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Quaternary diversification in a sexual Holarctic zooplankter, *Daphnia galeata*

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Abstract

The effects of Quaternary glacial range partitioning on the diversification of Holarctic biota remain unclear. Glacial refugial lineages may form vicariant species, hybrid products, or merge after secondary contact. Here, we assess the effects of Quaternary glaciation on a Holarctic sexual zooplankter, *Daphnia galeata*, with apparently marked dispersal capacity and a widespread hybrid lineage in the New World. We collected samples of this species from 148 Holarctic lakes, analysed the nuclear and mitochondrial gene sequences, and tested predictions for hypotheses that account for the origin and spread of the New World *D. galeata*. We detected five nuclear phylogroups and four mitochondrial phylogroups, most of which were restricted to either the New World or the Old World. The oldest mitochondrial phylogroup was restricted to Japan. One major mitochondrial clade was distributed throughout the Holarctic, but only four haplotypes were shared among continents, and analysis of molecular variance indicated significant structure at the continental level. Haplotype sharing among continents could largely be attributed to anthropogenic introductions. Mismatch distributions, haplotype networks, phylogenetic trees, longitudinal haplotype diversity erosion and coalescence analyses are consistent with colonization from an Old World and a New World refugium. Our nuclear and mitochondrial DNA sequence evidence supports the hypothesis that the New World *D. galeata* underwent introgression with *Daphnia dentifera*, with dispersal being enhanced by glaciation. We conclude that Quaternary glaciation had a pronounced effect on the diversification of a Holarctic sexual zooplankter.

Keywords: *Daphnia*, gene flow, glacial refugia, introgression, nuclear DNA, phylogeography

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Introduction

There is considerable evidence that the waves of Quaternary glaciation dramatically affected species distributions, but for many taxonomic groups the role of glacial cycles as sources of diversity remains unclear (Hewitt 2004). Older refugial hypotheses predicted that glacial cycles fragmented species' ranges and promoted reproductive isolation (Rand 1948). Secondary contact could further contribute to diversity by hybrid speciation (Stebbins 1985). Nevertheless, there is now palaeolimnological evidence that glacial cycles failed to partition tropical forests (Colinvaux *et al*. 2000), and genetic evidence that many proposed refugial species originated well before the Quaternary (Zink *et al*. 2004). Even where there is ample evidence that glaciers divided species' ranges, glaciation may actually reduce gradual reproductive isolation (Dynesius & Jansson 2000). In this scenario, highly dispersive (vagile) generalists are selectively favoured by repeated glaciation and there is insufficient time for reproductive isolation to develop among refugial lineages. Thus, secondary contact can often lead to a merging of refugial lineages.

There are, however, several vagile groups of sexual organisms whose biology may promote diversification by glacial vicariance. Aquatic invertebrates, for example, often possess strong dispersal capacity, imparted by wind and animal transport of desiccation-resistant dormant propagules (Cáceres & Soluk 2002; Figuerola *et al*. 2005; Louette & De Meester 2005). Still, local and regional studies of sexual cladocerans have revealed pronounced population structure (Hebert 1974; Boileau *et al*. 1992; Haag *et al*. 2006);

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and multiple geographical phylogroups (Taylor *et al*. 1998; Cox & Hebert 2001; Hebert *et al*. 2003; Penton *et al*. 2004; De Gelas & De Meester 2005). One explanation of this pattern is that priority effects from founder events, massive egg banks and rapid local selection limit effective gene flow despite marked dispersal (De Meester *et al*. 2002). With this scenario, populations from separate refugia should fail to merge, and deglaciated areas will become a mosaic of specialist populations that reflects colonization history (Haney & Taylor 2003; De Gelas & De Meester 2005). Unfortunately, understanding the genetic effects of glaciation on presently widespread northern species is hindered by the difficulty in sampling over vast Holarctic regions and acquiring combined nuclear and organellar gene sequence phylogenies (Hewitt 2004). The result is that little is known about the role of glaciation in the diversification of widespread Holarctic invertebrates — especially sexually reproducing diploids (Weider & Hobaek 2000).

Here, we investigate the role of Quaternary glaciation on the diversification of *Daphnia galeata*, a Holarctic sexual crustacean. Curiously, *D. galeata* populations in the formerly glaciated New World appear to be comprised of a lineage that has been exposed to nuclear gene transfer from *Daphnia dentifera* by hybridization (introgression; Taylor *et al*. 2005). This New World form has been called *D. galeata mendotae* or *D. mendotae* (Brooks 1957). Identification of hybrid lineages involving *D. galeata* is relatively straightforward because the hybridizing taxa are genetically divergent (estimated as Late Tertiary) and diagnostic, or near diagnostic, species markers are numerous (Schwenk *et al*. 2000). Considerable morphological, multilocus allozyme, amplified fragment length polymorphisms (AFLPs), microsatellite, mitochondrial DNA (mtDNA) sequence, nuclear ITS–RFLP (internal transcribed spacer–restriction fragment length polymorphism) and sequence and egg bank evidence indicate that local hybrids involving *D. galeata* are common, and often more abundant than parental taxa within lakes (Wolf & Mort 1986; Taylor & Hebert 1992, 1993; Schwenk 1993; Spaak & Hoekstra 1995; Spaak 1996; Taylor *et al*. 1996, 2005; Kerfoot *et al*. 1999; Schwenk *et al*. 2000; Giessler 2001; Gili *et al*. 2004; Hobaek *et al*. 2004; Jankowski & Straile 2004). The evidence for widespread introgression is based on unique geographical sharing of nuclear alleles in regions where the hybridization occurs (a geographical test with multiple allozyme loci and ITS rDNA) and a phylogenetic incongruence between morphology/mtDNA and nuclear loci (allozymes and ITS). These tests together with the abundance of hybrids and marked interspecies divergences make unsorted ancestral lineages an unlikely explanation for gene sharing (Taylor *et al*. 2005).

The details of how a sexual introgressed lineage of *D. galeata* came to occupy the glacial lakes of much of the New World are unknown. Unlike other zooplankton species, intercontinental comparisons of mtDNA sequences from *D. galeata* (12S rDNA, and COI) have revealed almost no divergence or geographical structuring (Taylor *et al*. 1996, 2005; Schwenk *et al*. 2000; see Fig. 1). There is clear

