dwelling taxon, *Daphnia pulex*. The rarer form bears resemblance to that of the ubiquitous lake-dwelling taxon, *D. pulicaria*. The two forms appear to be morphologically

and genetically disjunct but retain at very low levels the capacity to exchange genes. The sharpest possible descriptions of this system are threefold, although these need not be entirely mutually exclusive. First, the pond- and lake-like forms are indeed separate species that retain the capacity for interspecific hybridization. Second, the rarer lake-like form is the consequence of a colonization from a more permanent lake environment into newly created or newly vacated habitats in which specific adaptations must, and are taking place, such that the creation of a new, regionally sympatric species is currently detected as in progress. Or third, the pond- and lake-like forms represent two extremes (e.g. subspecies) of a single species that has pronounced population subdivision and whose members are in a kind of genetic tension with limited, but discernible gene flow.

To settle on which of these is the best description, we need both a detailed genetic characterization of the populations under study, and a frame of reference to delineate species. The former is presented in the current study. Regarding the latter, Avise (1994; p. 264) points out that one must 'obtain a suitable frame of reference by extensive sampling of intraspecific variation and by assessment of the magnitude and pattern of interlocus variation'. Without this, it is difficult to discriminate populations within a species from sympatric species, and to discriminate species members as distinct from interspecific hybrids. The frame of reference is provided in this case by extensive previous work that has identified two unique habitats, permanent lakes and temporary ponds, and has attempted to systematically assay for genetic variation within each. In presenting an argument for distinguishing *D. pulicaria* and *D. pulex* as separate species, Brandlova *et al.* (1972) put forth lakes and ponds as distinct ecological settings and identified a single morphological character, the reticulation pattern of the head, as segregating differentially between the two habitats within the pulex-like species. Otherwise these authors noted that *D. pulicaria* and *D. pulex* were nearly morphologically indistinguishable, but they did refer to physiological studies that suggested that the two forms fed on different-sized prey (Hrbackova-Esslova 1963) and showed reproductive optima at different temperatures (Brandlova *et al.* 1972). Later allozyme analyses found a great deal of genetic overlap between lake and pond forms but extracted a single allelic difference, *LDH*-F vs. *LDH*-S, as being diagnostic of *D. pulicaria* and *D. pulex*, respectively (Hebert *et al*. 1989, 1993; Cerny & Hebert 1993). Because *Daphnia* populations, especially in ponds, have been demonstrated to experience periodic selective sweeps that are correlated with allozyme allelic differences (Lynch 1983), it is reasonable to a first approximation to treat a single allozymic allelic difference as a diagnostic character of an ecological genetic cluster. Similar criteria have been used to distinguish among cryptic species, for example *Anopheles* mosquitoes (Narang *et al.* 1989). The *LDH*-F allele has been repeatedly demonstrated to be nearly fixed among north American lake populations of the *D. pulex* complex, including among the numerous lakes in the hills and mountains that surround the Willamette valley (Lehman *et al*. 1995; Crease *et al*. 1997; K. Spitze, unpublished data). Thus, this allozyme locus, along with associated alleles at other loci such as *HK* and *PEP*, serves as one independent frame of reference for our populations.

A second possible frame of reference are life-history characteristics, which should differ among ecological settings. Pond populations, in contrast to 'permanent' lake populations, have a high probability of drying up in any given year, experience greater daily temperature and light extremes, and support a different constellation of predators. These present pond populations with unique adaptation requirements that are likely to be manifest in characters such as mode of reproduction, size at birth and at maturity, and age and clutch size of first reproduction. Consequently we can link a distinct set of nuclear alleles with a distinct habitat to define two evolutionary trajectories that serve as acceptable references for the question of speciation.

