

Molecular systematics of European *Hyalodaphnia*: the role of contemporary hybridization in ancient species

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We examined phylogenetic relationships among *Daphnia* using mitochondrial DNA (mtDNA) sequences from the small subunit ribosomal RNA (12S), cytochrome *c* oxidase subunit I and nuclear DNA sequences from the first and second internal transcribed spacer representing 1612 base positions. Phylogenetic analyses using several species of the three main *Daphnia* subgenera, *Ctenodaphnia*, *Hyalodaphnia* and *Daphnia*, revealed that the *Hyalodaphnia* are a monophyletic sister group of the *Daphnia*. Most *Hyalodaphnia* species occur on one continent, whereas only three are found in North America and Europe. Endemicity of species is associated with variation in thermal tolerance and habitat differentiation. Although many species of the *Hyalodaphnia* are known to hybridize in nature, mtDNA divergence is relatively high (*ca.* 9%) compared to other hybridizing arthropods (*ca.* 3%). Reproductive isolation in *Daphnia* seems to evolve significantly slower than genetic isolation. We related these findings to what is known about the ecology and genetics of *Daphnia* in order to better understand the evolutionary diversification of lineages. The relationship of these data to phylogenetic patterns is discussed in the context of speciation processes in *Daphnia*.

Keywords: interspecific hybridization; *Daphnia*; phylogeny; speciation; reproductive isolation

1. INTRODUCTION

The evolutionary history of freshwater organisms has long attracted the interest of biologists: 'As lakes and river-systems are separated from each other by barriers of land, it might have been thought that fresh-water productions would not have ranged widely within the same country, and as the sea is apparently a still more formidable barrier, that they would never have extended to distant countries. But the case is exactly the reverse. Not only have many fresh-water species, belonging to different classes, an enormous range, but allied species prevail in a remarkable manner throughout the world' (Darwin 1859, p.380). For more than a century, this pattern was attributed to the exceptional dispersal capabilities of aquatic life (Mayr 1963). However, recent work has revealed that many of these cases of phenotypic congruence are not founded on genetic cohesion. Instead they reflect the phenotypic stasis of lineages showing marked genetic divergence (Hebert 1998).

The cladoceran crustaceans were long regarded as one of those aquatic groups whose component species showed both broad geographical distributions and limited regional diversification. However, recent analyses have revealed that many of the cladoceran taxa recognized by traditional taxonomists are actually an amalgam of genetically divergent species (Frey 1987; Hebert & Wilson 1994). This result has provided new motivation for investigations of

speciation mechanisms in these organisms. This work has now provided evidence for the importance of speciation linked to the range fragmentation of an ancestral species by physical barriers such as oceans or mountain ranges (Taylor *et al.* 1998). However, there is also evidence of other speciation processes. Interspecific hybrids are common between many closely allied cladocerans and one case of rapid speciation in these organisms has been linked to introgression (Taylor & Hebert 1993; Schwenk & Spaak 1995). There is also evidence that some species pairs have arisen in neighbouring sympatry through disruptive selection linked to their occupancy of habitats with divergent predation regimes and physical conditions.

Although there is now a suggestion of possible speciation mechanisms in the cladocerans, past studies had a limited geographic and taxonomic scope. The present study builds upon earlier work, which focused largely on North American taxa, by examining the affinities among members of the cladoceran subgenus *Hyalodaphnia*, which forms a dominant element of the daphniid fauna in permanent bodies of water throughout the Holarctic (Taylor *et al.* 1996). This group is an interesting target for analysis because its component taxa are exposed to all of the processes that have been implicated in the speciation of cladocerans. Some of its members have narrow distributions, often bounded by geographic barriers, suggesting their possible origin through allopatric speciation. In contrast, other species appear to be distributed across the Holarctic, raising questions about the recency and extent of gene exchange among their component populations (Hobæk & Wolf 1991; Schwenk *et al.* 1998; Taylor *et al.* 1998). Interspecific hybrids are also common between many of the species in this subgenus (Schwenk & Spaak

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1997). The frequent co-occurrence of these hybrids with their parental taxa provides a situation favourable for introgression and possibly also reticulate speciation (Taylor & Hebert 1993; Spaak 1996; Giebler *et al.* 1999). Finally, members of the subgenus occur in both lakes where they are exposed to fish predation and in ponds where fishes are absent, providing a possible venue for disruptive selection.

The present study had the primary goal of examining phylogenetic affinities between all of the species of *Hyalodaphnia* known from the Holarctic. In addition, we compared the extent of genetic divergence between hybridizing taxa of *Hyalodaphnia* and hybridizing populations of other invertebrates. These analyses jointly aim to advance understanding of the processes that have promoted diversification in these cladocerans.

2. MATERIAL AND METHODS

(a) Sampling

Daphnia were sampled from ponds and lakes and stored in ethanol until DNA was extracted. The sampling area ranged from northern (Iceland to Finland) to southern Europe (Portugal to Greece) (table 1). In addition, one specimen from Africa (Lake Tana) and several DNA sequences from North American species were included in the analysis (GenBank/European Molecular Biology Laboratory (Heidelberg) (EMBL) database). All recognized species of the subgenus *Hyalodaphnia*, four species of the subgenus *Daphnia* and two species of *Ctenodaphnia* were included (table 1). European *Daphnia* species were identified according to Flöbner (1972, 1993) and Flöbner & Kraus (1986).

(b) DNA amplification and sequencing

DNA was extracted using a standard protocol (Schwenk *et al.* 1998). Five to ten microlitres of the homogenate (total 100 µl) were directly subjected to a polymerase chain reaction (PCR). Both universal primers, i.e. cytochrome *c* oxidase subunit I (COI) (Folmer *et al.* 1994) and 18d-5' (Palumbi 1996) or *Daphnia*-specific primers, i.e. 12S (Taylor *et al.* 1996) and ITS2-R (D. J. Taylor, unpublished data), were used for PCR and automated sequencing. The internal transcribed spacer (ITS) fragment included a short piece of the ITS1 region, 5.8S rDNA and a large part of the ITS2 region. PCRs were performed with the following amplification conditions and temperature profiles.