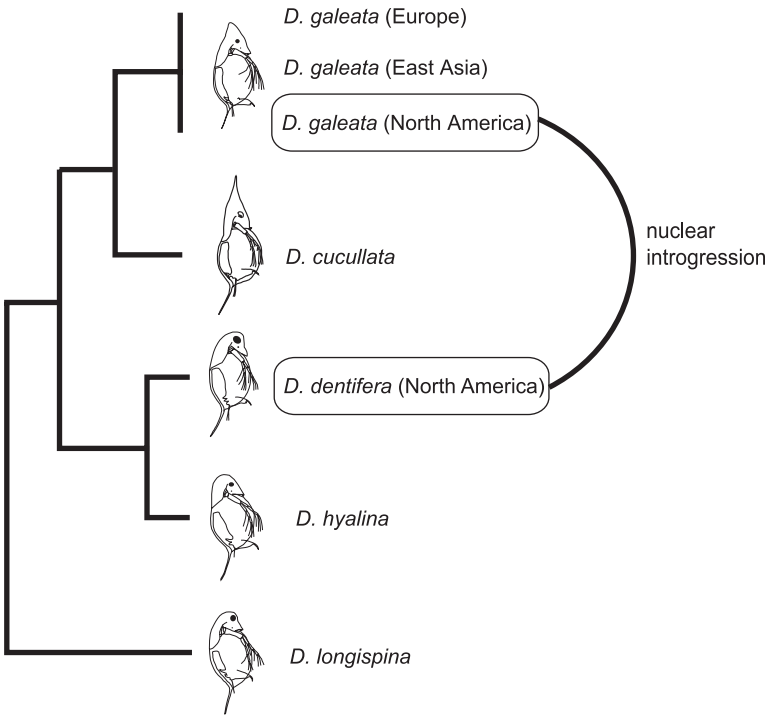

Fig. 1 Proposed species tree for the *Daphnia longispina* group (Taylor *et al*. 2005) showing the lack of regional differentiation in *Daphnia galeata* with conserved genes (COI and 12S rRNA) and the proposed nuclear introgression involving *D. galeata* and *Daphnia dentifera* in the New World. Cartoons represent lateral views of adult female specimens.

differentiation between nuclear markers of the Old and New World populations, but much of the nuclear gene structure differences can be attributed to introgression with *D. dentifera* in the New World (Taylor *et al*. 1996, 2005). Brooks (1957) hypothesized that *D. galeata* colonized from the Old World to the New World in the Holocene, where it may have hybridized with *D. dentifera* (Taylor & Hebert 1992). In this first hypothesis, rapidly evolving mtDNA gene sequences of New World populations should possess the genetic signature of a bottleneck consistent with Holocene expansion from an Old World refugium. A second hypothesis for the mtDNA sharing among continents is that *D. galeata* was anthropogenically introduced into the New World from the Old World in recent centuries or decades (Taylor & Hebert 1993). In this case, a bottleneck in genetic markers should also be detected, but the expansion should involve haplotypes that are shared between Old and New World. A third hypothesis is that *D. galeata* survived in separate refugia during the last glaciation and received genes from hybridizing with *Daphnia dentifera* in the New World (Taylor *et al*. 2005). This hypothesis predicts separate bottleneck and expansion patterns for each continent with sharing of *D. dentifera* clades only in the New World.

In this study, we aimed to test the genetic predictions of the above three hypotheses for the origins of *D. galeata*. We are improving on prior studies by carrying out geographically widespread sampling (148 Holarctic lakes) and using rapidly evolving mitochondrial and nuclear gene sequences. It is hoped that the results will provide insights into the role of Quaternary glaciation in the formation and geographical spread of a diploid sexual introgressed lineage.

Materials and methods

Sample collection

Daphnia galeata was collected from 148 Holarctic lakes (see Table S1 Supplementary material): 44 European lakes, 11 Siberian lakes (including two distant locations in Lake Baikal), 11 Japanese lakes and 82 North American lakes. The sister species, *Daphnia cucullata*, was collected from two European lakes. Two other related species, *Daphnia dentifera* and *Daphnia longispina*, were collected from 13 North American lakes and one European pond. All samples were preserved in absolute ethanol at room temperature, or frozen at –80 C. These specimens were morphologically identified according to Brooks (1957), Flössner (2000) or Taylor *et al*. (1996). The samples from Lake Erie (New York, USA) and Onondaga Lake (New York, USA) were included for the reconstruction of the mitochondrial phylogeny and haplotype network, but excluded for population analyses [i.e. analysis of molecular variance (AMOVA), mismatch distribution, coalescent analysis, haplotype diversity, nucleotide diversity, neutrality tests, gene flow indices and Mantel test], because previous allozyme and rRNA ITS study concluded that European populations were recently introduced in these populations (Taylor & Hebert 1993; Taylor *et al*. 2005). Likewise, the sample from Chuzenji Ko (Japan) and Nishino Ko (Japan) had a mitochondrial haplotype that belonged to an endemic North American clade. Because we could not exclude the possibility of recent anthropologic introduction into these lakes from North America, this haplotype was not used for the population analyses.

Extraction, PCR and sequencing

Total genomic DNA was extracted by using QuickExtract (Epicentre). Samples were homogenized in 30–50 L of the QuickExtract solution, incubated at 65 C for 2 h and 98 C for 10 min, and stored at –20 C. A *c*. 760-bp fragment of the nuclear protein-coding HSP90 gene was amplified using the primers (5′-TTACGAGTCCAGATGGGCTT-3′) and (5′-ATCCGTTATGAATCCCTGACTGA-3′) (Kotov *et al*. 2006). A *c*. 1000-bp fragment of the mitochondrial protein-coding NADH-2 (ND2) gene was amplified using the primers MetF3 (5′-GTTCATGCCCCATTTATAGGTTA-3′) and TrpR (5′-GAAGGTTTTTAGTTTAGTTAACTTAAAATTCT-3′) (Ishida *et al*. 2006). Each 50- L polymerase chain reaction (PCR) consisted of 5 L of extracted DNA, 10 × PCR buffer [50 mM KCl, 1.5 mg $MgCl_2$, 10 mM Tris-HCl pH 8.3, 0.01% (w/v) gelatin], 2 mM of each dNTP, 1 M of each primer and 1 U of *Taq* DNA polymerase. PCR temperature profiles for the nuclear HSP90 gene were: 40 cycles at 94 C for 30 s, 50 C for 30 s and 72 C for 1 min, and a final extension at 72 C for 5 min. PCR temperature profiles for the mitochondrial ND2 gene were: 40 cycles at 94 C for 30 s, 48 C for 30 s and 72 C for 1 min, and a final extension at 72 C for 5 min. Cloning was performed for HSP90 PCR products using the TOPO TA Cloning Kit (Invitrogen). For HSP90, PCR products were directly sequenced and then four or five colonies were sequenced. Sequences of HSP90 and ND2 were obtained in both directions by Genaissance Pharmaceuticals or Roswell Park Cancer Institute. Sequences were assembled and edited with SEQUENCHER 4.2 (Gene Code). Intron boundaries of the HSP90 sequences were identified by comparing arthropod HSP90 mRNA sequences (e.g. AY528900, AY423488) and by examining intron-splicing signature sequences. The HSP90 intron sequences were aligned with CLUSTAL W and then manually adjusted using SEAL 2.0 (Rambaut 1996). The HSP90 exon sequences and the ND2 sequences were manually aligned using SEAL 2.0.