When our detailed genetic analysis of the pond populations is placed within these frames of reference, we can clearly see that the lake-like forms that dominate the GI, MP, and PW ponds (Fig. 3; Table 3), and that populate others ponds such as SH in low frequency, are allied to, but distinct from, the *D. pulicaria* standard. The *LDH*-F allele is nearly absent from all ponds except MP, being detected only as 21% of the alleles from the SH population and 4.7% of the alleles in the PW population. Similarly, the *HK*-S allele, a secondary diagnostic for *D. pulicaria*, is absent from all but five of the ponds, is not found at all in MP, and is not found in GI or PW above 11%. Thus, by the allozyme definition, even the lake-like populations GI, MP, and PW do not simply harbour the *D. pulicaria* genotype, and these populations do not represent rare pond-dwelling populations of *D. pulicaria* as observed in Canada (Hebert *et al*. 1993). The distinct sequence characteristic of the 5′-flanking region of the *Dpu122* microsatellite locus in GI and PW individuals (Fig. 7) lends support to this assertion. Conversely, the pond-like populations do not genetically match *D. pulex* from other localities. The *MPI*-M allele predominates in these ponds (80%, Fig. 4) in contrast to the F allele that predominates in Midwestern *D. pulex* (90%, Hebert *et al*. 1989), and nucleotide sequence analysis of the entire mtDNA control region reveals a slightly higher similarity between the pond-like mtDNA genotypes and Oregon lake-like genotypes (average = 96.5%) than between Oregon pond-like mtDNA genotypes and Midwestern *D. pulex* (average = 96.0%; Lehman *et al*. 1995).

With these data, we can tentatively reject the hypothesis that the pure-pond populations consist of one species (e.g.

D. pulex or *D. arenata*), the pure-lake populations consist of another species (e.g. *D. pulicaria*) and the mixed populations are predominantly hybrids. In the mixed populations CP, OA, SH, and WM, a large majority (>91% even by the most conservative definitions) of individuals possess one complete set of genotypes or the other (Fig. 10). In contrast, past work on Midwestern US and Canadian populations has shown that hybridization within the pulex species complex is facile and common. For example, asexual clones that are described as pulex/pulicaria hybrids ('urban clones'), and that show heterozygous genotypes at allozyme loci such as *LDH*, *PEP*, and *AMY*, have been detected in ponds in frequencies of over 50% (Hebert & Crease 1980, 1983; Crease *et al.* 1989; Hebert *et al*. 1989). However, these studies describe a system in which *LDH* heterozygotes are common and there is a poor association between mtDNA genotype and allozyme genotype (Crease *et al*. 1989). In contrast, our study describes a system where *LDH* heterozygotes are rare and there is a very strong association between mtDNA genotype group and nuclear genotype group. Only in pond MP, where *LDH* heterozygotes are fixed, do we see any concrete evidence of hybridization. In addition, the 'urban' clones of the Midwest have been demonstrated to be the result of crosses between male *D. pulicaria* individuals and female *D. pulex* individuals, generating hybrids that possess a pulex mtDNA genome and a mixed lake/pond nuclear genome (Crease *et al.* 1989; T. Crease, personal communication). These clones are believed to be asexual as a consequence of a meiosis suppresser allele carried by some males (Hebert *et al*. 1989). This situation was almost never observed in our populations (once, in pond MV); the majority of potential cases of introgression (in ponds MP and WM) involve lake-like mtDNA in a hybrid or pond-like nuclear background. Along with 'urban' clones, Midwestern studies detected a large frequency of asexual 'forest' clones that are the result of the spread of the meiosis suppresser allele into pond populations without substantial hybridization (Hebert *et al*. 1989). With the exception of MP, we have no evidence for obligate asexual reproduction in these ponds (unpublished data), and we can tentatively conclude that they are instead undergoing cyclical parthenogenesis.

As evidence that hybridization between *D. pulex* and *D. pulicaria* is not the source of the GI and PW genotypes, we again considered the multilocus genotypes of individuals from these ponds (Fig. 10). Hybridization between two genetic assemblages, with various degrees of backcrossing to one or the other parental taxon, could easily occur in the absence of selection in species as similar as these two. Yet this would generate an introgression pattern characterized by a range of allelic frequencies across multiple loci. Without tight linkage one would not expect multiple individuals to have the same blocks of lake-derived and pond-derived alleles. However, this is exactly the pattern that we observe. The GI and PW individuals all have lake-diagnostic alleles at most loci but pond-diagnostic alleles at *LDH* and *HK*; there is no blending of genotypes at the population level (Fig. 10). Barring a highly improbable scenario in which lake-derived alleles introgressed into a pond population at all loci except *LDH* and *HK*, hybridization cannot explain the genetic composition of GI and PW.

