

Intercontinental allozyme differentiation among four holarctic *Daphnia* species

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Abstract

The species overlap between European and North American cladoceran faunas has been questioned from the very beginning of faunistic studies on Nearctic zooplankton and still remains unsettled. However, more recently, genetic techniques seem to overcome the limits of morphology as the basis for taxonomy. We analyzed 8–11 allozyme loci to assess the genetic differentiation between intercontinental counterparts of four daphniids: *Daphnia curvirostris*, *Daphnia obtusa*, *Daphnia pulex*, and *Daphnia pulicaria*. Despite their morphological likeness, all equivalents showed a large amount of genetic divergence between continents due to complete allelic substitutions and major gene frequency shifts. From an evolutionary perspective, this study shows that as in other organisms, geographic isolation seems to be sufficient to minimize gene flow to levels at which the origin of new daphniid species can be considered. From a practical point of view, a revision of Holarctic daphniid taxonomy should be considered.

The use of allozyme analysis in taxonomic studies on zooplankton has brought a new dimension to the traditional phenotypic approach by allowing the study of underlying patterns of genetic variability. Allozyme studies have proven invaluable in clarifying species boundaries in a local context, allowing the identification of sibling species and enabling the recognition of interspecific hybrids. Coupled with DNA sequencing studies, these investigations are now providing insights concerning both speciation processes and patterns of phylogenetic diversification.

The most intensive genetic studies on the freshwater zooplankton have examined cladoceran crustaceans, especially members of the genus *Daphnia*. Much taxonomic work has focused on the North American fauna, although detailed studies have also been completed in Australia (Hebert and Wilson 1994). These analyses have shown that there is no species overlap between these two continents, reinforcing the conclusions of earlier morphological work. Interestingly, there has been little effort directed toward examination of the genetic similarity between the European and North American faunas, an issue of more importance, because these faunas show an uncertain amount of overlap in species composition. Birge (1918) concluded that all species of *Daphnia* in North America were conspecific with European forms. Brooks (1957) reduced the extent of overlap, recognizing six North American endemics, but concluded that the remaining nine species had Holarctic distributions. A recent taxonomic revision of the North American fauna, driven by genetic analyses, raised the number of recognized taxa to 34 and increased the level of endemism to 75%, due largely to the discovery of new species apparently restricted to this continent (Hebert 1995). However, in the sole case where

detailed information was available, European populations of *Daphnia galeata* were found to be distinct genetically from supposedly conspecific populations in North America (Taylor and Hebert 1993a,b). Moreover, the inadvertent introduction of European *D. galeata* into the Laurentian Great Lakes revealed their reproductive incompatibility with North American populations, provoking recognition of the latter taxon as a distinct species, *Daphnia mendotae* (Taylor and Hebert 1993a). Although *D. galeata* remains the only carefully analyzed case, studies with a more limited scope have revealed substantial genetic divergence between supposedly conspecific populations in Europe and North America (Hebert 1987).

The growing number of genetic studies helps to destroy the former concept of zooplankton cosmopolitanism. As already pointed out by Frey (1987), the species that have been claimed to be cosmopolitan are being shown to be groups of or complexes of morphologically similar species instead, each member species of which has a much more restricted distribution than the group or complex as a whole. The present study involved a comparison of the genetic similarity between North American and European populations of four species of *Daphnia* thought to occur in small nonpermanent ponds or low fish-stocked habitats on both continents. Three of these species (*Daphnia curvirostris* Eylman, *Daphnia obtusa* Kurz, *Daphnia pulex* Linné emend. Leydig) were described from Europe, while *Daphnia pulicaria* Forbes was described from North America. All of these species are thought to be distributed widely on both continents, except *D. curvirostris*, which is common in Europe but is known from only a few sites in northwestern Canada.

Materials and methods

Populations sampled—This study involved a survey of *Daphnia* populations from pond and lake habitats in the temperate zone of North America and Central Europe. On the basis of current taxonomic treatises, populations were as-

Acknowledgments

Funding for this research was provided by the Natural Sciences and Engineering Research Council of Canada. We thank Derek J. Taylor, John K. Colbourne, Lawrence Weider, Alan J. Tessier, and two anonymous reviewers for their helpful suggestions and comments and Steve Schwartz for providing samples from Georgia.