Data analysis for sequences

Fifty *D. galeata* individuals from 50 Holarctic populations were used for nuclear HSP90 genealogy: 12 European

populations; two Siberian populations; six Japanese populations and 30 North American populations (see Table S1, Supplementary material). Five hundred fifty-one *D. galeata* individuals from 148 Holarctic populations were analysed for mitochondrial ND2 genealogy: 155 individuals from 44 European populations; 46 individuals from 11 Siberian populations; 43 individuals from 10 Japanese populations and 307 individuals from 83 North American populations. Three closely related species were used for outgroups: 13 *D. dentifera* populations; two *D. cucullata* populations and one *D. longispina* population. One individual per each population of the related species was analysed. Neighbour-joining analyses was conducted for nuclear HSP90 and mitochondrial ND2 alignments with Kimura 2-parameters, complete gap deletions and 1000 bootstrap replicates using MEGA 3.1 (Kumar *et al*. 2004). The uncorrected *p* distance was calculated with MEGA 3.1.

We inferred demographic properties of each major clade in each of six regions: Europe, Siberia, Japan, western North America (west of 110 W), central North America (between 110 W and 80 W), and eastern North America (east of 80 W). Haplotype diversity and nucleotide diversity were calculated with DNASP 4.10 (Rozas *et al*. 2003). The neutrality tests of Fu's F_S (Fu 1997) and Tajima's *D* (Tajima 1989) were performed with ARLEQUIN 3.01 (Excoffier *et al*. 2005). The F_{ST} and Tajima's *D* are expected to have large negative values for population expansion (other explanations are background selection and hitchhiking associated with selective sweeps). The significance of Fu's F_{ST} and Tajima's *D* were tested by random permutation using 1000 replicates in ARLEQUIN. The index of gene flow (F_{ST}) was calculated with DNASP. F_{ST} is the fraction of nucleotide diversity resulting from genetic variation between populations (Hudson *et al*. 1992). Screening for recombinant sequences in the HSP90 alignment was carried out using the single breakpoint algorithm of Kosakovsky Pond *et al*. (2006), which has a low rate of false positives.

One major mitochondrial clade was distributed throughout most of the Old World and the New World. To address the evidence of genetic differentiation and gene flow among geographical regions within the clade, we used the following four methods. (i) The computer program TCS 1.21 was used to estimate haplotype network of this clade that illustrates all connections that have a 95% probability of being the most parsimonious (Clement *et al*. 2000). If gene exchange is active throughout the distributed area, the haplotypes should be largely shared between the hemispheres. If gene flow was restricted, shared haplotypes should be undetected or limited to ancient haplotypes, and each regional group should form unique lineages (Omland *et al*. 2006). (ii) We estimated the proportion of total genetic variation that was explained by differences among geographical regions using an AMOVA (Excoffier *et al*. 1992) in ARLEQUIN with 1000 permutation replicates. While the hierarchical likelihood test for ND2 sequences selected the GTR + G with a gamma parameter of 0.485 (Nylander 2004), the best-fit model is not implemented in ARLEQUIN. We used the Tamura & Nei (1993) with a gamma parameter of 0.485 for AMOVA. (iii) A Mantel test (1967) was used to determine the relationship between genetic distance (pairwise F_{ST} from ARLEQUIN) and geographical distance to test an isolation-by-distance model. The geographical distance was calculated using the great-circle distance formula. The Mantel test was implemented in IBD 1.52 (Bohonak 2002) using 1000 randomizations. (iv) The migration rate between the New World and the Old World was estimated using LAMARC 2.0 (Kuhner *et al*. 2005), which is based on coalescent theory using a maximum-likelihood framework. LAMARC uses the Metropolis–Hastings Markov chain Monte Carlo sampling algorithm to simultaneously estimate effective population size (from theta = 2 *Nf* where is mutation rate per site and *Nf* is the female effective population size), growth rate (*g*) under the exponential growth rate, and migration rate ($M = m/$ where *m* is the per generation migration rate and is mutation rate per site). Since LAMARC recommends using 20–30 randomly chosen individuals per group, we randomly chose 30 individuals of the B clade from Old World and New World samples, respectively, and used them for analyses. The GTR model was selected for the substitution model (because LAMARC lacks the gamma parameter). LAMARC was run with 10 initial chains with 10 000 sampled genealogies each and two final chains with 200 000 sampled genealogies each. Eight replicate runs were used to examine the consistency of the result. Because the coalescent analysis did not consider the other endemic mitochondrial clade, estimated values of geographical structure were conservative.

To infer the population history of each major mitochondrial clade in each hemisphere, we used the frequency distribution of the number of pairwise differences among all sequences (i.e. the mismatch distribution). The mismatch distributions were calculated using ARLEQUIN based on pairwise sequence differences. Because pairwise sequence differences among geographically subdivided populations tend to produce multimodal patterns (Marjoram & Donnelly 1994), we separated Old World populations into European, Siberian and Japanese populations for this analysis. The bootstrap approach (1000 replications) was used to test the observed data with the simulated data under the models of pure demographic expansion and spatial expansion by comparing the sum of squared deviations (SSD) between the observed (SSD_{OBS}) and simulated (SSD_{SIM}) data. The model of pure demographic expansion assumes that unsubdivided populations suddenly expand in population size (Rogers & Harpending 1992). It estimates $Tau = 2ut$ where $u = m_T \cdot t$ is the estimated time of expansion, m_T is the number of nucleotide sequences under study, and is the mutation rate per time. The model of spatial expansion

assumes that subdivided populations expand the distribution range and increase the total number of the individuals (Ray *et al*. 2003; Excoffier 2004). It estimates *Tau* and M. The value of *Tau* is again 2*u*t. M is the number of migrants among neighbouring demes per time. The 95% confidence intervals of *Tau*, M and mismatch distributions were calculated using a parametric bootstrap approach with 1000 replicates.

Results

Nuclear genealogy

No HSP90 haplotype was shared between New World and Old World *Daphnia galeata* populations, while three HSP90 haplotypes were shared between *Daphnia dentifera* and New World *D. galeata* populations. The HSP alignment (781 bp) was comprised of 73 unique haplotypes from 30 *D. galeata* populations in the New World, 57 unique haplotypes from 20 *D. galeata* populations in the Old World, 30 unique haplotypes from 13 *D. dentifera* populations, six unique haplotypes from two *Daphnia cucullata* populations, and four unique haplotypes from *Daphnia longispina* populations (see Table S1, Supplementary material). Each individual had various numbers (1–5) of HSP90 haplotypes. The average genetic distance (*p*-distance) among haplotypes within each individual was 0.0075 ± 0.0017 (95% CI), while the average pairwise genetic distance between individuals was 0.0273 ± 0.0005. Presumed introns were 87–152 bp (Intron I) and 445–513 bp (Intron II). *D. galeata* sequences had between one and three deletions in Intron I, and between one and six deletions in Intron II. *D. dentifera* sequences had two deletions in Intron I and four or three deletions in Intron II. *D. cucullata* sequences had two or three deletions in Intron I and five deletions in Intron II. *D. longispina* sequences had two deletions in Intron I and five deletions in Intron II. No insertions or deletions were found in exons among the four species. Variable sites were detected in 166 positions.

