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Geographic and phylogenetic evidence for dispersed nuclear introgression in a daphniid with sexual propagules

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Abstract

The role of among-species gene flow in eukaryotic evolution remains controversial. Putative hybrid lineages are common in water fleas, but their ecological success is often associated with polyploidy and the production of asexual propagules. Advanced hybrid lineages with sexual propagules are expected to be geographically restricted because their successful dispersal is contingent on overcoming fertility complications, assimilation by parent taxa, and competition with parent taxa. Here we provide evidence that a diploid lineage of *Daphnia* has been formed by introgression between distantly related species and attained a broad distribution (Nearctic) despite its requirement for sexual propagules. The evidence is based on geographical discordance, phylogenetic discordance, recombinant genotypes and additive genotypes of the nuclear internal transcribed spacer regions (ITS) and mitochondrial DNA. Additive genotypes also provided evidence of hybridization between introduced European *Daphnia* and North American *Daphnia*. We argue that the unique biology of Holarctic lacustrine water fleas and the spatial separation of lineages during Pleistocene glaciation have promoted hybridization and its evolutionary consequences.

Keywords: cladocera, *Daphnia*, hybridization, introgression, speciation

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Introduction

> 'When we recollect how complicated are the relations of these instincts with coexisting species, both of the animal and vegetable kingdoms, it is scarcely possible to imagine that a bastard race could spring from the union of two of these species, and retain just so much of the qualities of each parent-stock as to preserve its ground in spite of the dangers which surround it.'
>
> Charles Lyell (1832)

Lyell (1832) viewed natural interspecific hybrids as common but dead-end lineages that fail to successfully disperse from the region of parental hybridization. Similarly, several modern theories (tension, mosaic and bounded hybrid superiority models) agree that hybridization is a local phenomenon where two species interbreed in a narrow or mosaic geographical zone and hybrid products are less fit than parent taxa outside of this hybrid zone (Moore 1977; Barton & Hewitt 1985; Rand & Harrison 1989). But, it is unclear how representative the hybrid zone models based largely on obligately sexual organisms are of natural hybrid systems and the evolutionary significance of hybridization remains controversial (Arnold 1997). Strictly asexual hybrids, for example, can be stabilized by clonal propagation, immediately incur fitness advantages from heterosis, and potentially replace parent taxa (Barton 2001). Still, the long-term evolutionary success of asexual lineages contradicts theory and empirical observations. Many other organisms, including a panoply of metazoan taxa, are capable of clonal and sexual propagation (Hebert 1987a). Hybrids that retain a functional mixed breeding system could possess the best of both evolutionary worlds: short-term stabilization from clonal production and long-term stabilization from sexual reproduction. Such a mixed breeding system should also enhance gene flow or introgression among hybridizing species (Ebert *et al*. 2002). In support of the link between breeding system and hybridization, Ellstrand *et al*. (1996) concluded from a comparative study that hybridization and its evolutionary consequences are concentrated in perennial plant taxa that possess mixed outcrossing and clonal breeding systems.

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Although no similar comparative studies of hybridization and breeding system exist for animals, it is clear that hybridization is extremely common in some groups with mixed breeding systems (Pejler 1956; Hebert 1985; Schwenk & Spaak 1995; McFadden & Hutchinson 2001; Vollmer & Palumbi 2004). Hybrid products, for example, are prevalent in all three subgenera of cyclically parthenogenetic water fleas of the genus *Daphnia* (Schwenk & Spaak 1995). In one of these complexes, the *Daphnia longispina* species group, hybrid products are diploid (Beaton & Hebert 1994), live in permanent waters, and appear to require sexual reproduction for the production of dispersing resting eggs (Taylor & Hebert 1993a; Spaak 1997; Schwenk *et al*. 2001). The existence of polyploid asexual lineages cannot be ruled out in a globally distributed complex, but the evidence (including population genetic studies from Arctic and alpine regions) indicates that such lineages must be rare in the *D. longispina* group compared to other cladoceran hybrid complexes (Hebert 1985; Little *et al*. 1997; Weider *et al*. 1999b; Adamowicz *et al*. 2002).

The *D. longispina* complex lacks defined hybrid zones. Seven of the species overlap in distribution and naturally hybridize with at least one other species over broad and sometimes intercontinental geographical areas (Wolf & Mort 1986; Taylor & Hebert 1992; Taylor & Hebert 1993b; Giessler *et al*. 1999; Hobaek *et al*. 2004) (Fig. 1). The most broadly distributed and aggressive hybridizer is *Daphnia galeata* Sars, 1864, a common zooplankter in Holarctic lakes (Brooks 1957; Keller & Conlon 1994; Flossner 2000). The distribution of *D. galeata* overlaps extensively with the distributions of all of the species except the Arctic denizen *Daphnia umbra* (Hobaek & Wolf 1991; Taylor & Hebert 1994; Taylor *et al*. 1996). Considerable morphological, multilocus allozyme, amplified fragment length polymorphisms (AFLP), microsatellite, mitochondrial DNA (mtDNA) sequence, and egg bank evidence indicate that natural hybrids are common, and often more abundant than parent taxa (Taylor & Hebert 1992; Schwenk 1993; Taylor & Hebert 1993a; Spaak & Hoekstra 1995; Spaak 1996; Taylor *et al*. 1996; Kerfoot *et al*. 1999; Schwenk *et al*. 2000; Giessler 2001; Gili *et al*. 2004; Hobaek *et al*. 2004; Jankowski & Straile 2004). There is also extensive evidence for ecological differences among the *D. longispina* complex species and their hybrids (Taylor & Hebert 1993d; Spaak & Hoekstra 1997; Spaak *et al*. 2000; Duffy *et al*. 2004; Jankowski & Straile 2004). Finally, there is allozyme evidence that humans have enabled hybridization as Palearctic *D. galeata* have likely been introduced by shipping into the lower Great Lakes region of North America, and have hybridized with native *Daphnia* (Taylor & Hebert 1993b).

Although the deeper relations of *Daphnia* are weakly resolved, phylogenetic relations for the major clades of the *D. longispina* complex are fairly robust. Evidence from morphology, 12S rRNA, 16S rRNA, COI, CytB, ITS-2 rRNA,