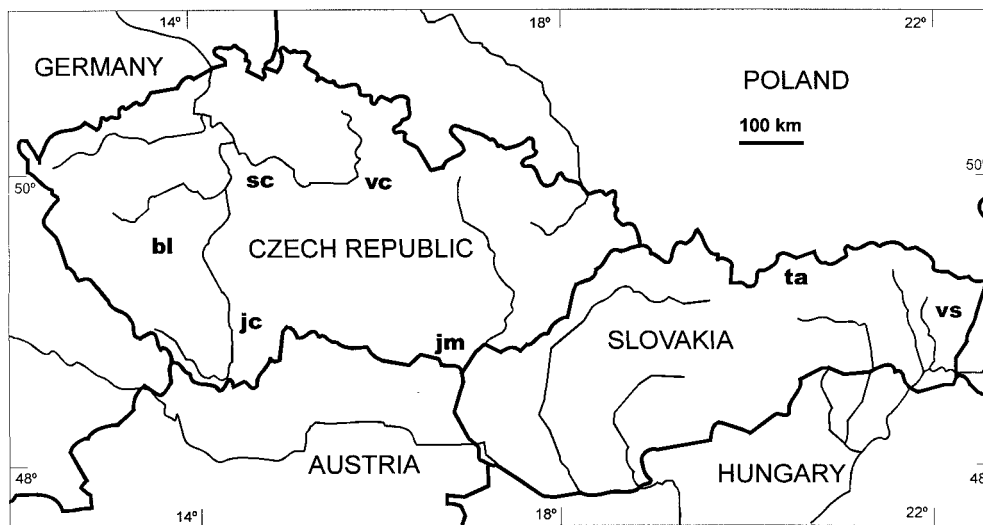


Fig. 1. Regions sampled in Central Europe with corresponding population codes.

signed to four species: *D. curvirostris* Eylman, *D. obtusa* Kurz, *D. pulex* Linné emend. Leydig, and *D. pulicaria* Forbes. For North American populations, population codes correspond with common abbreviations of U.S. states and Canadian provinces, and the region codes of European populations are shown in Fig. 1.

Daphnia curvirostris: A total of 534 individuals from 17 populations in Europe (Czech Republic, Slovakia) and 3 laboratory clones from the Canadian Arctic were analyzed. Most of the European populations inhabited small, often intermittent, lowland ponds, which is their typical habitat in this region (Hrbáček 1987), but one population (vc01) was obtained from the slow-moving river Labe. The North American clonal isolates were originally collected at Tuktoyaktuk, Northwest Territories, Canada, in July 1992 (T. van Raay, pers. comm.).

Daphnia obtusa: A total of 994 animals was analyzed including 862 individuals from 11 European populations and 132 individuals from 3 North American populations (Georgia). European populations were obtained from two distinct habitats: lowland temporary puddles represented by populations (coded bl##), sampled from habitats within 80 km of the type locality (Kurz 1874), and a second set of populations (ta##) from small ponds at high elevations (>1,000 m) in the Tatra Mountains. For the genetic distance assessment the pooled North American data of *D. obtusa* from Hebert and Finston (1996) are included.

Daphnia pulex: A total of 916 individuals were examined from 23 mid-European populations collected from a wide range of habitats, including natural flood pools along rivers (jc## but jc09), carp ponds (vs08), experimental concrete basins (sc03, 04), lakes (ta5#), and deep reservoirs (jc09). Allele frequencies in North American populations of *D. pulex* were obtained from Hebert et al. (1993). Although this paper employed a different allelic nomenclature, the following conversion rules permitted the reassignment of alleles to

the present system. The three alleles (1–3) reported at AO (aldehyde oxidase), FUM (fumarate hydratase), GOTs (glutamate-oxaloacetate transferase), MPI (mannose phosphate isomerase), and GPI (glucose-6-phosphate isomerase) correspond to the present **s**, **m**, and **f** alleles, while the four alleles (2–5) at PGM (phosphoglucomutase) correspond to the **s'**, **s**, **m**, and **f** alleles in this paper. Two other loci (LDH [lactate dehydrogenase], APK [arginine phosphokinase]) were monomorphic for the **m** allele, while no data were available for MDH (malate dehydrogenase), GOTm (mitochondrial GOT), or AMY (amylase).