- (i) 12S. A total volume of 50 µl with 200 µM dNTPs, 5 µl 10 × PCR buffer (10 mM Tris-HCl, pH 8.3, 50 mM KCl), 1.5 mM MgCl₂, 130 nM of each primer (5'-ATGCACTTTCCAGTACATCTAC-3' and 5'-AAATCGTGCCAGCCGTGCGC-3') and 0.5–1 unit of DNA polymerase from *Thermus aquaticus* (Taq) with 15% w/v trehalose was subjected to ten cycles of 1 min at 94 °C, 1 min at 53 °C and 1 min at 72 °C, 25 cycles of 45 s at 92 °C, 1 min at 53 °C and 1 min at 72 °C and one cycle of 6 min at 72 °C.
- (ii) COI. A total volume of 50 µl with 250 µM dNTPs, 4.5 µl 10 × PCR buffer (10 mM Tris-HCl, pH 8.3, 50 mM KCl), 2.2 mM MgCl₂, 350 nM of each primer (5'-GGTCAACAATCATAAAGATATTGG-3' and 5'-TAAACTTCAGGGTGACCAAAAATCA-3') and 0.5–1 unit of DNA polymerase from *Thermus aquaticus* (Taq) with 15% w/v trehalose was subjected to one cycle of 1 min at 94 °C, 40 cycles of 1 min at 94 °C, 1.5 min at 40 °C and 1.5 min at 72 °C and one cycle of 6 min at 72 °C.

- (iii) ITS. A total volume of 50 µl with 200 µM dNTPs, 5 µl 10 × PCR buffer (10 mM Tris-HCl, pH 8.3, 50 mM KCl), 1.5 mM MgCl₂, 300 nM of each primer (5'-CACACCGCCCGTCGCTACTACCGATTG-3' and 5'-CGGTGGTCGACACTTCGACACGC-3') and 0.5–1 unit of DNA polymerase from *Thermus aquaticus* (Taq) with 15% w/v trehalose was subjected to five cycles of 30 s at 94 °C, 30 s at 53 °C and 1 min at 72 °C, 35 cycles of 1 min at 92 °C, 1 min at 53 °C and 1.5 min at 72 °C and one cycle of 6 min at 72 °C.

PCR products were excised from agarose gels and purified using the Quiaex II gel extraction kit (Quiagen, Hilden, Germany) and *ca.* 20–50 ng per sample were subjected to cycle sequencing using the ABI Prism TaqFS dye terminator kit (Perkin-Elmer, Norwalk, CT, USA). Sequencing reactions were electrophoresed on an ABI 377 automated DNA sequencer.

(c) Alignment and data analysis

DNA sequences were initially aligned using ClustalV (Higgins *et al.* 1996). However, the alignments for 12S were refined using the secondary structures from the SSU rRNA database and DCSE software (De Rijk & De Wachter 1993; Van de Peer *et al.* 1994; Kjer 1995). In order to verify the results, gene products were ordinarily sequenced in both directions (or more than once) and the two or more strands were aligned with Sequencher for Windows 4.0.2 (Gene Codes Corp., Ann Arbor, MI, USA).

The phylogenetic signal in the sequences was assessed by calculating the *g*₁ skewness statistic from tree length distributions (100 000 random trees or exact distributions) (Hillis & Huelsenbeck 1992). Strongly supported clades were successively collapsed in order to examine whether the phylogenetic signal was present in deeper branches ('stopping algorithm') (Hillis 1991).

In order to select the model of DNA substitution that best fitted the data, we used the hierarchical likelihood ratio test approach (Huelsenbeck & Crandall 1997) implemented in the program Modeltest 1.05 (Posada & Crandall 1998). In order to reconstruct the phylogenetic relationships of the sampled sequences, a minimum evolution (ME) (Rzhetsky & Nei 1992) search was conducted (1000 replicates of random stepwise addition, tree bisection–reconnection branch-swapping) from a matrix of maximum-likelihood (ML) distances calculated using the parameter estimates under the best-fit models. In addition, maximum-parsimony (MP) trees were estimated. Confidence in estimated relationships was determined by 1000 bootstrap replicates (Felsenstein 1985). Bootstrap analysis and phylogeny reconstruction were performed using PAUP* v. 4.01b (Swofford 1998).

Estimation of divergence times using 12S variation was based on the calibration method of Lynch & Jarrell (1993). Since Lynch & Jarrell (1993) estimates become negative below 9% sequence divergence, we also used the calibration of Brower (1994), i.e. 2.3% per million years for 12S and COI and the calibration of Schlötterer *et al.* (1994), i.e. 1.2% per million years for ITS.

3. RESULTS

(a) Mitochondrial DNA variation

Approximately 500 bp of the 12S were sequenced for 41 *Daphnia* individuals. An additional 18 sequences were either obtained from the GenBank/EMBL database

Table 1. *Daphnia* species subjected to DNA sequencing of the 12S, COI and ITS regions

(Abbreviations for the taxa used in trees (label), the sampling location (latitude and longitude in decimal degrees) and the GenBank accession numbers for selected 12S sequences are provided. The DNA sequences curl and mid1 are from Colbourne & Hebert (1996).)