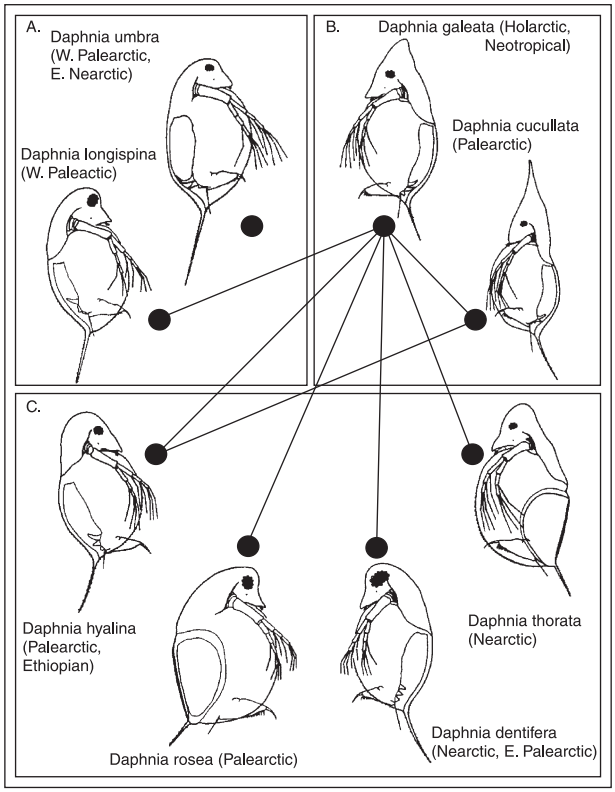

Fig. 1 A summary of the major clades of species in the *Daphnia longispina* complex and their natural hybridization patterns. Internal rectangles (A, B, C) indicate well-established clades based on genetic evidence (see text). Geographic ranges are given in parentheses and the line drawings represent adult females figured in lateral view (natural populations contain much more morphological variation than shown here). Lines connecting solid circles indicate species that naturally hybridize.

and multiple allozyme loci indicated three major clades (Schwenk 1993; Taylor & Hebert 1994; Taylor *et al*. 1996; Schwenk *et al*. 1998; Schwenk *et al*. 2000). The most basal clade contains *D. longispina* (*sensu* Taylor *et al*. 1996) and *D. umbra* (Fig. 1a). The *galeata-cucullata* clade (Fig. 1b) contains two species capable of producing pointed helmets and is the sister clade to *rosea/dentifera/thorata/hyalina* (Fig. 1c). There is little or no evidence for introgression of mtDNA among the complex, as population-level analysis of mtDNA have agreed well with morphological characterizations of species (Schwenk 1993; Taylor & Hebert 1993c; Taylor *et al*. 1996; Schwenk *et al*. 2000; Giessler 2001; Billiones *et al*. 2004; Hobaek *et al*. 2004).

In contrast to mtDNA and morphology, there is evidence that allozyme alleles have been introgressed. The evidence comes from recombinant genotypes at diagnostic loci (Wolf 1987; Taylor & Hebert 1992; Spaak 1996; Giessler 1997; Kerfoot *et al*. 1999; Schwenk *et al*. 2000; Giessler 2001; Duffy *et al*. 2004; Jankowski & Straile 2004), discordant phylogenetic trees (Taylor *et al*. 1996; Giessler *et al*. 1999; Giessler

2001), and discordant geographical distributions of alleles (Taylor & Hebert 1993b; Taylor & Hebert 1993c; Taylor *et al*. 1996). The geographical discordances involve alleles that are shared between species only where they co-occur and hybridize. The most dispersed introgression appears to occur in North America, where it has been proposed that *D. galeata* underwent nuclear introgression with *Daphnia dentifera* to form a new taxon, *Daphnia galeata mendotae* (Taylor & Hebert 1993b). The number of loci involved (at least six), and the divergent nonsister taxon relationship between hybridizing species, make the alternative explanations of shared ancestral alleles or single locus convergent evolution unlikely (Taylor *et al*. 1996). Coyne & Orr (2004) listed *D. galeata mendotae* (or Nearctic *D. galeata*) as one of only three animal species that may have originated via diploid hybrid speciation. Still, with the current evidence, the contributions of stochastic error to the geographical and phylogenetic discordance tests are difficult to assess.

In the present study, we genotyped the internal transcribed spacers of the nuclear ribosomal RNA (rRNA) loci and mtDNA from a wide array of Holarctic sites, many of which have been previously analysed for allozyme variation (Taylor & Hebert 1993b). The internal transcribed spacer (ITS) region has been shown to be phylogenetically concordant with mtDNA and morphology for the *D. longispina* group within Europe (Schwenk *et al*. 2000), and exhibit additivity for hybrids (Billiones *et al*. 2004; Hobaek *et al*. 2004). This gene region has not yet been examined in Nearctic or eastern Palearctic populations where extensive dispersed nuclear introgression has been proposed. Nor has the ITS region been examined for within-individual variation (by cloning) in *Daphnia*, an important step for ruling out shared ancient genotypes in this gene family (Vollmer & Palumbi 2004). Here we examine three predictions of hybrid lineage formation using direct genotyping and cloning: early generation hybrids should possess genotypes that are additive or recombined parental genotypes, ITS introgressants should show a well-supported phylogenetic discordance of ITS sequences with mtDNA and morphology; and putative ITS introgressants should be geographically restricted to regions where parental taxa are sympatric and hybridize.

Materials and methods

Sampling

Five hundred and ninety-eight individual *Daphnia* from 97 Holarctic lakes (see www.buffalo.edu/~djtaylor/publications) were analysed for ITS variation. Twenty-eight of these individuals from geographically distant sites (Table 1) or with unexpected restriction fragment length polymorphism (RFLP) patterns were chosen for ITS and mtDNA sequencing (13 *Daphnia galeata*, six *Daphnia dentifera*, one *Daphnia cucullata*, one *Daphnia thorata*, one *Daphnia umbra*, one *Daphnia longispina*, three *Daphnia rosea*, and two *Daphnia hyalina*). Each of the eight well-recognized species of the *D. longispina* complex was sampled (Taylor *et al*. 1996; Schwenk *et al*. 2000). *D. umbra* and *D. longispina* (*sensu* Taylor *et al*. 1996) were used as outgroups because there is strong independent evidence of their outgroup status to the B and C clades in Fig. 1 (Taylor *et al*. 1996; Schwenk *et al*. 2000). Hybrid specimens of *D. galeata* X *D. dentifera* and Nearctic *D. galeata* X Palearctic *D. galeata* were chosen from the same samples that had been previously well characterized by allozymes, morphology and mtDNA. We also attempted to sequence partial 12S rRNA from each individual used for ITS sequence analysis. However, for four ingroup populations, different individuals were used from the same sample for 12S and ITS (Table 1). Thirteen additional 12S sequences were taken from GenBank (two *D. longispina*, one *D. umbra*, three *D. galeata*, one *D. dentifera*, one *D. thorata*, two *D. hyalina*, one *D. rosea*, and two *D. cucullata* sequences, see Table 1).

DNA sequencing, cloning and genotyping

Using a CTAB (hexadecyltrimethylammonium bromide) (Doyle & Doyle 1987) or Quickextract protocol (Epicentre Technologies), DNA was extracted from frozen samples preserved in ethanol or acetone. For sequencing and initial RFLP's, the entire ITS1-5.8S-ITS2 region of rRNA genes was amplified using the 28SD2BR primer (5'-TTAGAAGG-AGTTTACCTCCCGCTTAGG-3') and the conserved 18SD primer (5'-CACACCGCCCGTCGCTACTACCGATTG-3'). The 28SD2R primer was designed to be specific for branchiopod crustaceans (a BLAST search revealed that only branchiopods and some insects had significant matches), making amplification of all authentic daphniid rRNA gene copies likely, while minimizing the possibility of nonarthropod contaminants. Each 50 μL reaction consisted of 1–5 μL template DNA, 1× PCR buffer [50 mM KCl, 1.5 mM $MgCl_2$, 10 mM Tris-HCl pH 8.3, 0.01% (w/v) gelatin], 2 mM each dNTP, 1 μM each primer, and 1 unit *Taq* DNA polymerase. Polymerase chain reaction (PCR) parameters were 35–40 cycles of 45–60 s at 94 °C denaturation, 45–60 s at 58 °C (50 °C for 12S) annealing, and 1–2 min at 72 °C extension, followed by 1 cycle of 7–20 min at 72 °C final extension. Partial 12S rRNA regions were amplified as in Taylor *et al*. (1996).