Daphnia pulicaria: A total of 3,779 specimens were analyzed from 63 populations. European samples were obtained mainly from lowland carp ponds or reservoirs in Bohemia, Moravia (sc##, jc10, jm##), and Slovakia (vs##). In North America, *D. pulicaria* was typically collected from lakes or large reservoirs (see Černý and Hebert 1993). Although *D. pulicaria* is morphologically distinct from *D. pulex*, their discrimination in North America is complicated by the occurrence of F₁ hybrids. However, past studies have revealed fixed allozyme differences that can be used in species diagnosis (Hebert et al. 1989). Only specimens homozygous for the **f** allele at the LDH locus were treated as *D. pulicaria* in this study; such populations ordinarily contained only individuals with a head shape similar to that of *D. pulicaria* (cf. Brandlová et al. 1972, =*D. schoedleri* sensu Brooks 1957).

Sampling and sample analysis—Most of the sites, especially lakes and reservoirs, were sampled in the spring or early summer when populations were likely represented by individuals hatched from sexually produced resting eggs and matched the Hardy-Weinberg equilibrium criterion. In larger water bodies, samples were ordinarily collected from a boat by towing a plankton net in the deepest part. However, a few of these habitats together with all shallow water bodies were sampled from shore. Whenever possible, at least 60 individuals of each species of *Daphnia* present in a sample were

frozen in liquid nitrogen. When larger samples were available, specimens were also preserved in formalin or 70% ethanol for morphological analysis.

To assess the genotypic structure of each population, at least 40 individuals were scored for allozyme variation at 8–11 commonly polymorphic loci. These loci included: AMY (the most cathodal of several migrating zones), AO (EC 1.2.3.1), APK (EC 2.7.3.3), FUM (EC 4.2.1.2), GOTs—supernatant GOT (EC 2.6.1.1), GOTm (EC 2.6.1.1), GPI (EC 5.3.1.9), LDH (EC 1.1.1.27), MDH (supernatant form) (EC 1.1.1.37), MPI (EC 5.3.1.8), and PGM (EC 2.7.5.1). Alleles were assigned a letter code according to their mobility: s''' (the slowest allele) $< s'' < s' < s < m$ (medium) $< f < f' < f''$ (the fastest). Two clones of *Daphnia*, i.e., North American *D. pulex* W2-8 (AMY, **mm**; APK, **mm**; FUM, **mf**; GOTs, **mm**; GOTm, **mm**; GPI, **mm**; LDH, **ss**; MDH, **mm**; MPI, **mm**; PGM, **mf**) and North American *D. pulex/D. pulicaria* hybrid W1-1 (AMY, **mm**; APK, **mm**; FUM, **mm**; GOTs, **mm**; GOTm, **mm**; GPI, **mm**; LDH, **sf**; MDH, **mm**; MPI, **mm**; PGM, **mm**) were run as references on all gels. All electrophoresis was carried out on Titan III cellulose acetate plates employing standard methods (Hebert and Beaton 1989).

Genetic relatedness—After calculating gene frequencies, the genetic relatedness of populations was evaluated using Nei's unbiased genetic distance (Wright 1978) in BIOSYS-1 (Swofford and Selander 1989). Analysis usually focused on a single species, but because *D. pulex* and *D. pulicaria* often hybridize in North America, these taxa were treated jointly. When multiple samples of a single species were collected at the same locality, only one was used in the genetic distance assessment.

The distance analysis of the *pulex-pulicaria* group was performed on eight loci because North American populations of *D. pulicaria* were not analyzed for the whole allozyme set (cf. Table 1), neither were the included data from Hebert et al. (1993). Similarly, only eight loci were used for the distance analysis of *D. obtusa*, allowing the data from Hebert and Finston (1996) to be included to strengthen the American data set.

The genetic relatedness among populations was evaluated using both (1) the unweighted pair-group (UPGMA) clustering method that groups populations into a hierarchical tree according to their mean genetic distance, and (2) multidimensional scaling (MDS) that yields a pattern of nonhierarchical variability (Lessa 1990). For the latter one, however, the original nonmetric distance matrix was transformed to the metric form (Chakraborty and Rao 1995) so that we could see how the total variance of distance matrix was partitioned among ordination axes. Both of these analyses were performed using statistical packages STATISTICA (Statsoft 1995) and NCSS (NCSS 1996).