label	morph	country	location	latitude	longitude	GenBank number
amb1	<i>ambigua</i>	Mexico	Vincent Aguirre Reservoir	—	—	—
AMB2	<i>ambigua</i>	France	Etang de Bellebouché	46.71° N	1.102° E	AF277283
CRI1	<i>cristata</i>	Belarus	Kroman	53.701° N	26.327° E	AF277281
CRI2	<i>cristata</i>	Lithuania	Utensis	55.45° N	26.006° E	AF277282
CUC1	<i>cucullata</i>	Czech Republic	Medlov Pond	49.74° N	17.083° E	AF277270
CUC2	<i>cucullata</i>	The Netherlands	Tjeukemeer	52.896° N	5.809° E	AF277271
CUC3	<i>cucullata</i>	Russia	Lake Glubokoc	55.75° N	36.155° E	AF277272
CUC4	<i>cucullata</i>	Slovenia	Lake Bled	46.367° N	14.091° E	AF277269
CUC8	<i>cucullata</i>	The Netherlands	Vechten	52.064° N	5.166° E	—
CUC11	<i>cucullata</i>	Russia	Lake Glubokoc	55.75° N	36.155° E	—
curl	<i>curvirostris</i>	Canada	Tuktoyaktuk	69.43° N	133.0° W	AF277280
C × G1	<i>cuc × gal</i>	The Netherlands	laboratory hybrids	—	—	—
C × G2	<i>cuc × gal</i>	The Netherlands	laboratory hybrids	—	—	—
den1	<i>dentifera</i>	Canada	Victoria Road Guelph	43.611° N	80.219° W	AF277277
den2	<i>dentifera</i>	Canada	Canal Flats Pond, BC	50.188° N	115.786° W	DDU34732
dub1	<i>dubia</i>	Canada	Little Wren Lake, ON	—	—	DDU34735
dub2	<i>dubia</i>	USA	Stormy Lake, WI	49.366° N	92.285° W	DDU34736
GAL1	<i>galeata</i>	Spain	Embalse de Valdecanas	39.802° N	5.468° W	AF277265
GAL2	<i>galeata</i>	Finland	Pyhajarvi	62.292° N	26.769° E	AF277266
GAL3	<i>galeata</i>	Germany	Obiger See	47.999° N	12.425° E	AF277268
GAL14	<i>galeata</i>	Hungary	Lake Balaton	46.814° N	17.67° E	—
GAL15	<i>galeata</i>	Iceland	Lake Myvatn	65.588° N	16.993° W	—
GAL16	<i>galeata</i>	Italy	Lago Maggiore	45.99° N	8.668° E	—
GAL17	<i>galeata</i>	Italy	Lago Maggiore	45.99° N	8.668° E	—
GAL19	<i>galeata</i>	Norway	Grimevatnet	60.346° N	5.413° E	—
GAL20	<i>galeata</i>	Norway	Grimevatnet	60.346° N	5.413° E	—
GAL21	<i>galeata</i>	The Netherlands	Tjeukemeer	52.896° N	5.809° E	—
GAL23	<i>galeata</i>	Portugal	Divor	39.581° N	7.732° W	—
GAL25	<i>galeata</i>	Portugal	Meimoa	39.594° N	7.799° W	—
GAL27	<i>galeata</i>	Sweden	Lake Norrviken	59.459° N	17.958° E	—
GAL28	<i>galeata</i>	UK	Loch Leven	56.202° N	3.383° W	—
GAL31	<i>galeata</i>	The Netherlands	Grote Brekken	52.884° N	5.703° E	—
G × H1	<i>gal × hya</i>	Spain	El Tobar	40.27° N	1.8° W	—
HYA1	<i>hyalina</i>	Ethiopia	Tana	12.023° N	37.33° E	AF277274
HYA2	<i>hyalina</i>	Spain	Zahillio	36.991° N	6.505° W	AF277275
HYA3	<i>hyalina</i>	Germany	Hartsee	47.935° N	12.372° E	AF277273
HYA8	<i>hyalina</i>	Spain	Dulce	36.978° N	6.484° W	—
HYA9	<i>hyalina</i>	Germany	Lake Constance	47.608° N	9.504° E	—
HYA20	<i>hyalina</i>	Spain	Villar Del Rey	39.181° N	6.865° W	—
HYA21	<i>hyalina</i>	Norway	Ringebu	61.547° N	10.115° E	—
lae1	<i>laevis</i>	Canada	Rondeau Pond, ON	—	—	DLU34734
log1	<i>longiremis</i>	Canada	Melville Peninsula Lake	67.516° N	84.6° E	DLU34737
LON1	<i>longispina</i>	Poland	Toporowy Staw	49.203° N	20.02° E	DLU34638
LON2	<i>longispina</i>	Norway	Finse Alpine area	60.544° N	7.431° E	AF277279
LON3	<i>longispina</i>	Norway	Myrdalsvatnet	59.991° N	10.792° E	AF277278
mag1	<i>magna</i>	USA	Sandhills Pond, NE	41.952° N	102.06° W	DMU34738
mail	<i>magniceps</i>	USA	Sacramento, CA	38.68° N	121.65° W	AF064158
men1	<i>mendotae</i>	Canada	North Bay Lake, ON	46.316° N	79.494° W	AF277267
men2	<i>mendotae</i>	USA	Center Lake, IN	41.315° N	85.737° W	DGU34649
men3	<i>mendotae</i>	Canada	Guelph Lake, ON	43.577° N	80.267° W	DGU34651
mid1	<i>middendorffiana</i>	Canada	Longstaff Bluff, NWT	68.96° N	75.21° W	—
par1	<i>parvula</i>	Canada	Trout Lake (North Bay)	46.324° N	79.363° W	AF277287
PAR2	<i>parvula</i>	France	Etang de Bellebouché	46.71° N	1.102° E	AF277286
PUL1	<i>pulex</i>	Spain	Amadorio	39.229° N	1.054° W	AF277284
PUL2	<i>pulex</i>	Finland	Mekkojarvi	62.761° N	30.961° E	AF277285
PUL4	<i>pulex</i>	Spain	Embalse La Jarosa	40.702° N	4.166° W	—
ROS1	<i>rosea</i>	Slovakia	Vysne Furkotske	49.139° N	20.021° E	DRU34642
ROS12	<i>rosea</i>	Germany	Ismaning	48.223° N	11.678° E	—
ROS2	<i>rosea</i>	Italy	Lago di Campo IV	45.166° N	8.119° E	DRU34643
ROS3	<i>rosea</i>	Germany	Ismaning	48.223° N	11.678° E	—
SIM1	<i>similis</i>	Spain	Mbrillo Observatorio	36.88° N	6.388° W	AF277288
tho1	<i>thorata</i>	USA	Flathead Lake, MT	47.998° N	114.124° W	DTU34641
umb1	<i>umbra</i>	Canada	Melville Peninsula Lake	69.637° N	83.702° W	DUU34639
umb2	<i>umbra</i>	Canada	Igloolik Lake	69.382° N	81.824° W	DUU34640
UMB3	<i>umbra</i>	Norway	Jotunheimen	61.575° N	8.509° E	AF277276
UMB4	<i>umbra</i>	Norway	Jotunheimen	61.575° N	8.509° E	—

(table 1) or directly from the authors (Colbourne & Hebert 1996; Taylor *et al.* 1998). Approximately 600 bp of the COI gene were sequenced for 30 individuals and alignments yielded 464 bp for 12S and 543 bp for COI.

The pairwise Kimura distances of 12S ranged from 0 to 33.1% and for COI from 0 to 24.5% within the *Hyalodaphnia* (with pairwise deletion of gaps and missing sites) (Kimura 1980). The divergence within species complexes, between *Daphnia galeata* and *Daphnia mendotae* and between *Daphnia hyalina* and *Daphnia rosea*, was below 1% for both genes. 12S divergence between sister taxa ranged from 0.23% (*Daphnia dentifera*–*Daphnia thorata*) to 22% (*Daphnia curvirostris*–*Daphnia laevis*), but most of the species comparisons resulted in values of between 10 and 15% (e.g. 9.8% *D. galeata*–*Daphnia cucullata* and 12% *Daphnia umbra*–*Daphnia longispina*). Intraspecific comparisons between Europe and North America revealed 0.2% sequence divergence for *Daphnia parvula*, 0.9% for *Daphnia ambigua*, 1.6% for *D. umbra* and 0.7% between African and European *D. hyalina*. However, the closely related North American species *D. thorata* and *D. dentifera* differed from their European sister taxa *D. rosea* and *D. hyalina* by 4.2 and 8.3% in 12S and COI, respectively.