A preliminary analysis indicated that ITS-RFLP's from the restriction enzyme *Rsa*I were informative for the species and hybrids examined in North America. However, in order to increase separation among RFLP bands, a shorter PCR fragment was obtained for each individual using a branchiopod specific primer (5.8SBF; 5'-ACCCTGAACGGT-GGATCACTAGGCTC-3') with the original branchiopod 28SD2BR primer. Gel-confirmed PCR products (1–5 μL) were exposed to *Rsa*I digestion for 3–6 h at 37 °C. Digestion products were then electrophoresed in 2.5% agarose gels in

Table 1 *Daphnia* specimens, sample locations, and GenBank accession nos for DNA sequences of 12S mtrRNA and ITS nrRNA regions*

Species*	ID	Location	Accession no.
D. cucullata	GER	Schierensee; Germany	ITS (AY730402); 12S (U34652‡)
D. cucullata	NE	Tjeukemeer; the Netherlands	12S (AF277271†)
D. dentifera	AK1a	Conners Lake, AK; USA	ITS (AY730384) 12S (AY730373)
D. dentifera	AK1b	Conners Lake, AK; USA	12S (AY730372)
D. dentifera	AK2a	Teller6 Pond, AK; USA	ITS (AY730383)
D. dentifera	AK2b	Teller6 Pond, AK; USA	12S (AY730374)
D. dentifera	BC	Canal Flats, BC; Canada	12S (U34732‡)
D. dentifera	IN1	Hammond Lake, IN; USA	ITS (AY730389)
D. dentifera	IN2	Wylund Lake, IN; USA	ITS (AY730387)
D. dentifera	NYa	Deep Lake, NY; USA	ITS (AY730390)
D. dentifera	NYb	Deep Lake, NY; USA	12S (AY730375)
D. dentifera	WI	Mud Lake, WI; USA	ITS (AY730391); 12S (AY730376)
D. galeata	AK1	Alder Pond, AK; USA	ITS (AY730378); 12S (AY730363)
D. galeata	AK2	Mirror Lake, AK; USA	ITS (AY730397); 12S (AY730365)
D. galeata	AK3	Summit Lake, AK; USA	ITS (AY730396); 12S (AY730367)
D. galeata	AK4	Weiner Lake, AK; USA	ITS (AY730379); 12S (AY730366)
D. galeata	CA	Los Carneros, CA; USA	ITS (AY730381); 12S (AY730368)
D. galeata	CZ	Slapy Reservior; Czech Republic	12S (U34647‡)
D. galeata	ENG	Ullswater; England	ITS (AY730401); 12S (AY730360)
D. galeata	GER	Bodensee; Germany	ITS (AY730399); 12S (AY730361)
D. galeata	IN	James Lake, IN; USA	ITS (AY730380)
D. galeata	JPN1a	Lake Biwa, Japan	ITS (AY730377); 12S (AY730359)
D. galeata	JPN1b	Lake Biwa, Japan	ITS (AY730398)
D. galeata	MI	Baseline Lake, MI; USA	ITS (AY730388); 12S (AY730369)
D. galeata	NB	Loch Lomond, NB; Canada	ITS (AY730382); 12S (AY730364)
D. galeata	OR	Lost Lake, OR; USA	12S (U34650‡)
D. galeata	SCO	Loch Oich; Scotland	ITS (AY730400); 12S (AY730362)
D. galeata	SPA	Embalse de Valdecanas; Spain	12S (AF277265†)
D. hyalina	AUS	Mondsee; Austria	ITS (AY730385)
D. hyalina	ETH	Tana; Ethiopia	12S (AF277274†)
D. hyalina	GER1	Kellersee clone; Germany	ITS (AY730394) 12S (U34644c)
D. longispina	NOR	Myrdalsvatnet; Norway	12S (AF277278†)
D. longispina	POLa	Nizny Toporowy Staw (Pond); Poland	ITS (AY730404)
D. longispina	POLb	Nizny Toporowy Staw (Pond); Poland	12S (U34638‡)
D. rosea	ITA	Lago Di Campo IV, Piedmont; Italy	12S (U34643‡)
D. rosea	SLO1	Rohacske pleso Dolni; Slovakia	ITS (AY730386); 12S (AY730371)
D. rosea	SLO2	Vsyne Furkotske; Slovakia	ITS (AY730395)
D. rosea	SW	Arosa; Switzerland	ITS (AY730393); 12S (AY730370)
D. thorata	MTa	Flathead Lake, MT; USA	12S (U34641‡)
D. thorata	MTb	Flathead Lake, MT; USA	ITS (AY730392)
D. umbra	NOR	Jotunheimen; Norway	12S (AF277276†)
D. umbra	NWT	Pond near Richards Bay, NWT; Canada	ITS (AY730403)

*as determined by morphology, mtDNA, & sometimes allozymes; †Schwenk *et al*. (2000); ‡Taylor *et al*. (1996).

the presence of ethidium bromide and photographed. To confirm that RFLP's were additive, cloned products (see below) of known hybrids were also exposed to RE analysis. For sequencing, the PCR products were purified by excision from agarose and centrifuged in Ultrafree DA agarose centrifugal units. Modified TAE buffer (0.1 mM EDTA and 40 mM Tris acetate) was used for electrophoresis. Purified PCR products from the ITS-5.8S-ITS2 region were cloned with the TOPO TA Cloning kit (Invitrogen). Target ITS DNA was amplified directly from the clones using vector primers (M13F & M13R, or T3 & T7 from Invitrogen) and the PCR protocol above, with the addition of a 10-min denaturation at 94 °C in the beginning to lyse the cells and inactivate nucleases. Positive clones were confirmed by size on agarose gels and the PCR products were purified as above.

Samples were cycle sequenced on a Stratagene RoboCycler thermal cycler using the ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction Kit. Forward

and reverse strands of the entire ITS region were sequenced with primer pairs previously mentioned (18D-5′- and 28sD2R) and internal primers from a conserved region of the 5.8S gene (5.8BR TAGGATTAGCGCACTTTGCTGC, 5.8BF ACCCTGAACGGTGGATCACTAGGCTC). In order to study within-individual sequence variation of ITS, 5–10 clones were sequenced using the 18SD-5′ primer from each of the completely sequenced *D. galeata*, *D. dentifera* and *D. thorata* individuals (save for Lake Biwa in Japan for which we sequenced just two clones). A total of 114 clones were sequenced and compared. Forward and reverse strands of 12S PCR products were directly sequenced with the PCR primers from Taylor *et al*. (1996). Sequence electropherograms were compared and assembled using Sequencher 4.1 (Gene Codes Corp.).