Results

The allelic frequencies for all examined taxa are summarized in Table 1.

Daphnia curvirostris: Both North American and European populations of *D. curvirostris* shared a unique **m** allele at the LDH locus that was not detected in the other three taxa. This allele was nearly fixed in all populations, although a rare **s'** allele was detected in homozygous or heterozygous conditions in 11 of 534 European individuals. Three other unique alleles were detected in this species (GPI— s''' , GOTm—**s**, AMY—**s'**), but each was present at low frequency. No F_1 hybrids (i.e., a heterozygote on both LDH and GPI loci) were detected between this species and *D. pulex*, although these taxa often co-occurred in European habitats.

Both the UPGMA and MDS analyses of genetic distances clearly separated North American clones and European populations, largely based on gene frequency differences at the MDH and GOTs loci (Figs. 2, 3). There was also substantial differentiation among European populations, especially as a result of the divergence of three East Slovak populations. However, the patterning of genetic divergence in Europe did not have a simple geographic explanation, as other Slovakian populations were grouped with Bohemian and Moravian ones.

Daphnia obtusa: *D. obtusa* did not possess any unique allozyme alleles to distinguish it from *D. pulex* and *D. pulicaria*. Although UPGMA analysis revealed considerable variation within European and American populations, the MDS analysis does not stress that as the differences along the second axis account for less than a third of variance covered by the first axis. Both analyses, however, showed a large amount of divergence between populations on the two continents (Figs. 4, 5). All European populations, including those from both Czech lowland and Slovakian mountain habitats, showed almost fixed differences at FUM, MPI, and GPI from their North American counterparts. As well, the GOTs locus showed a large shift in allele frequencies between continents.

Daphnia pulex and D. pulicaria: Both UPGMA and MDS analysis of genetic distances provided evidence for the occurrence of four major lineages among *D. pulex* and *D. pulicaria* from Europe and North America (Figs. 6, 7). These four lineages showed lower heterozygosity and genotypic arrays congruent with Hardy Weinberg equilibrium, suggesting that they represented single species populations. However, populations of *D. pulex* and *D. pulicaria* from Europe were apparently genetically divergent from their North American counterparts. Populations of *D. pulicaria* on the two continents were distinguished by an allele substitution at LDH and by major gene frequency shifts at AO and APK. North American populations of *D. pulex* were separated from European populations mainly due to differences at GPI (**s** allele dominant in European populations, versus **m** in Canada) and MPI (**f** allele in Europe versus **m** in Canada).

Despite the loss of the diagnostic difference at the LDH locus evident in North America, European populations of *D. pulex* and *D. pulicaria* were easily distinguished, due to their gain of a novel allele substitution at APK. Although these two species often co-occurred, no F_1 hybrids were detected, in contrast to the situation in North America. Considered

Table 1. Summary of gene frequencies for examined daphniid taxa.