A comparison of the 12S transition:transversion ratio (Ts:Tv) and pairwise 12S sequence divergence revealed a negative association, with saturation (Ts:Tv = 1) occurring at *ca.* 20% sequence divergence. Pairwise sequence divergence (Kimura 1980) of 12S and COI sequences for the same species (all *Hyalodaphnia*) indicated that the COI gene evolves *ca.* 1.3 times faster than the 12S gene ($y = 1.343x + 0.003$, $r = 0.93$ and $p = 0.002$).

(b) Mitochondrial DNA phylogenies

The g_1 -values for the 12S, COI and 12S + COI data sets ($g_1 = -0.477$ and $p < 0.01$, $g_1 = -0.666$ and $p < 0.01$, and $g_1 = -0.623$ and $p < 0.01$, respectively) indicated that the tree length distributions were more skewed than expected for random data, suggesting the presence of a phylogenetic signal. When strongly supported clades were collapsed (leaving one representative for *D. galeata*, *D. cucullata*, *D. hyalina*, *D. umbra*, *D. longispina*, *Daphnia dubia*, *D. laevis*, *Daphnia longiremis*, *D. parvula*, *D. ambigua* and *Daphnia similis* for 12S, *D. galeata*, *D. cucullata*, *D. hyalina*, *D. umbra*, *D. longispina*, *Daphnia pulex*, *D. ambigua* and *D. similis* for COI, and *D. galeata*, *D. cucullata*, *D. hyalina*, *D. dentifera*, *D. longispina*, *D. umbra*, *D. pulex*, *D. ambigua* and *D. similis* for 12S + COI) the signal showed a further increase ($g_1 = -1.083$ and $p < 0.01$, $g_1 = -0.586$ and $p < 0.01$, and $g_1 = -0.858$ and $p < 0.01$, respectively). Even when only the most divergent clades were included (leaving one representative for *D. galeata*, *D. dubia*, *Daphnia cristata*, *D. parvula* and *D. similis* for 12S and *D. galeata*, *D. hyalina*, *D. longispina*, *D. pulex* and *D. similis* for COI and 12S + COI), the signal remained significant ($g_1 = -0.374$ and $p < 0.01$, $g_1 = -0.165$ and $p < 0.01$, and $g_1 = -0.876$ and $p < 0.01$, respectively).

A comparison of different models of evolution indicated that the GTR + I + dG model (general time-reversible model) (Rodriguez *et al.* 1990) was best suited for the 12S and COI data sets (Posada & Crandall 1998). The hierarchical likelihood ratio tests of the 12S, COI and the COI + 12S combined data sets showed that the model fit improved significantly if the parameters describing base

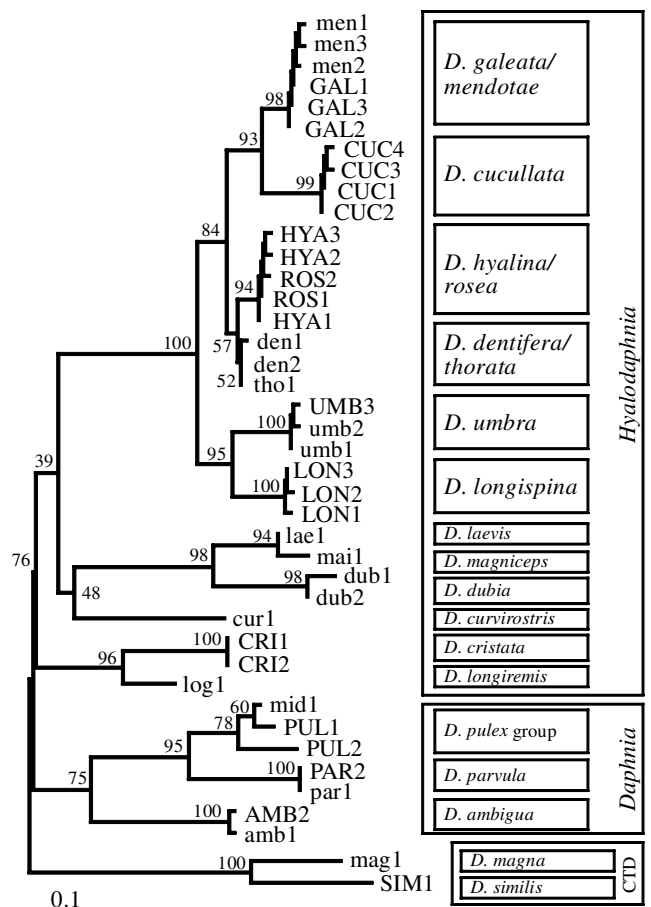


Figure 1. ME tree of *Daphnia* species based on 12S sequences. The model used to reconstruct the phylogeny was the general time-reversible model (Rodriguez *et al.* 1990). Branch lengths are drawn relative to ML distances and values above branches represent bootstrap values (1000 replicates). Abbreviations of individuals are given in table 1; upper-case characters represent European species (except HYA1, which occurs in Africa), whereas lower-case characters represent North American species. The tree has been rooted using the *Ctenodaphnia* (CTD) species *Daphnia magna* and *D. similis* as outgroups.

composition ($p < 0.001$), Ts:Tv ratio ($p < 0.001$), transition and transversion rates ($p < 0.001$), rates among sites ($p < 0.001$) and invariable sites ($p < 0.001$) were added.

MP (trees not presented) and ME analyses resulted in a topology corresponding to those in figures 1 and 2a, with the only variation being the placement of *D. curvirostris*, which was recovered either basal to the *D. laevis*–*magniceps*–*dubia* clade or as the sister taxon to the *Hyalodaphnia* clade. In these trees *Hyalodaphnia* is recovered as a monophyletic group. The best tree in which *Hyalodaphnia* appeared as a non-monophyletic group was significantly worse than our best tree estimates (Templeton test, p -value > 0.05) indicating that our data strongly support a monophyletic *Hyalodaphnia*. As a homogeneity-partition test (Farris *et al.* 1995) of both mtDNA data did not reveal significant differences in incongruence length ($p = 1$; 1000 replicates), we used the two data sets in a combined analysis. The combined tree of 12S and COI (figure 2b) possessed the same topology as the trees based on either 12S (figure 1) or COI sequences (figure 2a).