We can also tentatively reject the hypothesis that the pond system that we describe involves a single species with two genetically subdivided populations. There are two lines of evidence to support this position. First, the alternate forms (lake-like genotypes and pond-like genotypes) are sympatric at a very fine scale and still retain a dichotomous genetic make-up. In ponds SH and CP both genotypes coexist (and were concomitantly sampled) but show no evidence of interbreeding. In ponds WM and OA, if mtDNA genotypes #21 and #22 are counted as lake-like, the two genotypes also coexist and do show signs of reciprocal introgression, but even in these cases the nuclear alleles are almost exclusively pond-like, depicting the situation where infrequent hybridization events can lead to the introgression of mtDNA from one nuclear background into another (cf. Spolsky & Uzzell 1986; Lehman *et al*. 1991). Second, genetically intermediate forms are lacking (Fig. 10). If the two genotypic constellations were truly of the same species and following an interdependent evolutionary trajectory, then one would expect intermediate combinations of alleles that reflect persistent gene flow. However, only pond MP, where all individuals display a heterozygous genotype that is likely the consequence of obligate asexuality, begins to bridge the gap between the lake-like and the pond-like constellations (Fig. 5; Fig. 6). In contrast, the high positive F_{IS} values in the 'mixed' ponds seems to be the result of the existence of two noninterbreeding (and thus inbred) genetic assemblages.

As a consequence of the above reasoning, we feel that the most accurate description of the pond system is one in which permanent lakes act as a source of genetic material that periodically colonizes temporary ponds following floods and must then quickly adapt to an ephemeral habitat. To survive in a temporary pond setting, a lake-derived lineage would require several life-history alterations. For example, one critical adaptation involves the timing of the transition from an asexual to a sexual mode of reproduction. Temporary pond populations in western Oregon initiate a phase of sexual reproduction towards the end of the growing season in response to increasing photoperiod and other environmental cues. In contrast, lake-dwelling populations exhibit a reversed photoperiodic response and enter the sexual phase when the photoperiod is decreasing (Deng 1997). As a result, true

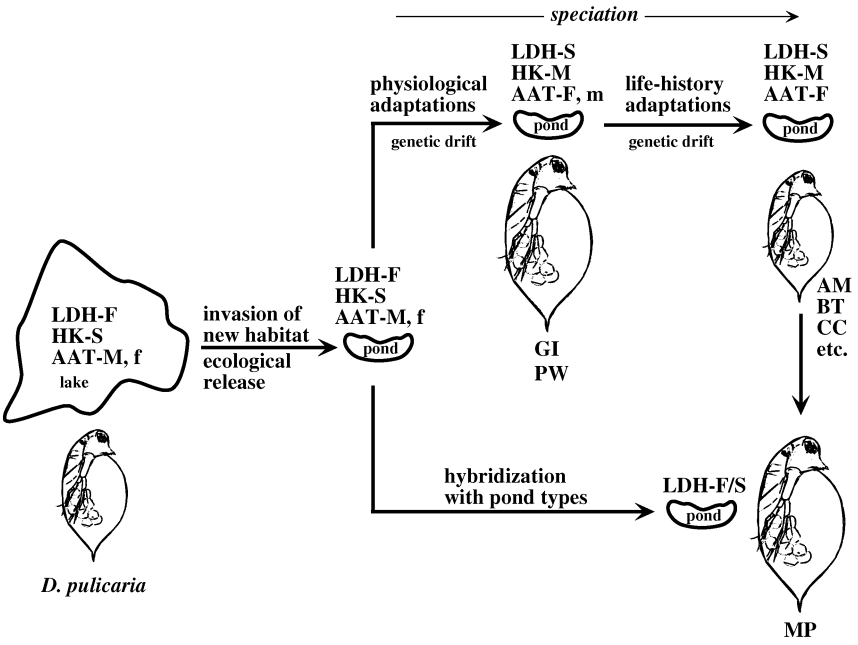


Fig. 11 Proposed model of speciation in *Daphnia pulex* species complex populations in Oregon. Flooding events allow colonization or invasion of pond habitats from lake-adapted sources. Adaptation of both physiological and life-history traits must occur for lake-derived lineages to survive in the ephemeral pond environment. Physiological adaptation is detected via shifts at the *LDH* and *HK* allozyme loci, which are either directly involved in pond adaptations or linked to loci that are. Life-history adaptations are exemplified by body size at maturity, which is typically small in *D. pulicaria*, and may undergo an ecological release upon invasion of ponds prior to regression during continued adaptation to pond existence. Genetic drift at neutral marker loci (e.g. mtDNA, microsatellites, and the allozyme locus *AAT*) accompanies all transitions. Hybridization in ponds between pond-adapted and lake-adapted forms generates heterozygous genotypes whose evolutionary trajectory may be truncated.

lake genotypes are maladapted to temporary pond environments and population persistence would require rapid adaptation. From their combined quantitative and genetic study of 17 of these ponds, Lynch *et al*. (1999) concluded that the strong correlation between the levels of population subdivision and broadsense heritabilities of some quantitative traits (most noticeably body size) was being driven by local adaptation to different environments. Moreover, Orr & Smith (1998) have recently reviewed the link between ecology and speciation, and note that several recent empirical studies have detected that very rapid evolution can be prompted by the colonization of novel environments by populations from a source species.