The neighbour-joining tree revealed no shared lineages between New World and Old World *D. galeata* populations, but a shared clade between *D. dentifera* and New World *D. galeata* populations. There were four major groups (O1, O2, N1, N2 and N3) and several lineages of *D. galeata* (Fig. 2A). Each major group were broadly distributed in either New World or Old World populations. O1 and O2 occurred only in Old World populations, while N1, N2 and N3 occurred only in New World populations. The O1 group was obtained from 15 populations: 10 European populations; two Siberian populations and three Japanese populations. The O2 group was obtained from 11 populations: five European populations; two Siberian populations and four Japanese populations. The NI group was obtained from 18 populations: six populations in the Western New World (west of 110 W), five populations in the Central New World (between 110 W and 80 W) and seven populations in the Eastern New World (east of 80 W). The N2 group was obtained from 11 populations: two populations in the Western, three populations in the Central and six populations in the Eastern. The N3 group was obtained from 12 populations: two populations in the Western, six populations in the Central and four populations in the Eastern.

Recombination screening of the HSP90 alignment identified the N2 alleles as recombinants between the New World N1 clade of *D. galeata* and the N3 clade of *D. dentifera*. A breakpoint for recombination was found at position 379 of the alignment of N1, N2 and N3 haplotypes. The sequences of intron I (87–152 bp) are shared between N2 and N3 haplotypes, while the sequences of intron II (445–513 bp) are shared between N1 and N2 haplotypes. We note that the putative recombinant N2 alleles were identical for both direct and cloned sequences.

Mitochondrial genealogy and population analyses

The ND2 alignment (929 bp) was comprised of 164 unique haplotypes from 553 *D. galeata* individuals of 148 Holarctic populations with 16 haplotypes of three closely related species. No deletions or insertions were found, and variable sites were detected in 411 positions. Neighbour-joining trees revealed four groups of *D. galeata* (A, B, C and D) (Fig. 2B). The A clade (43 unique haplotypes) and the B clade (117 unique haplotypes) were major mitochondrial clades. The C lineage (one haplotype) and the D clade (three unique haplotypes) were deeper, and the D lineage was more divergent than the C lineage. Seventeen per cent of the total genetic variation of all mitochondrial clades was explained by differences between the hemispheres (AMOVA, $P < 0.001$). Strong genetic structure was found among all populations of all clades ($F_{ST} = 0.713$), among the Old World populations of all clades ($F_{ST} = 0.665$), and among the New World populations of all clades ($F_{ST} = 0.726$).

The distributions of the A, C and D clades were geographically restricted, while the B clade was Holarctic (Fig. 3). The A clade was common in North America, rare in Japan and absent in Europe and Siberia. It was broadly distributed throughout North America and especially dominant in the northeastern and northwestern area of North America. One haplotype of the A clade was found in Japan, i.e. two natural lakes that were 2 km apart in Nikko National Park. The Japanese haplotype was not found in North America. The sole haplotype of the C clade was found in only one population in southern Sweden. The D clade was common throughout the Japanese archipelago; one population on Kyushu Island (Southern Area); three populations on Honshu Island (Central Area) and one population on Hokkaido Island (Northern Area). The clade consisted of three haplotypes and was endemic to Japan.

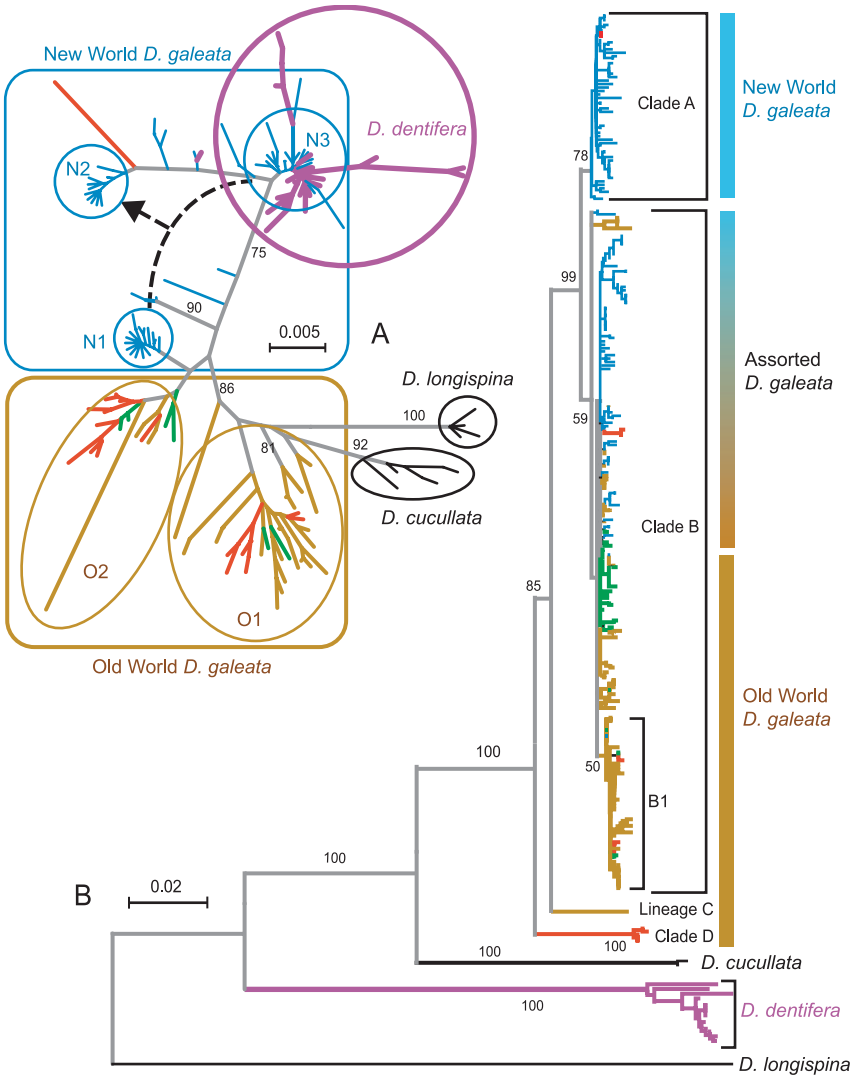

Fig. 2 Neighbour-joining phylograms of nuclear HSP90 gene sequences (A) and mitochondrial ND2 gene sequences (B). The HSP90 sequences are from a subset of specimens represented on the ND2 tree. The branch numbers are support values from nonparametric bootstrap replicates with thick branches having more than 70% bootstrap support. Colours represent either different *Daphnia* species or geographical locations (for *D. galeata*): purple (*D. dentifera*); black (*D. cucullata* and *D. longispina*); gold (European *D. galeata*); green (Siberian *D. galeata*); red (Japanese *D. galeata*) and blue (North American *D. galeata*). The coloured boxes or rectangles indicate New World clades (blue) or Old World Clades (gold). The black dashed arrow in the HSP90 phylogram indicates that N2 is a putative recombinant group between N1 and N3 (see Results from statistical tests of recombination).