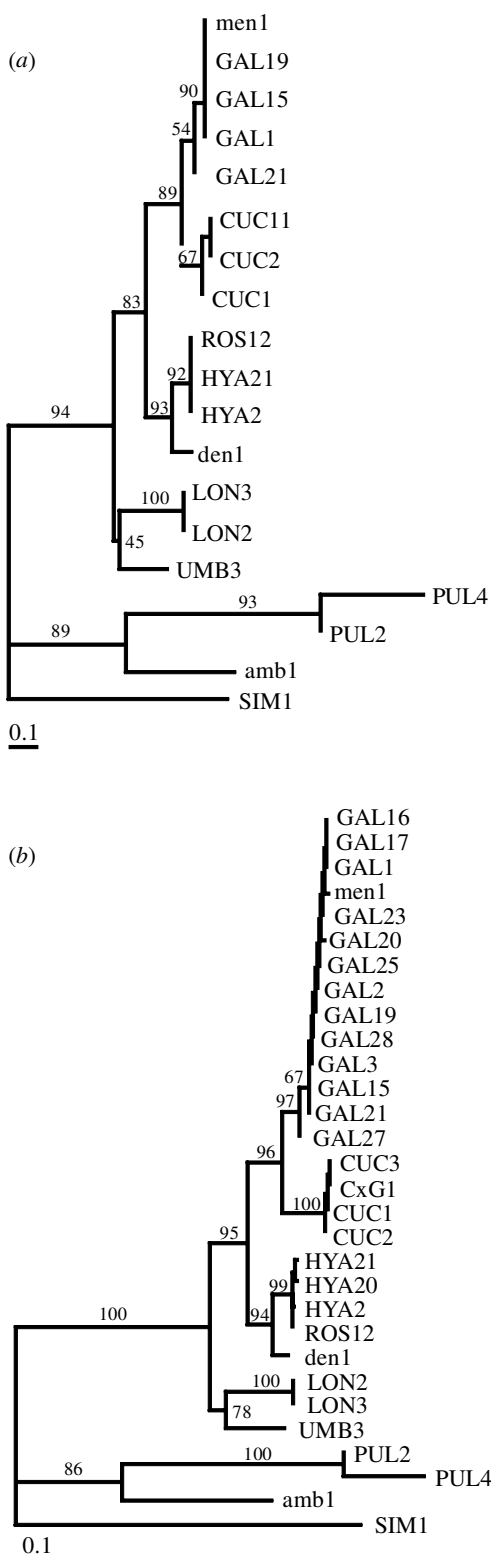


Figure 2. ME trees of the *Hyalodaphnia* species based on (a) COI and (b) COI plus 12S sequences. Five hundred and forty-three base pairs of the COI gene and 1008 bp of the COI/12S regions were analysed using the general time-reversible model (Rodríguez *et al.* 1990). Branch lengths are drawn relative to ML distances and values above branches represent bootstrap values (1000 replicates). Abbreviations are listed in table 1; upper-case characters represent European species (except HYA1 which occurs in Africa), whereas lower-case characters represent North American species. Both trees have been rooted using *D. similis* as an outgroup.

The genetic differentiation between several *D. galeata* clones from a large geographic area, ranging from Spain and Italy to Norway and Iceland, was on average 0.79% (0.35%). In comparison, the differentiation between North American *D. mendotae* and European *D. galeata* clones was 1.25% ($\pm 0.16\%$).

(c) ITS analyses

Approximately 650 bp of the ITS region were sequenced for 18 individuals from the subgenus *Hyalodaphnia*, of which 605 bp were used for phylogenetic analyses. Pairwise K80 distances (with pairwise deletion of gaps and missing sites) among all sequences ranged from 0 to 3.4%, with divergence between sister taxa ranging from 0.3% (*D. galeata*–*D. cucullata*) to 2.6% (*D. galeata*–*D. umbra*).

The g_1 -values for the ITS data set including gaps, treating them as missing data or weighting them according to their length, were statistically significant ($g_1 = -0.674$ and $p < 0.01$, $g_1 = -0.675$ and $p < 0.01$, and $g_1 = -0.6432$ and $p < 0.01$, respectively). When only the most divergent clades were included (leaving one representative for *D. galeata*, *D. cucullata*, *D. hyalina* and *D. umbra*) a signal was still present ($g_1 = -0.674$ and $p < 0.01$, $g_1 = -0.700$ and $p < 0.01$, and $g_1 = -0.70$ and $p < 0.01$). Although a phylogenetic signal was obtained in every case, the resolution of trees was highest when gaps were included. Comparison of different models of evolution revealed the F81 + dG model as the most suited for the ITS data.

A number of insertions and deletions were present within the ITS region involving either single-base substitutions or length variation in 1–2 bp repeats (figure 3). In order to assess the effect of gaps on the phylogenetic reconstruction, ITS sequences were subjected to three different parsimony analyses. In the first analysis, gaps were considered as single events regardless of their length and eight most parsimonious trees (MPT) were obtained. In the second analysis, each position, base pair or gap, was treated as an independent character state. Eight MPTs were obtained that were very similar in topology to the cladograms obtained from the previous analysis (figure 3). In the third analysis, all gaps were excluded, which resulted in thousands of MPTs. Although the MPTs and the strict consensus trees were poorly resolved, they did not contradict the relationships in trees obtained when information on gaps was included. Thus, the inclusion of gaps as characters substantially increased the amount of phylogenetic information.

The phylogenetic relationships between *Hyalodaphnia* species identified using the ITS region were consistent with the branching topology of trees based on mitochondrial DNA. *Daphnia cucullata* and *D. galeata* were recognized as being more closely related to each other than either was to *D. hyalina* (or *D. rosea*). However, the unresolved 12S + COI *D. hyalina* and *D. rosea* clade resulted in two monophyletic groups based on the ITS sequences. One clade was composed of two *D. rosea* and one *D. galeata* × *D. hyalina* hybrid and one *D. hyalina* individual, whereas the second clade was composed of three individuals of *D. hyalina* (figure 3).

Two interspecific hybrids, *D. cucullata* × *D. galeata* and *D. galeata* × *D. hyalina*, clustered together with one of their