Locus		<i>D. curvirostris</i>		<i>D. obtusa</i>		<i>D. pulex</i>	<i>D. pulicaria</i>	
		Europe	N. America	Europe	N. America	Europe	Europe	N. America
GPI	<i>n</i>	534	18	546	132	916	1,707	2,303
	<i>s'''</i>	0.001						
	<i>s''</i>	0.017				0.002		
	<i>s'</i>	0.959	1.000	0.999		0.020	0.001	0.001
	<i>s</i>	0.022		0.001	0.955	0.975	0.029	0.094
	<i>m</i>	0.001			0.045	0.003	0.876	0.645
	<i>f</i>						0.094	0.257
APK	<i>f'</i>						0.000	0.003
	<i>n</i>	411	18	311	66	717	1,682	356
	<i>s'</i>			0.003				
	<i>s</i>					0.001		
	<i>m</i>	1.000	1.000	0.997	1.000	0.999		0.862
PGM	<i>f</i>						1.000	0.138
	<i>n</i>	533	18	588	66	899	798	2,273
	<i>s'</i>	0.060				0.002		0.039
	<i>s</i>	0.168				0.051		0.036
	<i>m</i>	0.576	0.500	0.816	1.000	0.929	0.999	0.748
	<i>f</i>	0.175	0.500	0.184		0.017	0.001	0.177
LDH	<i>f'</i>	0.021				0.001		
	<i>n</i>	533	18	311	110	833	983	2,292
	<i>s'</i>	0.012				0.001		
	<i>s</i>			1.000	1.000	0.999	1.000	
	<i>m</i>	0.988	1.000					
AO	<i>f</i>							1.000
	<i>n</i>	488	18	288	105	868	1,003	2,309
	<i>s''</i>				0.438	0.325	0.043	
	<i>s'</i>	0.145			0.005	0.341	0.749	
	<i>s</i>	0.027			0.029	0.271	0.103	
	<i>m</i>	0.243	0.333			0.058	0.104	0.010
	<i>f</i>	0.118		1.000	0.529		0.002	0.990
FUM	<i>f'</i>	0.467	0.667			0.006		
	<i>n</i>	408	18	461	132	680	512	2,312
	<i>s'</i>			1.000				
	<i>s</i>	0.005						
	<i>m</i>	0.995	1.000		0.023	0.999	1.000	1.000
MPI	<i>f</i>					0.001		
	<i>n</i>	534	18	613	110	862	1,704	2,290
	<i>s'</i>							0.002
	<i>s</i>			0.992		0.002		0.003
	<i>m</i>	0.035		0.008		0.110	0.868	0.991
	<i>f</i>	0.021			0.141	0.727	0.132	0.003
	<i>f'</i>	0.945	1.000		0.859	0.159		
MDH	<i>f''</i>					0.001		
	<i>n</i>	521	18	310	109	727	872	N/A
	<i>s</i>	0.031	1.000	0.008		0.001		N/A
	<i>m</i>	0.969		0.992	0.665	0.998	0.999	N/A
GOTs	<i>f</i>				0.335	0.001	0.001	N/A
	<i>n</i>	455	18	575	88	763	516	2,314
	<i>s'</i>	0.008		0.866	0.012			
	<i>s</i>	0.056	1.000	0.134	0.733	0.004		
	<i>m</i>	0.910			0.256	0.904	0.998	0.986
GOTm	<i>f</i>	0.026				0.092	0.002	0.014
	<i>n</i>	369	18	574	88	763	516	N/A
	<i>s</i>	0.008						N/A
	<i>m</i>	0.992	1.000	1.000	1.000	0.995	1.000	N/A
AMY	<i>f</i>					0.005		N/A
	<i>n</i>	481	8	408	132	665	856	N/A
	<i>s''</i>			0.668	0.185		0.940	N/A
	<i>s'</i>	0.064						N/A
	<i>s</i>	0.038		0.332	0.815	0.019	0.060	N/A
	<i>m</i>	0.845	0.625			0.978		N/A
	<i>f</i>	0.052	0.375			0.003		N/A

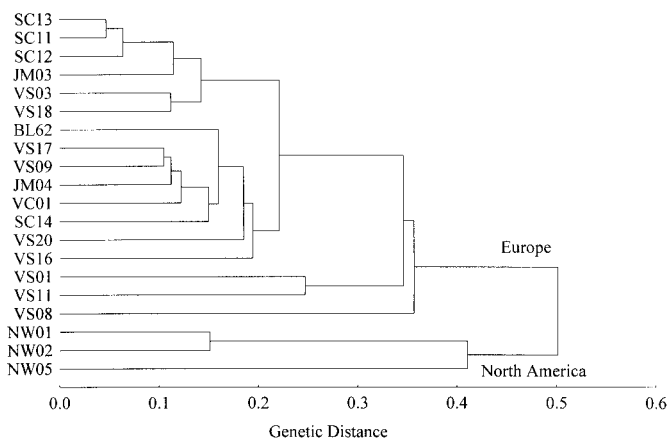


Fig. 2. UPGMA tree of Nei's genetic distances among populations of *D. curvirostris*.

individually, mid-European populations of both *D. pulicaria* and *D. pulex* showed modest genetic divergence. The most genetically distinctive population of *D. pulex* (jc10) was fixed for the GPI-s" allele that was otherwise rare. Interestingly, this population occupied a very unusual habitat for *D. pulex*, the most bottom waters of the 40-m deep Rimov Reservoir.