The B clade was common throughout the Holarctic and fixed in European and Siberian populations (except for one haplotype of the C clade in Europe). One major subclade was found in the B clade and denoted as the B1 subclade in Figs 2 and 3. The B1 subclade consisted of 29 unique haplotypes from 52 populations: 42 European populations, 5 Japanese populations, 5 Siberian populations, and one population in Onondaga Lake (New York, USA). Previous allozyme studies showed that Onondaga Lake was the location where European populations were recently introduced (Taylor & Hebert 1993; Taylor *et al*. 2005). Thus, the B1 subclade was essentially an Old World group. The nucleotide diversity of the B clade had a significant geographical trend in North America: a decrease from west to east ($P < 0.05$), because the differences in the nucleotide diversity between the western and central North America and between the central and eastern North America were more than twice lager than standard deviations of them (Table 1). The nucleotide diversity of the B clade was higher in the Old World than in the New World. The diversities of the A clade showed no clear longitudinal pattern in North America.

Four haplotypes of the B clade were shared between Old World and New World samples. One shared haplotype (Haplotype 1 in Fig. 4) was the centre of the starlike haplotype network in the clade. One other haplotype (Haplotype 2 in Fig. 4) was one mutation from the shared centre of the starlike network, suggesting a parallel mutation. The other haplotypes (Haplotypes 3 and 4 in Fig. 4) of Old World samples were found from the introduced populations of Onondaga Lake and Lake Erie (New York, USA). 17.1% of the total genetic variation of the B clade was explained by differences between the hemispheres (AMOVA, $P < 0.0010$). The coalescent analyses consistently estimated positive values of the growth rate for Old and New World samples, and larger values of theta (proportional to effective

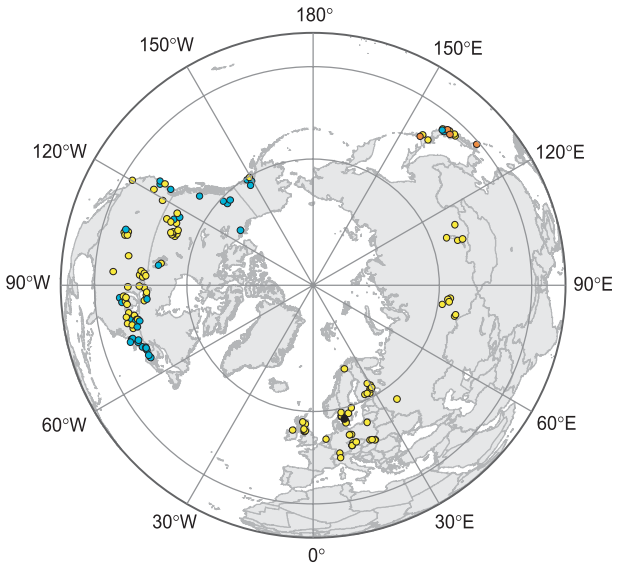

Fig. 3 Map showing the Holarctic sampling and the mtDNA genetic structure of *Daphnia galeata* (see Fig. 2B). Blue dots represent the endemic New World clade; yellow dots represent the Holarctic B clade. The black dot is the C lineage in Scandinavia and the orange dots represent the D clade, which is endemic to Japan.

population size) for Old World samples than New World samples. However, the migration rate between the hemispheres was variously estimated (Table 2).

Three haplotypes of the B clade were shared among Old World populations: Haplotypes 1 and 3 in Fig. 4 were shared by European and Siberian populations and Haplotype 5 was shared by Siberian and Japanese populations. 11.4% of the total genetic variation of Old World B clade samples was explained by differences of geographical groups of Europe, Siberia and Japan (AMOVA, $P < 0.001$). The Mantel test revealed a significant positive relationship between genetic and geographical distances among Old World populations ($r = 0.22$, $P < 0.001$).

Nuclear phylogroups (N1, N2, N3, O1 and O2) and mitochondrial phylogroups (A, B and D) were randomly associated in each continent. The nuclear N1 group consisted of six populations of the mitochondrial A clade and 11 populations of the B clade. The N2 group consisted of four A populations and seven B populations. The N3 group consisted of six A populations and six B populations. The O1 group consisted of nine B populations and two D populations. The O2 group consisted of 14 B populations and one D population.

Mismatch distributions

Mismatch distributions of the B clade in the New World fit the expected distributions of both sudden expansion and spatial expansion models (Fig. 5). P ($SSD_{SIM} > SSD_{OBS}$) indicated that the spatial expansion model was a better fit. Furthermore, the observed frequency of identical sequences was graphically more similar to the simulated frequency based on the spatial expansion model. The observed mismatch distributions of the A clade in the New World

Table 1 Lists of properties for the mitochondrial A and B clades in each geographical region: Old World (Europe, Siberia and Japan) and New World (Western, Central and Eastern). The properties are: populations (Pop); individuals (Ind); haplotypes (H); haplotype diversity (π_H) with standard deviation (SD); nucleotide diversity (π_N) with standard deviation; the statistical significance of the negative values of Fu's F_S and Tajima's D; and the indices of gene flow (F_{ST}). 'NA' and 'NS' in the lists mean not available and not significant, respectively

	Pop	Ind	H	π_H (± SD)	π_N (± SD)	Fu' F_S	Tajima's D	F_{ST}
Clade A								
Old World	2	4	1	0	0	NA	NA	NA
Europe	0	0	0	NA	NA	NA	NA	NA
Siberia	0	0	0	NA	NA	NA	NA	NA
Japan	2	4	1	0	0	NA	NA	NA
New World	39	128	42	0.941 (± 0.009)	0.00529 (± 0.00024)	$P < 0.001$	$P < 0.005$	0.826
West	14	42	36	0.908 (± 0.027)	0.00541 (± 0.00052)	NS	NS	0.794
Central	7	18	16	0.693 (± 0.114)	0.00272 (± 0.00092)	NS	$P < 0.05$	0.923
East	18	68	34	0.890 (± 0.019)	0.00437 (± 0.00028)	NS	NS	0.770
Clade B								
Old World	62	224	97	0.969 (± 0.004)	0.00676 (± 0.00022)	$P < 0.001$	$P < 0.01$	0.570
Europe	44	154	49	0.953 (± 0.008)	0.00652 (± 0.00029)	$P < 0.001$	NS	0.530
Siberia	11	46	20	0.942 (± 0.015)	0.00538 (± 0.00038)	$P < 0.05$	NS	0.324
Japan	6	24	7	0.815 (± 0.046)	0.00587 (± 0.00089)	NS	NS	0.953
New World	49	158	46	0.953 (± 0.008)	0.00522 (± 0.00026)	$P < 0.001$	$P < 0.005$	0.695
West	17	53	16	0.927 (± 0.013)	0.00616 (± 0.00031)	NS	NS	0.658
Central	26	80	29	0.916 (± 0.021)	0.00474 (± 0.00037)	$P < 0.005$	$P < 0.001$	0.718
East	6	25	8	0.853 (± 0.041)	0.00273 (± 0.00033)	NS	NS	0.533