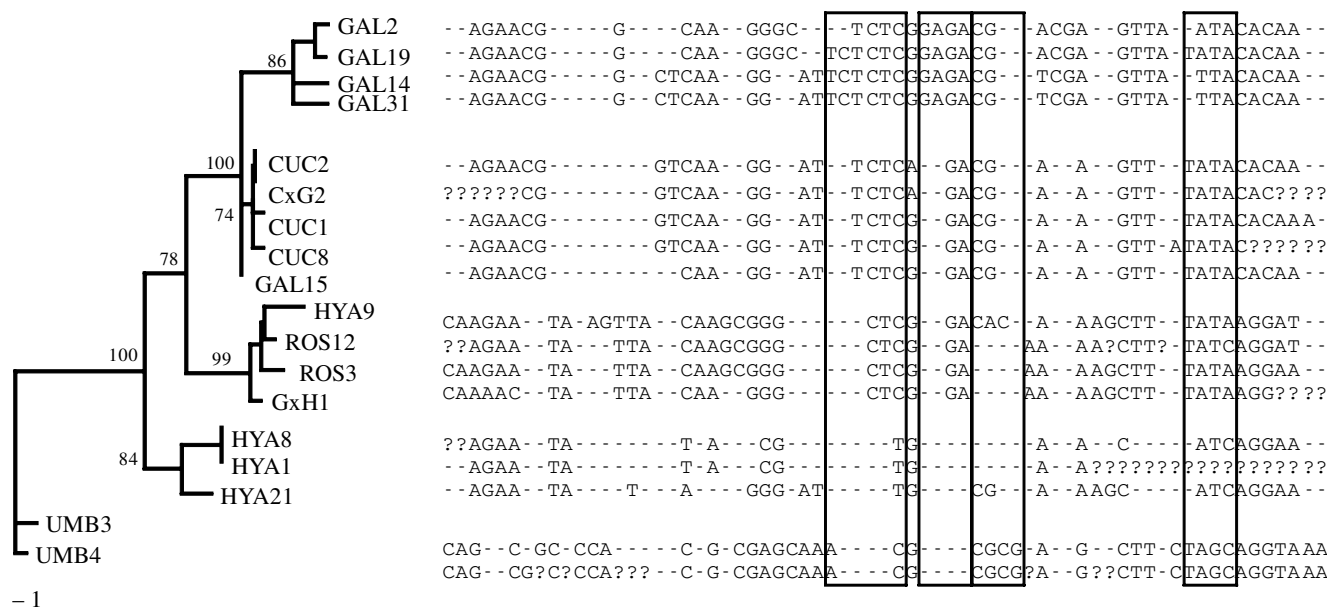


Figure 3. One of the eight ITS MPTs (tree length = 114, confidence interval = 0.771 and retention index = 0.878). Transitions were weighted equal to transversions and gaps were treated as a fifth base. The scale bar indicates the number of steps supporting each branch (branch lengths are drawn proportionally) and values above branches represent bootstrap values (1000 replicates). Variable sites and repetitive motifs (2 bp repeats) are highlighted in boxes. Two sequences of *D. umbra* (UMB3 and UMB4) were used to root the tree.

Table 2. Divergence times (Myr) and 95% confidence limits (in parentheses) for different *Daphnia* lineages

(Lynch & Jarrell (1993) calibration (marked with asterisks) was applied for 12S divergences over 9%. Brower (1994) calibration was applied to 12S divergences below 9% and to the COI data set. Schlötterer *et al.* (1994) was applied to the ITS data. Uncorrected distances are based on pairwise comparisons with pairwise deletion of gaps and missing sites. ML distances were estimated according to the best-fit model.)

split	12S		COI	ITS
	uncorrected	ML distances	uncorrected	uncorrected
<i>Hyalodaphnia</i> – <i>Daphnia</i>	187.58 (104.37–925.26)*	infinite*	9.820 (8.55–10.61)	—
GAL/men/CUC/HYA/den/ Umb/LON-lae/mai/dub/cur	158.08 (87.95–779.72)*	1252.78 (697.05–6179.34)*	—	—
cur-dub/mai/lae	115.93 (64.50–571.84)*	735.97 (409.50–3630.20)*	—	—
GAL/men/CUC/HY/den– Umb/LON	63.26 (35.20–312.04)*	143.76 (79.99–709.09)*	—	2.43 (1.47–3.29)
GAL–LON	56.0541 (31.19–276.49)*	—	8.390 (8.33–8.55)	—
CUC–HYA	38.28 (21.30–188.81)*	137.97 (76.77–680.52)*	6.150 (5.98–6.32)	1.52 (1.19–1.95)
CUC–GAL	2.57 (1.43–12.67)*	43.58 (24.25–214.96)*	4.461 (4.32–4.71)	0.39 (0.00–0.84)
GAL–HYA	3.73 (3.70–3.79)	33.63 (18.71–165.87)*	6.070 (5.90–6.23)	1.56 (1.18–1.80)
den/tho–HYA/ROS	1.79	2.01	3.25 (3.23–3.28)	—
HYA–ROS	0.43	0.44	0.360 (0.32–0.40)	1.26 (0.79–1.95)
AMB2–amb1	0.39	0.40	—	—
HYA1–HYA2/HYA3	0.29	0.30	—	0.39 (0.00–0.78)
GAL–men	0.28 (0.10–0.49)	0.28	0.310 (0.24–0.40)	—
PAR–parl	0.10	0.10	—	—

parental species. The F₁ hybrid C × G2, a laboratory cross between a female *D. cucullata* and a male *D. galeata*, has been subjected to a number of restriction digests that differentially cut the ITS region (data not shown), confirming the presence of both *D. galeata*- and *D. cucullata*-specific ITS alleles.

(d) *Estimation of divergence times*

Given the molecular clock calibration used, the major split between *Daphnia* and *Hyalodaphnia* species was estimated to have occurred more than 100 million years

(Myr) ago (table 2). The three main clades within the *D. longispina* ((i) GAL, men, CUC, HYA, den, UMB and LON, (ii) Lae, mai, dub and cur, and (iii) CRI and log) diverged more than 87 Myr ago. The oldest lineage within the *Hyalodaphnia* is *D. curvirostris* (more than 64 Myr), whereas species such as *D. rosea*, *D. galeata* and *D. dentifera* show divergence times of ca. 1 Myr. Intraspecific comparisons between continents have also revealed recent splits of 0.10 Myr for *D. parvula*, 0.28 Myr for *D. galeata*, 0.39 Myr for *D. ambigua*, 0.68 Myr for *D. umbra* (North America and Europe) and 0.29 Myr for *D. hyalina* (Africa and Europe).

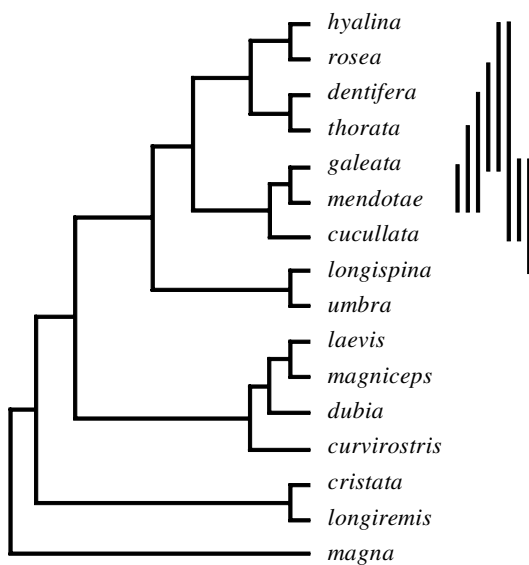


Figure 4. Phylogenetic relationships of all *Hyalodaphnia* species based on nuclear (Taylor & Hebert 1994; Taylor *et al.* 1996; this study) and mitochondrial (Schwenk 1993; Colbourne & Hebert 1996; Taylor *et al.* 1996; this study) genetic variation. Bars connect species that are known to hybridize (for a review, see Schwenk & Spaak 1995).