Phylogenetic analysis

Sequences were exposed to BLAST analysis, aligned with CLUSTALX (Thompson *et al*. 1997) under default settings, then manually adjusted. Ambiguously aligned sites were culled by GBLOCKS 0.91 with the allowed gap positions set to 'with half' and the remaining parameters set to default for DNA (Castresana 2000). Phylogenetic analyses were performed in PAUP* version 4.0b10 (Swofford 2000) and MRBAYES 3.1 (Huelsenbeck & Ronquist 2001). Models were determined with likelihood ratio tests as implemented in MODELTEST 3.06 (Posada & Crandall 1998). For the optimality criteria of maximum parsimony (MP) and maximum likelihood (ML) heuristic searches were conducted with tree-bisection-reconnection branch swapping and 10 random sequence taxon additions. Branch support was estimated by nonparametric bootstrapping with 1000 pseudoreplicates and fast stepwise addition and Bayesian posterior probabilities. Priors for Bayesian analysis were default but the covarion parameter was set to 'yes'. Starting from a random tree, four Monte Carlo chains ran simultaneously for 1 million generations. A sample frequency of 1 in 100 gave 10 000 trees, and then a portion of the early trees were conservatively removed after inspection for convergence on the Markov chain. A 50% majority rule consensus tree was calculated from the remaining trees. Branch support was the proportion of trees that contained the clade, and represented the posterior probability of the existence of that clade given the data and model of evolution.

Tests of statistical significance of the difference in tree topologies were carried out in PAUP* using the SH test (Shimodaira & Hasegawa 1999) with RELL bootstrapping (1000 replications). Tests for recombination were carried out by the permutation and BLAST-like Karlin–Altschul methods implemented in GENECONV 1.81 (Sawyer 1989). GENECONV examines the nucleotide substitution distribution among sequences for significant clusters that might indicate recombination. The null hypothesis was no recombination.

Results

Alignment

No ambiguous sites were identified for the 12S rRNA alignment by GBLOCKS, leaving the length at 440 nt. In contrast, the ITS–PCR product and sequences contained extensive length variation, particularly between *Daphnia longispina* and the other taxa. The ITS1 region of *D. longispina* differed from the other taxa in that it contained three large tandem repeats that were 131, 202, and 226 base pairs (bp) long. There was a 17 bp spacer between the repeats that was not found in the other taxa. The repeats differed mostly by indels, and in fact, the first and second repeats were identical except for insertions and deletions. When gaps were pairwise deleted, p-distances between the tandem repeats were 0.00, 0.07, and 0.04 substitutions per site. To facilitate alignment of *D. longispina* with the other sequences, a majority consensus was used for the three repeats and inserted in place of them. The CLUSTALX alignment of nuclear sequences from the ITS1-5.8S-ITS2 region of ribosomal DNA was then 1709 nucleotides long. The alignment was made available on the internet at http://www.treebase.org. GBLOCKS identified 26% of the sites as ambiguous and removed them leaving alignment length at 1272. When the outgroups were removed, GBLOCKS identified 17% of sites as ambiguous.

mtDNA species reference tree

The three historically well-supported clades (see Fig. 1) were supported in the present 12S rRNA tree (Fig. 2). Holarctic *Daphnia galeata* was monophyletic. Within the *Daphnia dentifera*/*Daphnia rosea* clade, a European and North American clade was apparent, but there was a lack of resolution between *D. rosea*/*Daphnia hyalina* and *D. dentifera*/*Daphnia thorata*. The optimal model found by MODELTEST had parameters for three substitution types, base frequencies, and the proportion of invariable sites (Tamura-Nei + I). Two best trees of 184 steps were found from MP searches and these differed only in one branch among closely related *D. galeata*. One best ML tree was found that had a likelihood score of –ln L = 1482.19694. Markov chain Monte Carlo (MCMC) convergence of likelihood scores appeared to occur before the 2500th tree and hence only subsequent trees were included in the Bayesian support estimate.

Discordance of ITS phylogeny with species reference clades

The ITS sequences matched best with bosminid water fleas in a BLAST analysis (no daphniid water flea sequences were available for ITS in the database). The ITS phylogeny (Fig. 3) contained three main clades, but there were some discordances from the expected traditional clades. The discordances

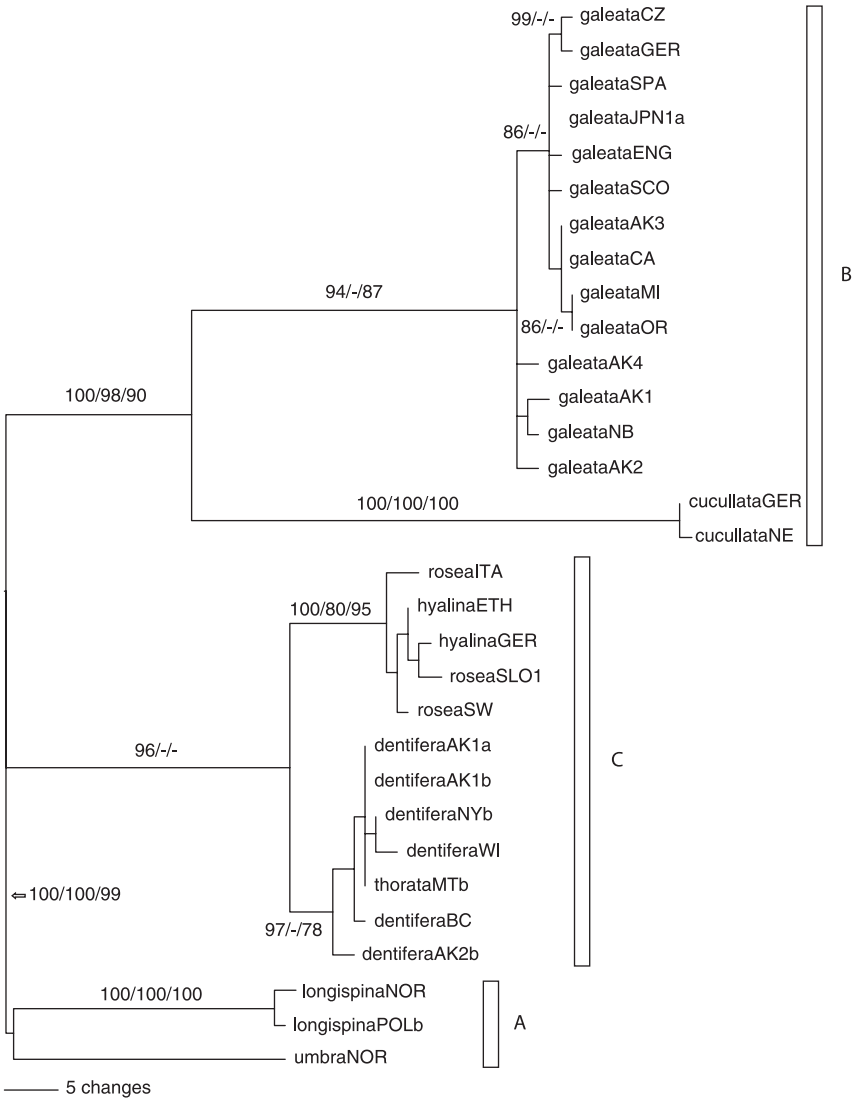

Fig. 2 A phylogram based on one of two best maximum parsimony (MP) trees from the 12S mtrRNA gene sequences of the *Daphnia longispina* complex. Numbers on the branches are Bayesian posterior probabilities, maximum likelihood (ML) nonparametric bootstrap values, and MP nonparametric bootstrap values. Specimen locations are detailed in Table 1. Boxes A, B, and C indicate the major clades pictured in Fig. 1.