Discussion

This study has provided the first comprehensive genetic information on the four species of *Daphnia* that dominate central European carp ponds and lakes with low fish densities. An earlier study (Hebert et al. 1989) of genetic divergence in a few populations of *D. pulex* and *D. pulicaria* from

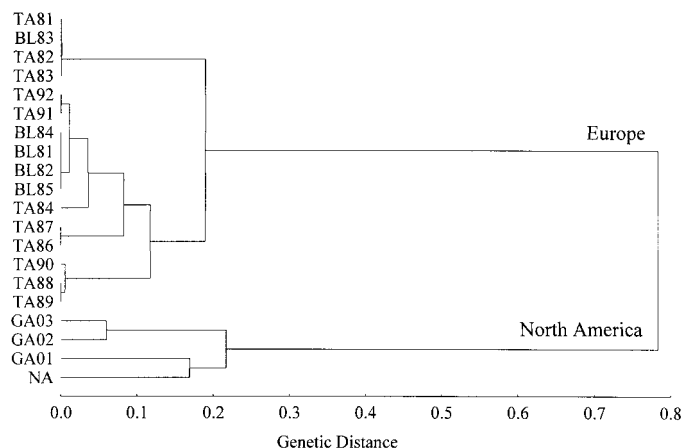


Fig. 4. UPGMA tree of Nei's genetic distances among populations of *D. obtusa*. NA—data from Hebert and Finston (1996a).

carp ponds in Bohemia revealed divergence in gene frequencies at GOT and AMY, similar to those observed in this study. This earlier investigation also suggested that interspecific hybrids between mid-European populations of these two taxa were either rare or absent (Hebert et al. 1989). The present study extends this result by discovering the presence of a diagnostic substitution at APK, and together with other loci verifying the absence of hybrids between these species in mid-Europe. However, the APK heterozygotes often have been found in obligatory asexual populations of the *D. pulex* and *D. tenebrosa* complex from arctic and subarctic regions of Eurasia, from which many clones are thought to be of multiple hybrid origin (Weider and Hobaek 1997). Our further results indicate that such arctic hybrids persist even in the temperate regions of Central Europe as glacial relicts

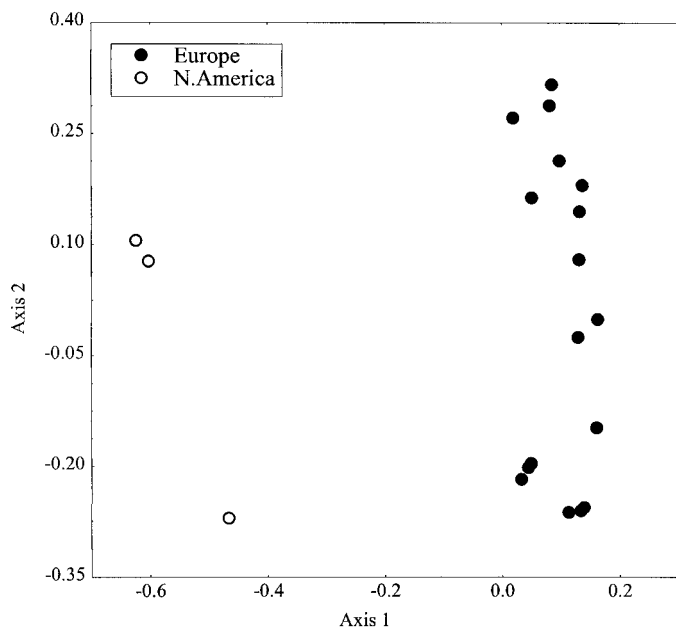


Fig. 3. Multidimensional scaling of Nei's genetic distances among populations of *D. curvirostris*. Axis 1 explains 22.3% and axis 2 15.9% of the total variance of distance matrix.