The ITS calibration is based on uncorrected distances with pairwise deletion of gaps and missing sites. However, if gaps were included, sequence divergence increased by a factor of 4.6 on average. Thus, nuclear and mitochondrial divergence times are in most cases within the same order of magnitude (table 2).

4. DISCUSSION

(a) *Phylogenetic affinities*

Fifteen species of *Hyalodaphnia* are known from the Holarctic region (figure 4). Earlier phylogenetic studies indicated that the nine North American species belong to four species complexes showing deep genetic divergences (Colbourne & Hebert 1996; Taylor *et al.* 1996). Two of these complexes (*curvirostris* and *longiremis*) were represented by a single taxon, while the *laevis* complex included three species (*dubia*, *laevis* and *magniceps*). The *galeata* complex was most diverse, being represented by four taxa (*dentifera*, *mendotae*, *thorata* and *umbra*). Seven species of *Hyalodaphnia* occur in Europe. The present study has established that four endemic taxa (*cucullata*, *galeata*, *longispina* and *rosea*) are also members of the *galeata* complex, while another species in this group, *D. umbra*, occurs in both Europe and North America. The present study also confirms that *D. curvirostris* is a member of the *Hyalodaphnia* and establishes that *D. cristata*, an endemic species, is a second member of the *longiremis* complex. Collectively, these results indicate that the European fauna not only lacks any endemic species complex, but also any representative of the *laevis* complex.

Aside from clarifying taxonomic affinities, the present analysis of phylogenetic relationships, based on joint consideration of 12S and COI sequences, revealed branching orders and overall patterns identical to those obtained by earlier work (Schwenk 1993; Colbourne & Hebert 1996; Taylor *et al.* 1996), which examined a subset

of the taxa and a single gene (12S or *cyt b*). The results confirm that the subgenus *Hyalodaphnia* constitutes a monophyletic group which is comprised of four species complexes. The most speciose of these complexes includes seven closely allied taxa (*cucullata*, *dentifera*, *galeata*, *hyalina*, *mendotae*, *rosea* and *thorata*) and two more divergent lineages (*longispina* and *umbra*). Three of the species pairs in the *galeata* complex were not distinguished by the mtDNA analysis. *Daphnia galeata* and *D. mendotae* share identical mtDNA sequences, but show marked nuclear divergence, reflecting the origin of the latter species through introgressive hybridization (Taylor & Hebert 1993). Other species pairs showed more limited nuclear divergence, but could still be discriminated either allozymically (*dentifera* and *thorata*) or via both allozymes and ITS sequences (*hyalina* and *rosea*) (Taylor *et al.* 1996).

The joint analyses of mtDNA and nuclear markers carried out in this study established that European populations of *Hyalodaphnia* can be reliably assigned to either a recognized species or to an interspecific hybrid. However, there was frequently disagreement for members of the *galeata* complex between these assignments and those based on morphology. Variation in helmet shape has traditionally been used to discriminate between members of this assemblage with *D. hyalina* and *D. galeata* being distinguished from *D. longispina* and *D. rosea* by their more prominent helmets. The present study has established that many lineages initially assigned to *D. longispina* or *D. rosea* because they lacked helmets were actually *D. galeata* or *D. hyalina*. This result indicates that the latter species show such a high level of variation in the expression of this trait that the lack of a helmet is of little taxonomic value. Further studies need to ascertain whether this variability arises as a response to environmental conditions or whether it reflects the introgression of genes from unhelmeted members of the complex. Aside from making clear the need for identifying other morphological differences permitting reliable discrimination of these species, the genetic reassignments indicate the need for a reappraisal of species distributions. For example, *D. longispina* has been regarded as a very widely distributed species in Europe, but genetic studies have only detected its occurrence in Poland and Scandinavia (Taylor *et al.* 1996; Schwenk *et al.* 1998).

The present study also confirms that two North American species of the *D. pulex* group, *D. ambigua* and *D. parvula*, also occur in Europe (Dumont 1974; Flößner & Kraus 1976). Both species show limited divergence between continents, supporting the hypothesis of a recent Transatlantic invasion.

(b) *Endemicity in the Hyalodaphnia*

In contrast to earlier studies, which presumed that many daphniids had broad geographic distributions, only three (*curvirostris*, *longiremis* and *umbra*) of the 15 species of *Hyalodaphnia* occur in both North America and Europe. Our comparison of the European and North American faunas suggested that patterns of endemicity are linked to variation in thermal tolerances among species. Two of the three cold stenotherms (*longiremis* and *umbra*, but not *cristata*) occur on both continents. The only other species (*curvirostris*) with a Holarctic distribution is restricted to the Arctic in North America, but occurs more widely in Europe. The remaining species are not only restricted to

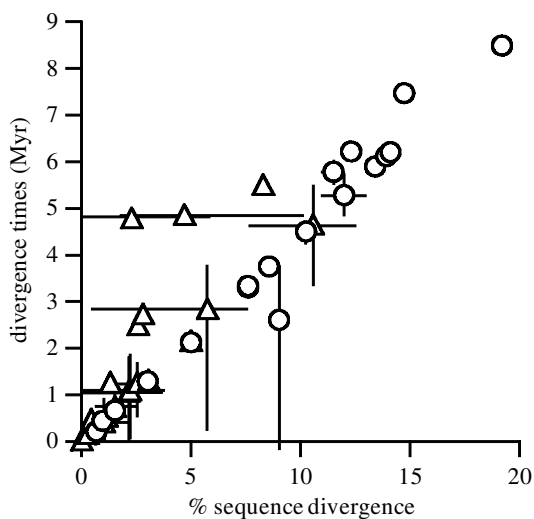


Figure 5. Plot of divergence times (in Myr) between hybridizing arthropod species. Triangles represent hybridizing arthropod species and circles represent hybridizing *Hyalodaphnia* species (electronic Appendix A on The Royal Society Web site). Estimations of divergence times are based on genetic distances from mtDNA sequences between two or more hybridizing species. Values other than those for *Hyalodaphnia* were taken from the literature (see electronic Appendix A). The divergence times for *Hyalodaphnia* are from Colbourne & Hebert (1996), Taylor *et al.* (1996) and table 2.

one of the continents, but to more mesic regions. For example, members of the *galeata* complex, whose distributional limits coincide with the northern boundary of the boreal forest, are each found on only a single continent. Admittedly the taxa in North America and Europe are closely allied. The most striking endemism involves the three North American species of the *laevis* complex and these taxa show the least cold tolerance as their northern range limits coincide with those of the deciduous forest ecozone.