involved species that hybridize. Namely, some North American and Japanese *D. galeata* grouped closely with *D. dentifera*; *Daphnia cucullata* grouped within European *D. galeata*, and some *D. rosea* and *D. hyalina* specimens grouped with *D. galeata* (instead of with *D. dentifera*). The monophyly of *D. galeata* was rejected by an SH test ($-\ln L_{MLtree} = 3416.85715$, $-\ln L_{MLmonophyly} = 3488.47381$, difference in $-\ln L = 71.61666$, $P < 0.001$). In contrast, the monophyly of *D. dentifera* was not rejected by an SH test ($-\ln L_{MLtree} = 3416.85715$, $-\ln L_{MLmonophyly} = 3429.89828$, difference in $-\ln L = 13.04113$, $P = 0.124$). The optimal model found by MODELTEST had parameters for two substitution types and two among-site rate parameters (K80 + gamma + I). 2737 best trees of 259 steps were found from MP searches (gaps = missing). One best ML tree was found ($-\ln L = 3416.85715$). MCMC convergence of likelihood scores appeared to occur before the 2000th tree and hence only subsequent trees were included in the Bayesian support estimate.

Because the inclusion of distantly related outgroups clearly reduced the number of unambiguous sites in the alignment and provided an uncertain root, we also carried out the phylogenetic analyses after excluding outgroups (not shown). The optimal model found by MODELTEST again had parameters for two substitution types and two among-site rate parameters (K80 + gamma + I). 877 best trees of 129 steps were found from MP searches (gaps = missing), but these differed only within the major clades. One best ML tree was found ($-\ln L = 2727.7313$). MCMC convergence of likelihood scores appeared to occur before the 2000th tree. Midpoint rooting produced the same root location as outgroup rooting. The exclusion of outgroups revealed a similar tree to the full analyses, but some of the support values, particularly those indicating a relationship between

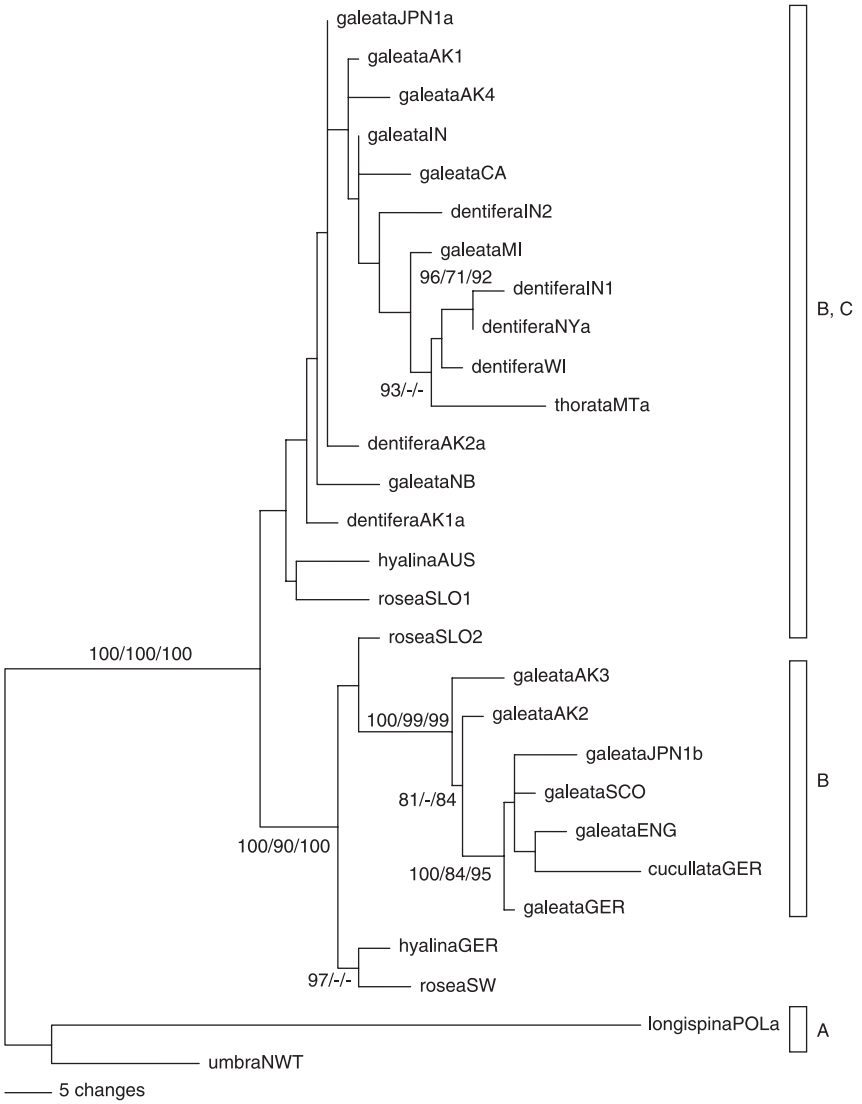

Fig. 3 A midpoint rooted phylogram based on a maximum parsimony (MP) tree from the ITS-1, 5.8S, and ITS-2 rRNA gene sequences of the *Daphnia longispina* complex. Numbers on the branches are Bayesian posterior probabilities, maximum likelihood (ML) nonparametric bootstrap values, and MP nonparametric bootstrap values. Specimen locations are detailed in Table 1. Boxes A, B, and C indicate the major clades pictured in Fig. 1.

sympatric *D. galeata* and *D. dentifera* were strengthened (100/81/87: Bayes PP/ML/MP).

Within-individual sequence variation and recombination

Intragenomic variation was summarized in a phylogram that included all changes among clones including indels as characters (see www.treebase.org). The alignment was 615 characters long and included 114 sequences. There were two major clades that agreed with those from the analysis of the complete region. The average within-individual sequence variation was low at 0.31% (range = 0%–1.94%). Only one individual from a nonhybrid population (*D. galeata* AK1 from Summit Lake, AK) contained clones that were placed in more than one of the two robust major clades (1.94% divergence). The sharing of major clades was consistent with the additive RFLP patterns observed for this population.

The permutation tests as implemented by GENECONV were presented in Table 2. The alignment was restricted to *D. galeata* and *D. dentifera*, because these species were the best sampled and proposed to undergo introgression. All of the significant fragments involved *D. galeata* from clade B (nonintrogressed) and a sequence from clade C (*D. dentifera* and putatively introgressed *D. galeata*). Indeed every *D. galeata* sequence in clade C showed a significant fragment match. In contrast only four of the seven *D. dentifera* sequences had significant fragments in GENECONV. All but two of the fragments involved the 3′ end of ITS-1 and 5.8S. However, the lowest *P*-value involved the ITS-2 region (positions 1039–1507 in the alignment of Japanese and Alaskan *D. galeata*). Phylogenetic evidence for recombination was found for *D. hyalina*/*D. rosea*. Three of these sequences group with clade B (*D. galeata*/*cucullata*) when ITS-1 was included. However, when the ITS-1 region was excluded, the five *D. hyalina*/*D. rosea* sequences formed a monophyletic group (tree not shown).