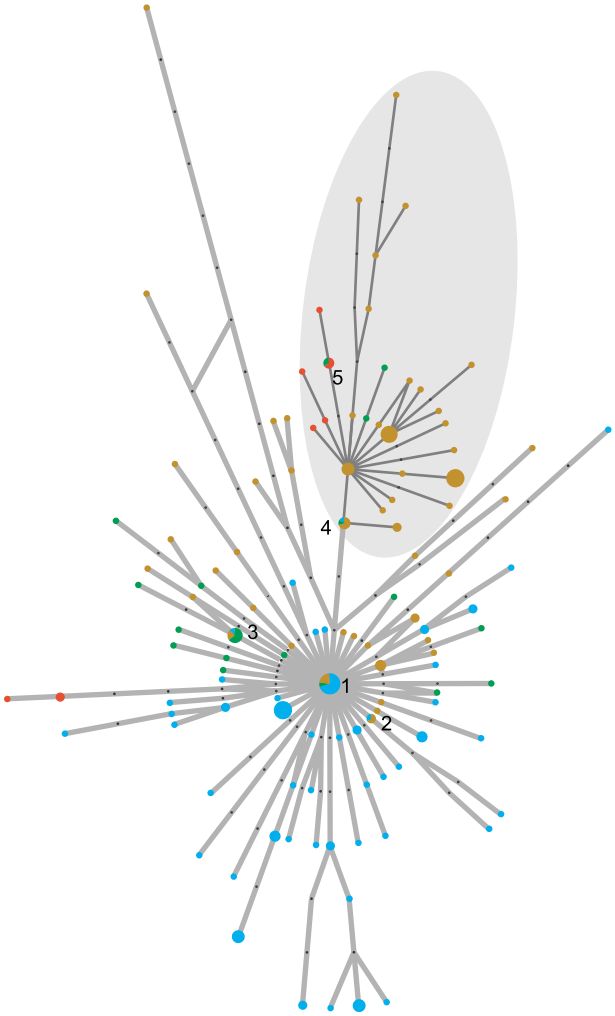

Fig. 4 A haplotype network of the mitochondrial B clade in *Daphnia galeata* based on ND2 nucleotide sequences. Haplotypes are coloured according to geographical region: gold (Europe), green (Siberia), red (Japan) and blue (North America). Grey dots represent inferred haplotypes. The dot size is proportional to the number of different populations with the identical haplotype. The grey region indicates the B1 subclade and numbered haplotypes are shared among multiple regional groups.

also fit the expected distribution of a spatial expansion model (SSD_{OBS} = 0.0029) better than a sudden expansion model (SSD_{OBS} = 0.0047). Estimated *Tau* (based on spatial expansion model) of the A clade in North America was larger than that of the B clade in North America (Table 3). A Mantel test revealed a nonsignificant relationship between genetic and geographical distances among each New World population of the A and B clades.

Because the populations of the Old World consisted of three geographical regions, i.e. Europe, Siberia and Japan, the observed mismatch distributions were obtained from each region. The European B clade fits the expected distribution of a sudden expansion model (SSD_{OBS} = 0.0027) better than a spatial expansion model (SSD_{OBS} = 0.0063). On the contrary, the Siberian B clade fits the expected distribution of a spatial expansion model (SSD_{OBS} = 0.0059) better than a sudden expansion model (SSD_{OBS} = 0.0068). The observed frequency of identical sequences was graphically more similar to the simulated frequency based on the spatial expansion model. The expansion patterns of the Japanese B clade were ambiguous.

Discussion

The genetic evidence indicates that the effects of Quaternary glaciation on *Daphnia galeata* have been pronounced, but complicated. None of the three proposed biogeographical hypotheses (expansion from a single Old World refugium, recent introduction into the New World, or postglacial expansions from multiple refugia) is sufficient to explain the observed genetic diversity. Instead, it is likely that nuclear introgression among distantly related species, anthropogenic introductions, Holocene recolonizations from multiple glacial refugia, and mtDNA introgression among refugial lineages have interacted to produce the present-day genetic diversity in *D. galeata*.

The effect of anthropogenic introductions on population structure

Prior investigators, using allozymes and ITS–RFLP, concluded that European *D. galeata* had been introduced into the lower Laurentian Great Lakes basin via ships' ballast water (e.g. Lake Erie and Onondaga Lake in New York, USA), and subsequently hybridized with New World *D. galeata*. We found that Onondaga Lake contained *D. galeata* with the B1 mtDNA clade — a group that is otherwise restricted to the Old World. Lake Erie also contained *D. galeata* that shared an mtDNA haplotype with European *D. galeata*. So, both lakes with independent evidence of introductions contained mtDNA haplotypes consistent with recent European introduction of *D. galeata* into North America.

But, recently introduced European haplotypes (the second hypothesis in the Introduction) seem limited in North America to the Great Lakes basin. With the exception of Onondaga Lake and Lake Erie, only two other haplotypes are shared with European populations. One haplotype is the ancestral haplotype (the centre of the starlike network; Fig. 4). The other shared haplotype may be explained by independent mutations because the haplotype is just one step from the ancestral haplotype. As well, the erosion of nucleotide and haplotype diversity from west to east in North American Clade B is counter to the expected diversity pattern of the second hypothesis where the Great Lakes are the source. The age of the expansion of the shared B clade between the New World and the Old World also seems too old for an anthropogenic introduction. Using *Tau* = 2ut