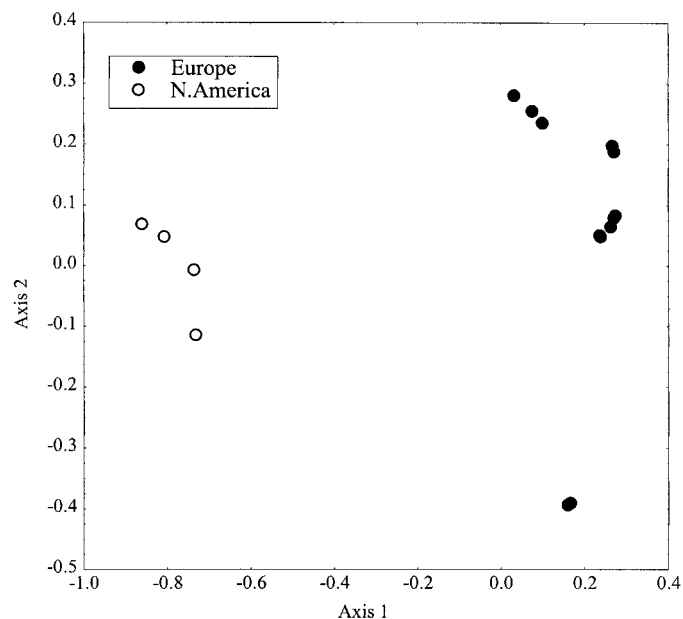


Fig. 5. Multidimensional scaling of Nei's genetic distances among populations of *D. obtusa*. Axis 1 explains 60.8% and axis 2 17.9% of the total variance of distance matrix.

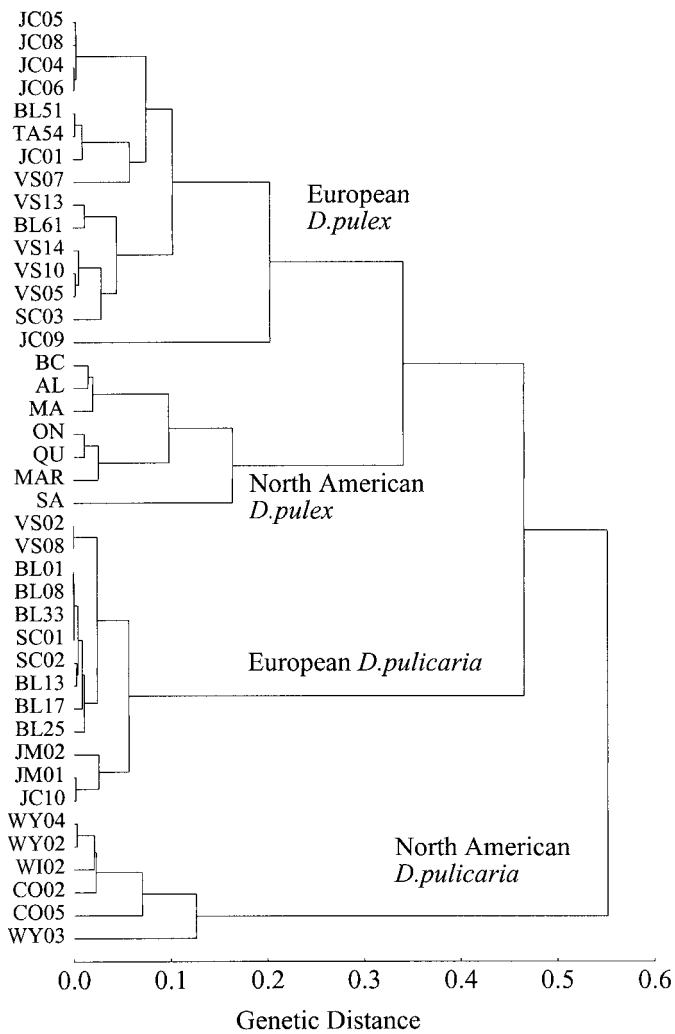


Fig. 6. UPGMA tree of Nei's genetic distances among populations of *D. pulex* and *D. pulicaria*. AL, BC, MA, MAR, ON, QU, and SA represent *D. pulex* data from Canadian provinces (Hebert et al. 1993).

inhabiting remote alpine lakes in the Tatra Mountains, Slovakia (Černý et al. in prep.).

The results of study examining patterns of genetic diversity in *D. pulex* from the Baltic region (Ward et al. 1994) suggest that these populations share allelic arrays with mid-European populations at AMY, LDH, MPI, and GPI, but there does appear to be divergence at the MDH locus with northern populations ordinarily fixed for a slower mobility allele than those in central Europe. Some continental patterning also has been observed in North American *D. obtusa*, and gene composition of populations from Georgia examined in this study fit well into the Eastern subgroup (Hebert and Finston 1996).