Table 2 Test results for recombination (from Geneconv 1.81) in the ITS regions of *Daphnia galeata* and *Daphnia dentifera*. Global fragments are listed if permutation *P*-value < 0.05. Clade letters are from Figs 1–3 and location abbreviations are from Table 1

Clade C sequences	Clade B sequences	Permutation *P*-values	Bonferroni corrected Karlin–Altschul *P*-values	Fragment begin	Fragment end	Fragment length	No. polymorphisms	Totl. Diff.
galeataJPN1a	galeataAK3	0.0001	0.00080	1039	1507	469	34	48
galeataIN	galeataAK2	0.0001	0.00129	382	823	442	33	48
galeataAK1	galeataAK2	0.0001	0.00282	382	823	442	33	46
dentiferaNYa	galeataGER	0.0003	0.00606	606	823	218	21	63
galeataMI	galeataJPN1b	0.0003	0.00722	493	777	285	26	53
dentiferaNYa	galeataJPN1b	0.0004	0.00891	606	777	172	20	64
galeataMI	galeataGER	0.0004	0.01160	493	823	331	27	50
dentiferaIN1	galeataJPN1b	0.0005	0.01456	606	729	124	17	70
dentiferaIN1	galeataGER	0.0009	0.01929	606	729	124	17	69
dentiferaNYa	galeataAK2	0.0020	0.02706	606	823	218	21	58
galeataIN	galeataJPN1b	0.0023	0.03556	584	777	194	22	55
dentiferaAK2	galeataAK3	0.0024	0.03578	1248	1550	303	26	48
galeataIN	galeataGER	0.0027	0.03648	584	823	240	23	53
galeataAK1	galeataJPN1b	0.0047	0.06260	584	777	194	22	53
dentiferaIN1	galeataAK2	0.0054	0.07298	606	729	124	17	64
galeataCA	galeataAK2	0.0070	0.09244	382	681	300	24	48
galeataIN	galeataAK3	0.0075	0.09405	343	681	339	28	42
galeataNB	galeataJPN1b	0.0085	0.10743	594	777	184	21	53
galeataNB	galeataAK2	0.0087	0.10830	594	823	230	22	51
galeataNB	galeataGER	0.0087	0.10830	594	823	230	22	51
galeataAK1	galeataGER	0.0090	0.11317	584	823	240	23	49
dentiferaNYa	galeataSCO	0.0094	0.11778	659	823	165	15	68
galeataCA	galeataAK3	0.0131	0.17174	343	681	339	28	40
galeataAK1	galeataAK3	0.0199	0.23056	343	681	339	28	39
galeataAK4	galeataAK2	0.0200	0.23340	430	698	269	21	50
galeataMI	galeataAK2	0.0305	0.32811	584	823	240	23	45
galeataMI	galeataENG	0.0490	0.48674	493	679	187	17	56

The geographical pattern of ITS — RFLP genotypes

The five common RFLP patterns (Fig. 4) showed either the expected association with hybridization (Fig. 5) or marked geographical structure expected from ITS introgression (Fig. 6). More specifically, the most common genotypes were C1 for *D. dentifera* (88 of 131; Fig. 5a), C2 for Nearctic *D. galeata* (226 of 262; Fig. 6) and B for Palearctic *D. galeata* (85 of 86 specimens Fig. 6). In contrast, the B genotype was rare in Nearctic *D. galeata* (5 of 262) outside of Lake Erie and Onondaga Lake (i.e. sites where Palearctic *D. galeata* have likely been introduced). Sequence inspection revealed that the C2 RFLP pattern was a potential recombinant between the *D. dentifera*-specific genotype (C1) and the Palearctic *D. galeata* pattern (B) as it shared both the *Rsa*I cut sites of B and C1. The C2 pattern was also found in *D. dentifera* at a frequency of 17% (23 of 131) and in Japanese *D. galeata* (Figs 5, 6). 43 of 47 hybrids between Nearctic *D. galeata* and *D. dentifera* contained either the additive genotype C1 + C2 (30 of 47) or the putative recombinant genotype C2 (13 of 47; Fig. 5b). Two hybrid individuals were cloned and the RFLP's of the clones segregated into either C1 or C2. The

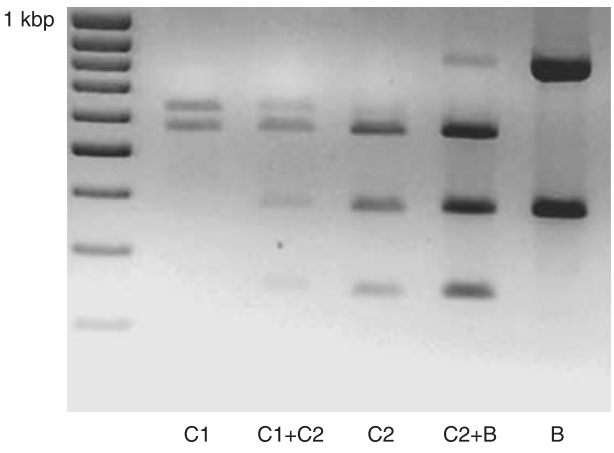

Fig. 4 A photograph of an agarose gel with the five main RFLP patterns found by digesting the ITS rRNA region from individuals of the *Daphnia longispina* complex with the *Rsa*I restriction enzyme. Lane one is a size ladder with rungs from 200 to 1000 bp. Lane C1 is from *Daphnia dentifera*, lane C1 + C2 is from a hybrid between *D. dentifera* and Nearctic *Daphnia galeata*; lane C2 is from Nearctic *D. galeata*; lane C2 + B is from a Nearctic X Palearctic hybrid of *D. galeata* from Lake Erie; and lane B is from a Palearctic *D. galeata* from Coniston Water, England.