This pattern of endemism is, in a sense, expected. Polar environments on the two continents are very closely juxtaposed and migrants adapted to these settings need not traverse inhospitable terrain in order to move between continents. In contrast, the intercontinental exchange of species restricted to the temperate zone requires either Transatlantic migrations or heroic trans-polar journeys. These results suggest that cold-tolerant taxa are the vehicles for lineage exchange between North America and Europe. Because groups lacking such clades will be effectively isolated from future contact, warm stenotherms should show marked divergence between North America and Eurasia. However, when viewed from a larger geographical perspective, these taxa may show less insularity. For example, migration between the temperate zones of North and South America and between similar environments in Europe and Africa may be common enough to sustain the cohesion of lineages on these pairs of continents. The present study has, for example, revealed very limited genetic divergence between European populations of *D. hyalina* and one African lineage. Similarly, although not yet the subject of genetic analysis, members of the *D. laevis* complex occur on both of the New World continents.

(c) Hybridization within the *Hyalodaphnia*

Past studies have shown that many species pairs within the genus *Daphnia* are capable of hybridization (Schwenk & Spaak 1997). Moreover, the resultant hybrids are often both locally common and broadly distributed. This prevalence of hybrids is linked, at least in part, to the maintenance of reproductive compatibility despite marked genetic divergence. For example, an examination of levels of mtDNA sequence divergence between other pairs of hybridizing arthropods shows an average of 2.7% (range 0.01–10%) divergence versus an average of 9.4% (range 0.6–19.2%) among species of *Daphnia* which hybridize (figure 5). The present results make it possible to place the findings of past observations of hybridization between species of *Hyalodaphnia* in a broader phylogenetic context. They demonstrate that none of these reports involve members of different species complexes. In fact, all cases of hybridization in the *Hyalodaphnia* involve species belonging to the *galeata* complex (figure 4).

The prevalence of hybrids and their frequent co-occurrence with their parental taxa sets the stage for introgression and potentially also for reticulate speciation. North American *D. mendotae* has clearly originated in this fashion, apparently as a result of the Pleistocene invasion of North America by *D. galeata*. Although its mitochondrial genome reflects this origin, its nuclear genome was rapidly reconfigured as a result of introgression from the North American endemic *D. dentifera*. The importance of this process is now signalled by the striking difference in the phylogenetic position of *D. mendotae* when examined for mitochondrial and nuclear DNA markers. The present study has failed to reveal any other similar case of discordance in the European fauna as phylogenies based on ITS were congruent with those based on analysis of mtDNA. However, recent studies on the *D. galeata* complex have revealed discordant phylogenies based on allozymes, randomly amplified polymorphic DNA analyses, morphology and mitochondrial DNA (Schierwater *et al.* 1994; Giebler *et al.* 1999). Since nearly every species of the *D. galeata* complex produces interspecific hybrids and backcrosses (figure 4) and several species co-occur in the same habitat, gene flow across species boundaries is facilitated. Considering the evolutionary age of hybridizing *Daphnia* species, it is conceivable that cycles of glaciation and invasion of new habitats enabled repeated secondary contact of previously isolated gene pools and subsequent hybridization and introgression. This result is important because it suggests that the impacts of hybridization in provoking speciation may be most potent when species come into secondary contact.

(d) Habitat diversity and the *Hyalodaphnia*

Members of the genus *Daphnia* occur in waters showing broad variation in salinity, turbidity and permanence (Hebert 1995). However, species of *Hyalodaphnia* only occur in a limited segment of this range of habitats. They are absent from saline and turbid waters and also from ephemeral ponds. Although the *Hyalodaphnia* are restricted to permanent waters with low ion concentrations and low levels of suspended solids, there is evidence of niche diversity. Some of these differences may have arisen in situations of allopatry, such as the differing

thermal tolerances that are now linked to the varying latitudinal distributions of certain species. However, other taxa show broad regional sympatry, but rarely co-occur because of divergent ecological tolerances. For example, in the temperate regions of North America, *D. dentifera* occurs in ponds and small lakes, while *D. mendotae* occurs in larger lakes. Within Europe, *D. cucullata* dominates lakes where fish predation is intense, while *D. galeata* occurs in lakes where exposure to these predators is less. Allied species such as *D. rosea* tend to be dominant in ponds lacking fishes entirely. The regional sympatry of these lineages, coupled with their differing habitat preferences, suggests that some of these species pairs may have arisen as a result of disruptive selection.

Within the European fauna, there is one pair of closely allied species that show a clear difference in habitat occupancy; *D. rosea* is found in ponds and small lakes while *D. hyalina* occurs in large lakes. These species show no mtDNA divergence, suggesting that they have recently shared a common ancestor. However, there is evidence of modest divergence of gene frequencies at both allozyme and ITS loci. This divergence in their nuclear genomes may reflect incipient genetic isolation, arising as a consequence of disruptive isolation on the blocks of genes that enhance fitness in these two settings.

(e) *Modes of speciation*

Introgressive hybridization may provide variation for adaptation to new environments and facilitate the origin of evolutionary innovation (Lewontin & Birch 1966). However, such a scenario requires two conditions: divergent selection and genetic isolation after the formation of hybrids or recombinants. Recent studies on the ecological differentiation of species have shown that, although a number of taxa co-occur in syntopy, species are ecologically differentiated (Weider 1993). Although reproductive isolation appears to evolve slowly in *Daphnia* and hybrids are prevalent in many habitats, the gene pools of most *Daphnia* species remain distinct. This fact suggests that the fitness of advanced generation hybrids and back-crosses is ordinarily low. The two cases of reticulate speciation through introgressive hybridization within the *Hyalodaphnia* appear linked to the secondary contact of species that diverged in allopatry.

Allopatric differentiation appears to play a very important role in *Daphnia*. Most species appear endemic to a single continent, apparently because intercontinental gene flow is rare. It is clear that the opportunities for genetic isolation are not a simple consequence of varied dispersal abilities. Instead, the isolation of populations is affected by continental positions, ecozone distributions and the environmental tolerances of lineages. This diversity of influences suggests that patterns of allopatric speciation may show marked singularities among groups in response to their varied exposure to these agents. Resolving the role of allopatric divergence is further complicated by the possibility that some species pairs arose initially as a result of disruptive selection linked to microallopatry. Identification of the factors important in promoting speciation is made difficult because many of the speciation events occurred in deep time, as evidenced by the marked genetic divergence between clades. Hence, many of the ecological, distributional or genetic differences that

now exist between clades may have arisen after the speciation event. This fact suggests the particular importance of detailed investigations on the genetic structure of groups such as the *galeata* complex, which show evidence of recent diversification.

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As this paper exceeds the maximum length normally permitted, the authors have agreed to contribute to production costs.

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