This study provides the first allozyme comparisons between European and North American populations for several species of *Daphnia*. As a general result, this analysis shows that genetic divergence is more pronounced between populations from different continents than among those from a

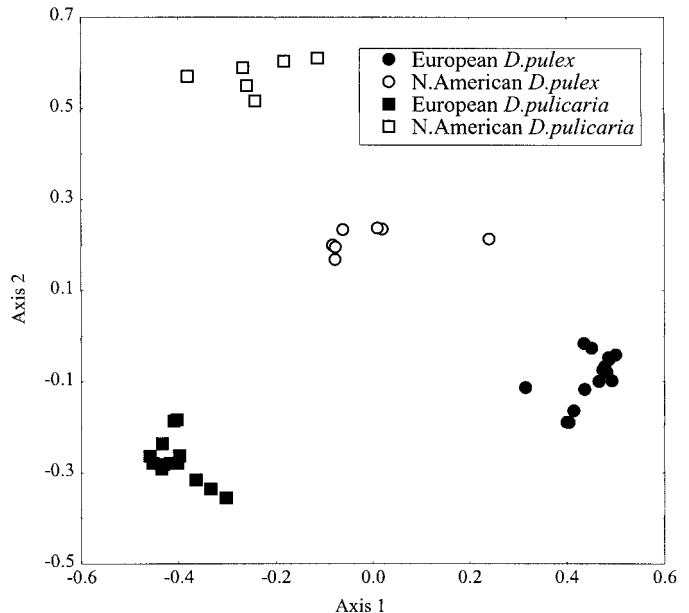


Fig. 7. Multidimensional scaling of genetic distances for populations of *D. pulex* and *D. pulicaria*. Axis 1 explains 44.9% and axis 2 explains 27.6% of the total variance of distance matrix.

single continent, even when the latter lineages are separated by thousands of kilometers.

At a practical level, the marked intercontinental divergence makes it possible to recognize cases in which populations have been transferred from one continent to another. While an earlier use of this approach revealed that the Laurentian Great Lakes have been colonized by European *D. galeata* (Taylor and Hebert 1993b), the present study provided no new evidence of intercontinental transfers. However, this might be just a matter of sampling effort because the occurrence of another North American daphniid invader, *Daphnia parvula*, has been reported recently from many water bodies in mid-Europe (Černý pers. obs.; Kořínek pers. comm.).

From a theoretical perspective, the discovery of regional divergence suggests that gene flow between populations on different continents has not been sufficient to prevent their differentiation. In time, such divergence would be expected to lead to speciation, suggesting that geographical isolation may play the same key role in the origin of new daphniid species as in other organisms. Indeed there is an uncertainty that derives from the failure of this study to survey populations across the Palearctic. As a result, it might be possible that European and North American populations are linked by genetic intermediates in Asia that might be suggested by occurrence of several common mtDNA *D. pulex* haplotypes across northern Eurasia (Weider and Hobaek 1997). On the other hand, it seems clearly to be inappropriate to neglect the likelihood that important biological differences are associated with this gene pool divergence. For example, North American populations of *D. pulex* and *D. pulicaria* often hybridize, while their European counterparts do not.

This study has shown that substantial genetic divergence

regularly exists between zooplankton taxa that share a species epithet but not a continent. The failure to recognize the distinct status of these taxa is clearly inappropriate, if such lineages are irrevocably launched on independent evolutionary trajectories, as a consequence of their genetic divergence. Recent phylogenetic studies that have confirmed that daphniids show both slow morphological differentiation and frequent character convergence had shown the risks in grouping taxa that show morphological affinity (Colbourne et al. 1997). Unfortunately, there is no simple protocol that can permit the determination of the acquisition of species status. A more thorough examination of patterns of genotypic diversity in Eurasian populations would provide a better sense of the degree of their genetic divergence from allied North American forms. As well, the use of additional measures of genetic affinity such as DNA sequencing studies can provide a more secure assessment of phylogenetic relationships among lineages. Such investigations might, for example, reveal that European and North American forms sharing an epithet are not sister taxa. Finally, tests of reproductive isolation—in cases of allopatry—would be particularly useful. The detailed analysis of faunas in contact zones between Eurasia and North America, such as Beringia, that has been already started by Weider and Hobaek (1997) seems to be particularly useful. Finally, given their amenability to experimental analysis, breeding tests could examine the fitness of both F_1 and advanced generation hybrids between lineages from different continents.

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Received: 25 September 1998

Amended: 6 April 1999

Accepted: 22 April 1999