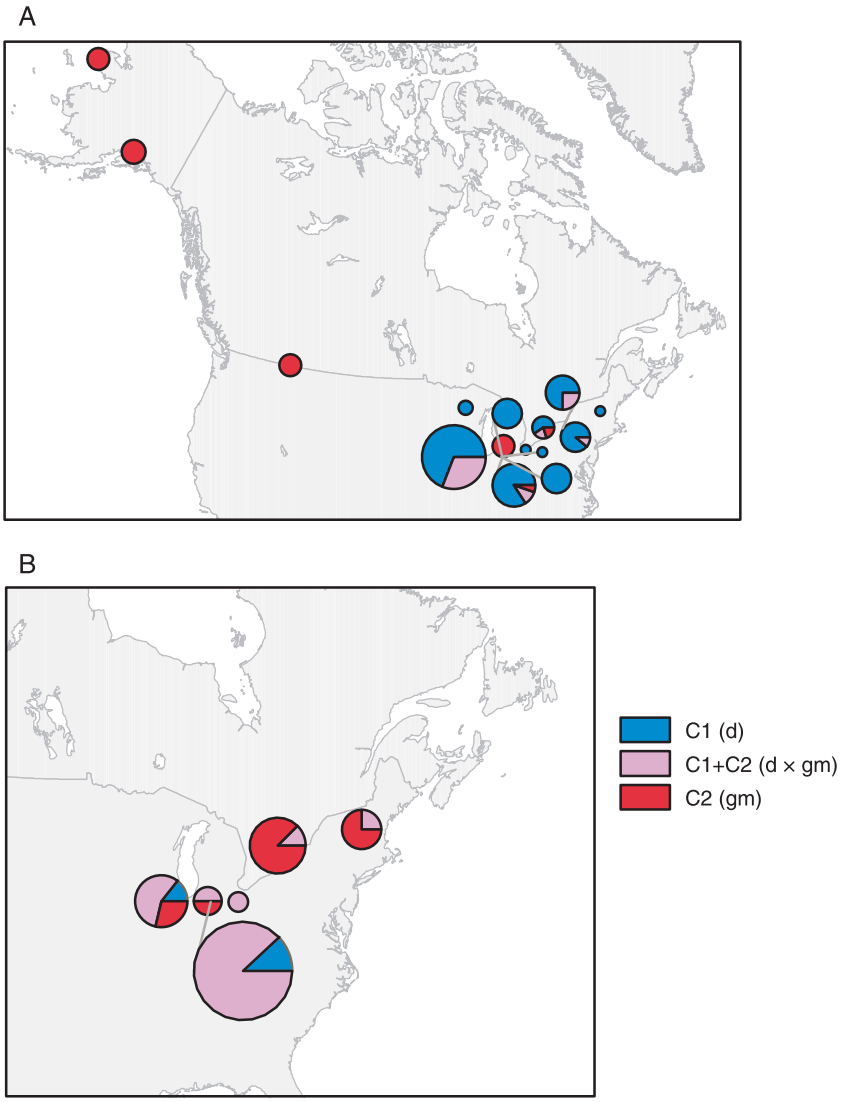

Fig. 5 Maps showing frequencies of ITS-RFLP genotypes in (A) *Daphnia dentifera* and (B) *Daphnia dentifera* × *Daphnia galeata* hybrids. The legend associates the fill patterns with RFLP patterns found in Fig. 4. Lowercase letters in parentheses indicate the taxon for which the RFLP pattern had the greatest frequency (d = *D. dentifera*, gm = *D. galeata mendotae* = Nearctic *D. galeata*). The size of the pies is proportional to the sample size from the sites ranging from 1 to 42 for A and 1–25 for B. Pies were staggered to prevent overlapping.

putative Nearctic X Palearctic *D. galeata* patterns (B + C2) were found throughout North America and Asia, but were most common (17 of 26) in the Great Lakes basin (i.e. in Lake Erie, Grenadier Pond and Onondaga Lake where hybrid swarms between Palearctic and Nearctic *D. galeata* had been previously reported). Sequence analysis did reveal that at least one of the uncommon distant populations from the Great Lakes with the B + C2 pattern (Loch Lomond NB) acquired the B allele by an independent loss of the restriction site.

Discussion

The success of hybrid products depends largely on whether they can escape from hybrid zones as introgressants or new species. For some animals, hybrid success has likely been aided by polyploidy and the production of asexual propagules or resting eggs (Hebert 1987b; Weider *et al*. 1999a; Weider *et al*. 1999b). Our evidence is consistent with the hypothesis that diploid introgressants with sexually produced propagules can also attain intercontinental geographical ranges and ecological success. The phylogenetic and geographical discordances, additive and recombinant ITS sequences, and prior genetic evidence are consistent with the hypothesis that introgressants involving *Daphnia galeata* and regionally distributed species are common in the Holarctic. Indeed, *D. galeata*, which is a dominant component of Holarctic freshwater zooplankton, seems to be an introgressant involving *Daphnia dentifera* throughout its North American and Japanese range.

Phylogenetic and geographical evidence for introgression

The main competing hypothesis to introgression in the evolutionary analysis of hybrid systems is shared ancestral alleles (Barton 2001). Vollmer & Palumbi (2004), for example, provided evidence that some shared ITS lineages in hybridizing

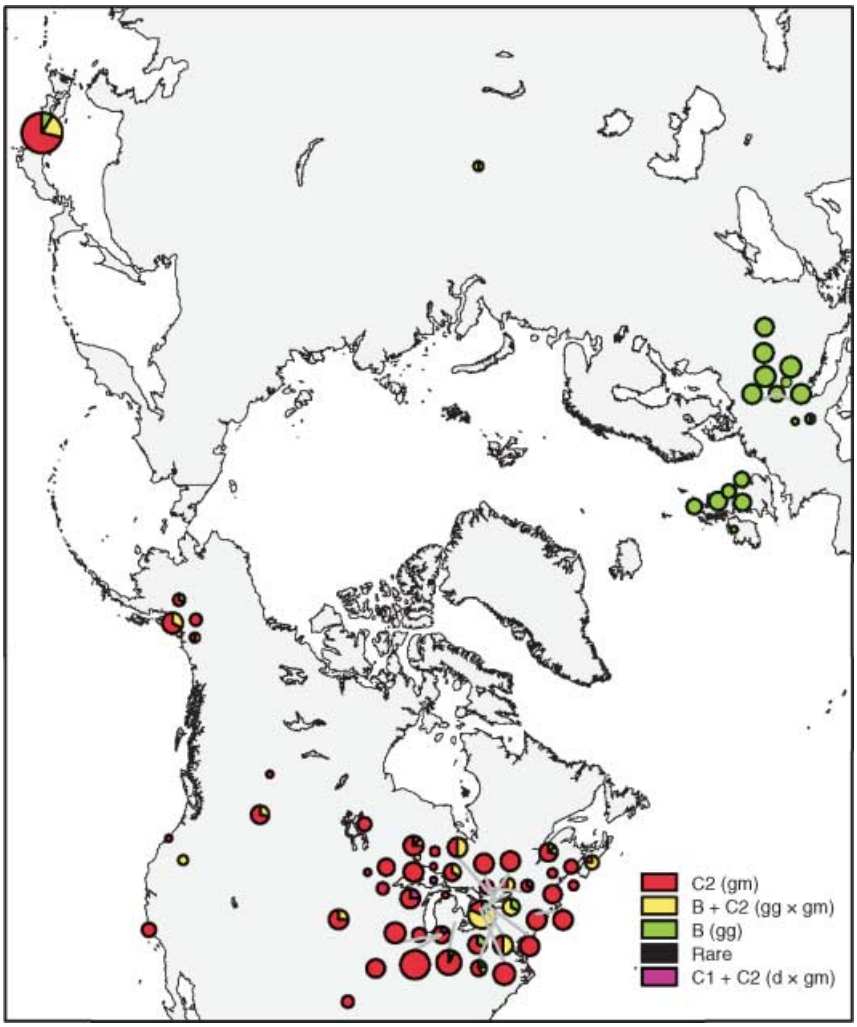

Fig. 6 Map showing frequencies of ITS-RFLP genotypes in Holarctic populations of *Daphnia galeata*. The legend associates the fill patterns with RFLP patterns found in Fig. 4. Lowercase letters in parentheses indicate the taxon for which the RFLP pattern had the greatest frequency (d = *Daphnia dentifera*, gm = *D. galeata mendotae* = Nearctic *D. galeata*, gg = *D. galeata galeata* = eastern Palearctic *D. galeata*). The size of the pies is proportional to the sample size from the sites ranging from 1 to 35 (Lake Biwa, Japan). Pies were staggered to prevent overlapping. Note the discordance in genotype frequencies between Europe and North America + Japan.