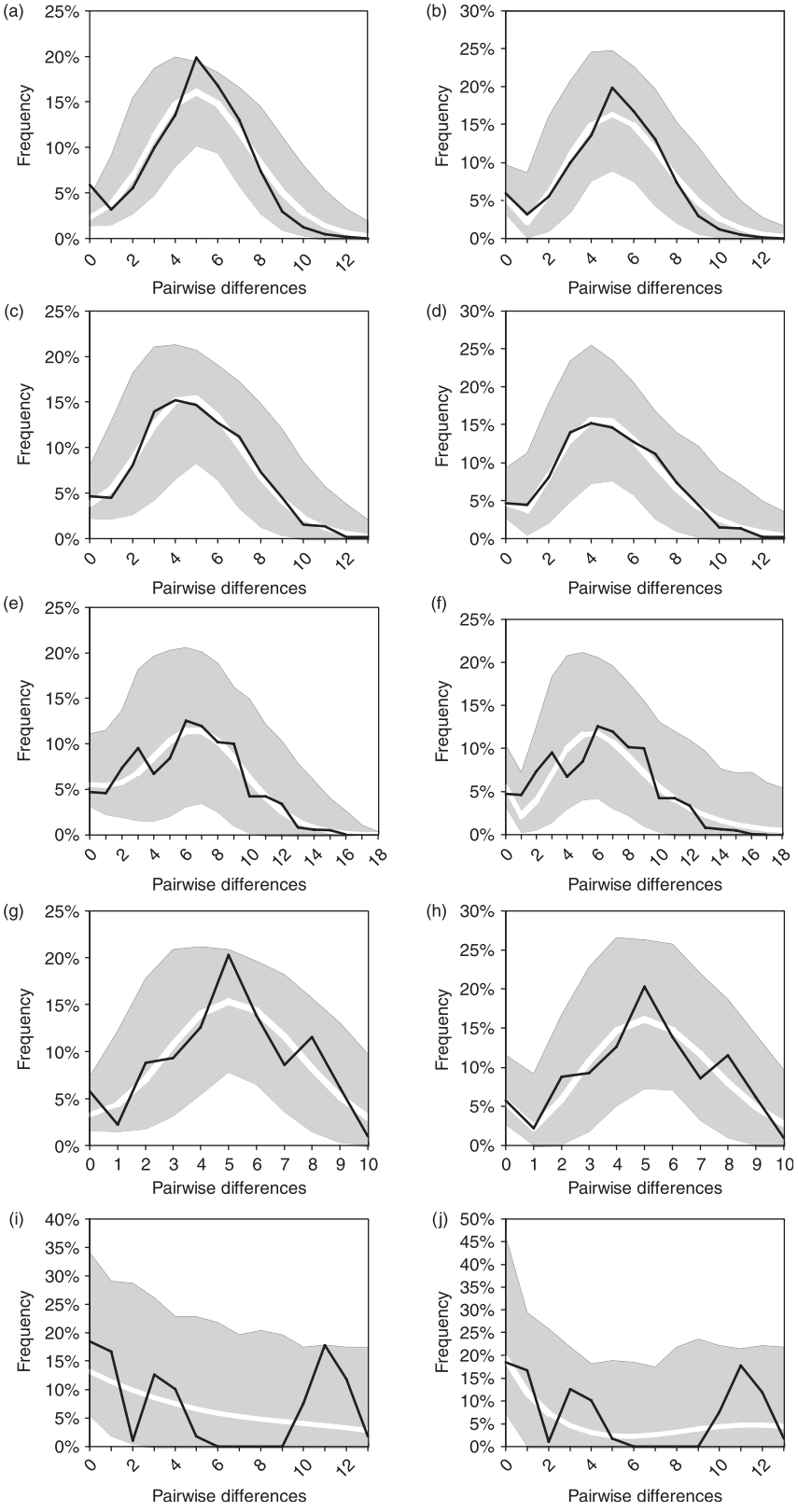

Fig. 5 Results of the mismatch distribution of the mitochondrial A clade in North America (a, b), the mitochondrial B clade in North America (c, d), the B clade in Europe (e, f), the B clade in Siberia (g, h) and the B clade in Japan (i, j). Black lines represent the observed distribution. White lines and the grey zone represent expected distributions and the 95% credible interval of the sudden expansion model (a, c, e, g, i) or the spatial expansion model (b, d, f, h, j).

Table 2 Outputs of the coalescent analysis for the mitochondrial B clades, estimating values of Theta (proportional to effective population size), migration rate from New World to Old World (NW to OW) and from Old World to New World (OW to NW), and exponential growth rates

		Theta		Migration		Growth	
		Old World	New World	NW to OW	OW to NW	Old World	New World
1st	Best value	0.0807	0.0158	205.44	0.01	1121.50	314.91
	95% upper	0.1577	0.0291	351.85	78.61	1410.95	967.50
	95% lower	0.0501	0.0092	106.67	0.00	802.88	–587.29
2nd	Best value	0.1297	0.0246	0.00	206.15	1688.93	396.45
	95% upper	0.3740	0.0402	52.94	362.23	2405.85	631.90
	95% lower	0.0520	0.0158	0.00	103.09	919.27	135.05
3rd	Best value	0.1906	0.0585	0.00	305.80	1150.34	1059.93
	95% upper	0.5136	0.1012	39.07	488.96	1845.53	1575.40
	95% lower	0.0806	0.0356	0.00	175.61	582.87	781.40
4th	Best value	0.0761	0.0605	0.00	157.93	872.10	541.95
	95% upper	0.1988	0.1858	149.49	347.47	1399.69	1073.86
	95% lower	0.0251	0.0306	0.00	80.64	87.69	294.26
5th	Best value	0.0468	0.0304	185.95	0.00	526.26	390.86
	95% upper	0.0844	0.0597	318.60	58.64	771.30	958.05
	95% lower	0.0276	0.0166	96.54	0.00	275.03	–343.54
6th	Best value	0.0313	0.0283	0.00	290.52	3.58	740.40
	95% upper	0.0667	0.0604	42.37	488.45	440.74	1352.67
	95% lower	0.0144	0.0183	0.00	154.95	−630.94	508.62
7th	Best value	0.0622	0.0684	17.40	386.25	466.98	2040.41
	95% upper	0.1932	0.1704	100.20	647.72	888.35	2534.72
	95% lower	0.0263	0.0332	0.00	170.74	−110.00	1643.15
8th	Best value	0.1003	0.0379	0.00	273.38	679.91	1260.23
	95% upper	0.2762	0.0686	132.72	498.33	1276.93	1721.60
	95% lower	0.0281	0.0114	0.00	132.26	−669.09	826.88

Table 3 Estimated parameters of the population expansion of *Daphnia galeata* from the sudden expansion model and the spatial expansion model

	SSD_{OBS} (*P* value)	*Tau* (95% CI)	Theta 0 (95% CI)	Theta 1 (95% interval)
Sudden expansion model				
Clade B in Old World	0.00478 (0.368)	8.47 (4.68–12.12)	0.000 (0.000–2.450)	23.3 (14.6–177.5)
Clade B in New World	0.00072 (0.803)	4.81 (2.77–7.67)	0.574 (0.000–2.391)	28.1 (14.8–718.1)
Clade A in New World	0.00775 (0.000)	5.69 (3.53–7.02)	0.000 (0.000–2.102)	72.4 (27.1–1539.9)
Spatial Expansion Model				
Clade B in Old World	0.00957 (0.098)	5.98 (3.73–9.62)	24.7 (14.6–59.0)	2.153 (0.000–8.544)
Clade B in New World	0.00065 (0.932)	4.02 (2.33–6.76)	23.8 (10.3–123.6)	0.003 (0.000–2.473)
Clade A in New World	0.00570 (0.373)	5.59 (2.99–7.03)	20.0 (10.3–174.0)	1.196 (0.000–4.796)

and assuming a divergence rate of 2.0% per million years for arthropod mitochondrial protein-coding genes (DeSalle *et al*. 1987), the expansion times are estimated to be 150 000 years BP (80 500–189 000 years BP in 95% CI) and 108 000 years BP (62 700–182 000 years BP in 95% CI) for North American populations of the A clade and the B clade, respectively. Still, the estimated expansion times could be overestimated by more than 10 times because of the tendency of mtDNA clocks to overestimate recent divergences (< 0.2 million years BP) (Simon *et al*. 2005). Thus, the spatial expansion of North American populations (A and B clades) might have occurred after the last Ice Age (< 15 000 years BP), but probably not in the past few hundred years of European contact in North America.

Another potential introduction involves the endemic New World Clade A being detected in Nikko National Park (Chuzenji-Ko and Nishi-no-Ko), Japan and nowhere else in the Old World. Some fishes have been recently introduced into Chuzenji-Ko from North America, e.g. *Oncorhynchus mykiss* (rainbow trout), *Salvelinus namaycush* (lake trout) and *Salmo trutta* (brown trout). New World *D. galeata* of the A clade may have been introduced into the lakes with the New World fishes.