If speciation is occurring continuously, one would expect that at any point in time many stages of the speciation process would be captured in the genetic and ecological make-up of the populations (Fig. 11). In our survey of 20 pond populations in western Oregon we can detect four such stages. In pond MP, the lake-derived and the pond-derived forms have hybridized, producing the equivalent of asexual 'urban' clones detected elsewhere. These hybrids could result in evolutionary dead-ends that have a short-term advantage as a consequence of losing the cost of sexual reproduction (Crease *et al*. 1989; but see Lynch & Gabriel 1983), but whose lineage will eventually expire in the highly unpredictable ephemeral pond environment. In ponds CP, OA, SH, and WM, the lake- and pond-like lineages are coexisting with minimal gene flow between them. These lineages were found in the same shallow pond, often in the same collection throw of a plankton net. Thus, they are competing genotypic assemblages, a common paradox in planktonic fauna (Hutchinson 1961; Hebert & Crease 1980). Here it is notable that the pond-like genotypes are always in significantly higher frequencies ($P < 0.001$, $P < 0.001$, $P < 0.005$, χ^2-tests of independence for allozymes, microsatellites, and mtDNA, respectively), suggesting these individuals enjoy a competitive advantage in the pond environment, but that they have not excluded alternative genotypes because the relative fitnesses of genotypes may be unstable in this environment (Hebert & Crease 1980). In the ponds GI and PW, the lake-like genotype is not found coexisting with a pond-like genotype, perhaps because the colonization of these ponds was from a lake source into an unoccupied habitat during a flooding event. Here, the lake-like ancestry is evident at many loci, but adaptations to the pond niche may be underway and evident, for example, in the fixation or near-fixation of the *LDH*-S and *HK*-M alleles, which may in fact signal linkage to pond-specific adaptations, similarly observed in Midwestern pond populations (Lynch 1983). We note that the *LDH*-S and *HK*-M alleles *are* present in low frequencies in Oregon lakes (Crease *et al*. 1997) providing a source of genetic variation on which selection can act. Finally, in the remaining 13 populations, the pond-like genotype dominates, and these populations can be described as the oldest lineages that have fully adapted to the ephemerality of pond life (Fig. 11).

It is of relevance that the lake-like and mixed populations are all to the north, closer to the mouth of the Willamette River where it empties into the Columbia River

(Fig. 1), while the pond-like populations are all more southern or on the coast. In fact, the ponds that show the highest incidence of nonhybrid lake-like genotypes (GI, PW, SH, and WM) are all less than 2 km from the Willamette river and are the four most northern populations. Before several dams were constructed on the Willamette and its tributaries, periodic major floods of the Willamette occurred on roughly a 20–100 years cycle (US Army Corps of Engineers 1980). These tended to swell the river over its banks more dramatically in the north, in the vicinity of the present-day cities of Corvallis, Salem, and Portland, an observation that would account for the predominance of the younger, lake-derived populations in the north.

In this paper, we have established independent frames of reference from which to examine evolutionary trajectories. By combining data from several genetic systems we can postulate a directionality for gene flow (lakes to ponds) and thus avoid circular argumentation (Avise 1994; p. 269). We have described a dynamic system in which adaptive peak shifts can occur in fragmented populations of a ubiquitous organism. We propose that the *LDH* and *HK* allelic characteristics of GI and PW are reflective of peak shifts for these populations, and that their pond habitats have contributed to a reproductive isolating mechanism from their regionally sympatric lake congeners. The result appears to be the advent of populations that attain a level of genetic and phenotypic cohesion that could ascribe them with species status (Templeton 1989). This phenomenon could explain the reciprocal paraphyly that is often observed among lake- and pond-dwelling forms in the pulex species complex (Lehman *et al*. 1995; Van Raay & Crease 1995; Crease *et al*. 1997). In following the implication of Bush (1994), the taxonomic nomenclature of the evolving entity (i.e. *D. pulex* vs. *D. arenata*) will not be debated here; instead we hope that this paper will serve as a convincing example of how the simultaneous analysis of data from multiple genetic systems can illuminate the ecology of speciation.

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