corals predate the divergence of the species involved. Nevertheless, shared ancestry is an unlikely explanation for the observed genetic patterns in the present study of a *Daphnia* hybrid complex for a number of reasons. The biogeographical pattern where *D. galeata* possesses the shared *D. dentifera* C2 RFLP pattern only when sympatric with *D. dentifera* (North America and East Asia) is consistent with introgression, but unpredicted by shared ancestry. The near genetic identity (often < 5 steps) between hybridizing *D. galeata* and *D. dentifera* in the rapidly evolving ITS region is also more consistent with introgression than with sharing of an ancient genotype. Moreover, water fleas with similar population sizes and life histories to *Daphnia galeata* show reciprocal monophyly for ITS genotypes where the mtrRNA divergence is only about 2% (Haney & Taylor 2003). Incomplete lineage sorting of ancestral ITS types is therefore unexpected between *D. galeata* and *D. dentifera* with mtrRNA divergences of about 9%. Finally, independent multiple single-copy nuclear loci (allozymes) show a striking agreement with ITS in the phylogenetic discordance, heterozygosity of hybrids and geographical discordance (Taylor *et al*. 1996).

We did find other discordances in addition to *D. galeata*/*D. dentifera*. One of these involved some *Daphnia rosea* and *Daphnia hyalina* specimens that grouped with the *D. galeata* clade. These are putative recombinants between *D. galeata*/*cucullata* type of ITS and the *D. rosea*/*hyalina*/*dentifera*/*thorata* type of ITS. The ITS-1 tree clearly places some *D. rosea*/*D. hyalina* within the *D. galeata* clade, but the ITS-2 region places them as basal to the *D. rosea*/*hyalina*/*dentifera*/*thorata* clade. *D. thorata* and *D. cucullata* appeared in their expected major clades based on mtDNA, but could not be resolved from other species. As with allozymes, the lack of phylogenetic resolution in these cases is difficult to interpret. Reticulate evolution and recombination may be common in the ITS region of this syngameon and the evolution of this gene may be better represented by a network than a tree.

It is also difficult to concoct a molecular evolution or PCR artefact scenario that is a more likely explanation of the patterns than introgression. We minimized contamination

and pseudogene amplification by using branchiopod specific primers. We cloned and directly genotyped PCR products to assess within-individual sequence variation. We did find that the ITS rRNA arrays are incompletely homogenized in *Daphnia* (with differences usually comprised of a few substitutions or indels), but it is unlikely that a process other than hybridization can explain the marked phylogenetic and geographical discordances and close agreement with the additive patterns and discordant biogeography found at other nuclear loci.

Hybridization and dispersed introgression

The high frequency that hybrids can attain relative to parent taxa may have aided introgression in these *Daphnia*. Hybrid vigor in *Daphnia* has been demonstrated experimentally and proposed many times (Hebert *et al*. 1982; Taylor & Hebert 1993d; Ebert *et al*. 2002). For samples where hybrids between *D. galeata* and *D. dentifera* were previously characterized by nine or more informative allozyme loci, mtDNA, and morphology, the expected additive ITS pattern prevailed. This finding agrees with the already strong evidence for geographically widespread hybridization between *D. galeata* and *D. dentifera*. Further evidence for introduction and hybridization of European *D. galeata* in the lower Great Lakes basin is also provided by the ITS data. The finding of a high frequency of additive types and European *D. galeata* homozygotes agrees with the allozyme evidence for introduction and hybridization in the lower Great Lakes region (Taylor & Hebert 1993b). The rare presence of the European-type B alleles at sites distant from the Great Lakes basin in North America (i.e. Alaska) may reflect dispersal from Eurasian *D. galeata*, secondary introductions, independent mutations, or unconverted genotypes.

The age of the proposed nuclear introgression in North American *D. galeata* is equivocal, but the balance of the available genetic evidence indicates dispersed introgression instead of widespread ongoing introgression. For example, although almost all of the North American *D. galeata* group with *D. dentifera*, there are no *D. galeata* sequences that are identical to *D. dentifera* sequences. Moreover, *D. galeata* appears to share the same derived recombinant RFLP pattern throughout much of North America, rather than being a simple heterozygote of species-specific genotypes. Still, the sharing of the same recombinant pattern does not necessarily indicate a single introgressed lineage because gene conversion can be biased and occur rapidly. Further evidence for dispersed introgression comes from other nuclear loci. North American *D. galeata* form a monophyletic genetic cluster that is the sister group to *D. dentifera* with allozymes (Taylor *et al*. 1996). Also, as expected from dispersed introgression, there is no evidence that local *D. dentifera* allozyme alleles at PGI, LDH, and PGM have been introgressed into sympatric *D. galeata* (Taylor & Hebert 1993d). Phylogeographical sequence analyses of proposed introgressed allozyme loci should yield further insights about the timescale of introgression.

Conclusions

Our evidence further bolsters the hypothesis that North American *Daphnia galeata* (*D. galeata mendotae*) arose from diploid hybridization with *Daphnia dentifera*. The evidence agrees with the recombination hypothesis where hybrids backcross with a parent taxon to create a lineage with restored fertility (Buerkle *et al*. 2000). As there is no evidence of mtDNA transfer among lineages, backcrossing would have involved male hybrids with *D. galeata* females. But how could this lineage then avoid genetic assimilation with *D. dentifera* or *D. galeata*? For all demonstrated cases of diploid hybrid speciation, ecological differences between the parent taxa and the hybrid taxa have been found and invoked as an isolating mechanism (Rieseberg *et al*. 2003). There are clear ecological differences between introgressed *D. galeata* and *D. dentifera* today (Brooks 1957; Taylor & Hebert 1993d; Duffy *et al*. 2004), but it is unclear how introgressed lineages of *D. galeata* remained isolated from nonintrogressed *D. galeata*. The ITS evidence from the present study and the allozyme evidence from Taylor & Hebert (1993b) indicate that Eurasian *D. galeata* has been introduced into the Great Lakes basin and now hybridizes with North American *D. galeata mendotae*. However, there is little evidence of fusion or gene flow beyond the Great Lakes basin after decades of contact, suggesting the existence of some reproductive isolation. It is possible that glaciation could have created a large geographical separation between the introgressant lineage and *D. galeata* (different ends of North America or even the Holarctic) permitting vicariance to finish speciation. If true, then *D. galeata mendotae* represents an unusual case where sympatric (hybridization) and allopatric (vicariance) processes have interacted to form a diploid species.

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