Why the potential introductions have failed to spread on both continents remains a mystery, but there are theoretical reasons for containment. Populations may be founded by a few individuals that rapidly multiply and deposit large egg banks. These founders will also rapidly acquire local adaptations that might impart a homefield advantage or monopolization effect (De Meester *et al*. 2002). Effective gene flow among occupied lakes should be rare unless a lake is disturbed or an invading propagule has an unusually fit genotype (e.g. hybrid vigour). Spectacular spread after introduction has been attained by *Daphnia lumholtzi*, *Bythotrephes longimanus*, and *Eubosmina coregoni* in North America, but their invasion sites may be considered unoccupied by similar cladocerans. We note that the extremely high F_{ST}s (0.713) that we observe in *D. galeata* are consistent with the predictions of the monopolization hypothesis.

Postglacial expansion from multiple refugia and mtDNA gene flow

Several results are inconsistent with a single glacial refugium for *D. galeata*. First, nuclear HSP90 haplotypes and older lineages appear to be continent-specific (save a single Japanese individual). One of the endemic North American clades cannot be explained by introgression with *Daphnia dentifera* because it groups basal to Old World *D. galeata*. Second, although one phylogroup was shared for mtDNA, we detected several continent-specific phylogroups (A, B1, C and D). The large regional mtDNA clades (A and B) showed significant expansion patterns in the mismatch distributions that are consistent with expansion from separate refugia. Thus, it is likely that *D. galeata* colonized from at least two refugia.

The locations of the glacial refugia for the widespread clades are unknown. But for *D. galeata* present-day distribution, ecology, and population genetics patterns indicates an Old World and a New World refugium. In the Old World, southern Beringia (and the southern Japanese Archipelago) had a moderate climate during the Quaternary ice ages. Indeed, several species of ancient daphniids are endemic to this region (Ishida *et al*. 2006; Kotov *et al*. 2006), suggesting the existence of long-term freshwater refugia. The oldest detected mtDNA lineages of *D. galeata* are also endemic to Japan. Haplotype and nucleotide diversities were higher in the Old World than in the New World, indicating the origin of the B clade in the Old World. The decreases in diversity statistics from west to east in North America are also consistent with an expansion of the B clade from Beringia. Finally, the centre of the New World B clade contains Alaskan (Beringian) haplotypes, consistent with a Beringian origin. In North America, the most likely region for a refugium is the Atlantic coastal plain. *D. galeata* is rare in natural lakes of all other temperate refugial regions today (Brooks 1957). The dominance of the A clade in eastern North America and the existence of basal haplotypes in Maine, supports the Atlantic refugium hypothesis.

The sharing of the mitochondrial B clade between the New World and the Old World may have resulted from secondary contact and mitochondrial introgression during the Holocene maximum. During each Quaternary ice age, Eastern Siberia and Alaska were united, and the Bering Strait disappeared due to lowered sea levels. Temperate *D. galeata* may have expanded into central Beringia during the Holocene thermal maximum, when the temperature was warmer than at present. Pollen analysis indicates that forests of *Populus* (poplar, aspen) were established *c*. 13 100–11 600 years BP in central Beringia (now in western Siberia) (Kaufman *et al*. 2004). We note that the time of this warming period is consistent with the estimated age of the New World B clade of *c*. 10 800 years (see above). Even though we detected sharing of the mtDNA clade B, significant intercontinental genetic divergence was detected with AMOVA. The among-continent population structure supports restricted gene flow between continents. The pronounced Holarctic genetic structure of sexual *D. galeata* stands in contrast to the pattern found in asexual Holarctic *Daphnia* (Weider *et al*. 1999), where among-continent sharing of mtDNA-RFLP haplotypes was common. It is unknown if this difference reflects a greater capacity for long-distance colonization in asexual lineages or the reduced haplotype resolution for RFLPs compared to DNA sequences.

Widespread New World nuclear introgression: evidence from HSP90 sequences

We detected geographically widespread nuclear introgression in a nuclear protein-coding gene, HSP90 from *D. dentifera* to *D. galeata* in the New World. The unique sharing of the HSP90 clade between New World *D. galeata* and *D. dentifera* is consistent with postglacial introgression of *D. dentifera* alleles into the genome of *D. galeata*. The sharing is widespread in North America, where *D. dentifera* and *D. galeata* both occur, but undetected in Europe where *D. dentifera* is absent. Moreover, ITS nrDNA and allozymes show a very similar geographical pattern of sharing with *D. dentifera*, providing concordant evidence for introgression (Taylor *et al*. 1996, 2005). One apparently unique HSP90 clade to the New World is likely a recombinant with a single breakpoint between introgressed *D. dentifera* alleles and New World *D. galeata* alleles (N1 clade). The possession of several unique HSP90 alleles in the *D. dentifera* clade by New World *D. galeata* may indicate either mutational derivatives since the formation of an introgressed lineage or multiple ongoing introgression events involving alleles that we failed to sample in *D. dentifera*. More geographical and genomic sampling of *D. dentifera* is necessary to rule out ongoing introgression. Still, the presence of introgressed

D. galeata in vast areas of north central and northeast North America, where *D. dentifera* is absent or rare (Brooks 1957), supports the dispersal of introgressants. The most parsimonious explanation of colonization that is consistent with the mtDNA and nDNA evidence is expansion from an Atlantic refugium involving an introgressed form of *D. galeata*. By forming a continent-wide region of lakes free of parental species, Quaternary glaciation may have facilitated the stabilization and spread of an introgressed lineage that resulted from contact between Pliocene-diverged species.

Conclusions

We conclude that the Quaternary glaciations may dramatically contribute to the diversification of vagile, sexually reproducing aquatic invertebrates. We found evidence that much of the Holarctic has been colonized by the postglacial spatial expansion of *Daphnia galeata* from two glacial refugia. There is evidence of secondary contact between the two refugial lineages of mtDNA in the New World from natural dispersal and from anthropogenic introductions. But, neither of the hybridization events resulted in the merging of refugial lineages despite our estimates of recent divergence. The New World lineages appear stabilized because intercontinental gene flow barriers such as the Bering Strait and tundra have emerged, and because priority effects from initial colonization may be strong. We note that nuclear gene sequences are useful in understanding the origins of Holarctic diversity because they provide unique insights about the outcomes of hybridization and, in the present case, they show greater geographical structure than the mtDNA sequences. Here, we found evidence that Quaternary glaciation may have aided in the stabilization and expansion of a nuclear introgressed lineage involving species that originally diverged in the Late Tertiary. Finally, we emphasize that widespread geographical sampling (though challenging) is critical in understanding Holarctic diversity because present day populations may be part of spatial expansions from glacial refugia on separate continents and because humans have unwittingly and cryptically moved aquatic species among continents.

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Supplementary material

The supplementary material is available from
http://www.blackwellpublishing.com/products/journals/suppmat/MEC/MEC3160/MEC3160sm.htm

Table S1 The lists of sampling locations, geographical position, IDs of ND2 and HSP90 sequences, number of individuals analyzed for ND2 sequences, and number of cloned colonies analyzed for HSP90 sequences.

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