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Local human pressures influence gene flow in a hybridizing *Daphnia* species complex

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Abstract

Anthropogenic environmental changes are considered critical drivers of the genetic structure of populations and communities through, for example, the facilitation of introgressive hybridization between syntopic species. However, the mechanisms by which environmental perturbations trigger changes in the genetic structure of populations and communities, such as the processes that determine the directionality of hybridization and patterns of mitochondrial introgression over many generations, remain largely unexplored. In this study, the changes in genetic structure of hybridizing members of the *Daphnia longispina* species complex were reconstructed over the last 100 years for three large temperate lakes under strong anthropogenic pressures via palaeogenetic analyses of resting egg banks. Drastic changes in the genetic structure of the *Daphnia* community, associated with hybridization events between *D. longispina* and *D. galeata* and subsequent introgression, were detected in Lakes Geneva and Bourget. In Lake Bourget, these changes were induced by the successful establishment of *D. galeata* with rising phosphorus levels and reinforced by the sensitivity of *D. longispina* to fish predation pressure. In Lake Geneva, the pattern of hybridization during eutrophication is more likely a function of the original taxonomic composition of the species complex in this lake. Lakes seem to require at least a meso-oligotrophic status to allow *D. galeata* populations to establish and accordingly no *D. galeata* genotypes were found in the egg bank of oligotrophic Lake Annecy. In contrast to the generally assumed pattern of unidirectional hybridization in this species complex, bidirectional hybridization was recorded in Lakes Geneva and Bourget. Our results also demonstrate complex genetic trajectories within this species complex and highlight the irreversibility of changes in the genotypic architecture of populations driven by local human pressures. Finally, we show that extensive hybridization and introgression do not necessarily result in a large and homogenous hybrid swarm.

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Introduction

Species often experience spatio-temporal heterogeneity in their environment. The capacity to adapt to local environmental conditions can be constrained by gene flow among populations, which is controlled by

dispersal (Bohonak & Jenkins, 2003; Farkas *et al.*, 2015). Gene flow is, however, not only a function of dispersal but also of the success of migrants in their new habitats (De Meester *et al.*, 2002). When range expansions of genetically divergent populations result in spatial overlap, hybrid zones may develop following the successful establishment of migrants, contact and cross-fertilization (Barton & Hewitt, 1989). First considered as an exception, hybridization has been shown to be widespread with important but often contrasting evolutionary consequences (e.g. Mallet, 2005; Abbott *et al.*, 2013). Interspecific hybridization and introgression, that is the spread of foreign genetic material into a genome through backcrossing, may contribute to the diversification and adaptation of species (e.g. Arnold, 2006; Arias *et al.*, 2008) or, contrarily, lead to the collapse of existing species, known as speciation reversal (e.g. Taylor *et al.*, 2006; Seehausen *et al.*, 2008; Hasselman *et al.*, 2014). The evolutionary consequences of hybridization and introgression depend on various factors, that is directionality of hybridization and the maintenance as well as the extent of the hybrid zones, which can be affected by the anthropogenic alterations of species habitats (Lamont *et al.*, 2003; Marie *et al.*, 2010; Hasselman *et al.*, 2014). Such considerations call for more attention to the genetic consequences of anthropogenic changes and the mechanisms by which environmental perturbations affect gene flow among populations.

Species-rich complexes that involve (i) relatively young lineages with various degrees of reproductive isolation, (ii) sharp genetic subdivision between populations, (iii) potential for hybridization and introgression and (iv) high dispersal capacity provide good models for studying the evolutionary forces that drive the genetic structure of populations (Schwenk & Spaak, 1995; Delmotte *et al.*, 2002; Bohonak & Jenkins, 2003; Abbott *et al.*, 2013). Such species complexes are found within the genus *Daphnia* spp. Most *Daphnia* spp. are cyclical parthenogens with long periods of asexual reproduction alternating with periods of environmentally induced sexual reproduction (Hebert, 1980), which allows them to avoid deleterious conditions either by escape in space, that is dispersal, or in time, that is diapause (Bilton *et al.*, 2001; Havel & Shurin, 2004).

Within the genus *Daphnia*, members of the *D. longispina* species complex commonly hybridize in many European lakes where species occur in syntopy (Schwenk & Spaak, 1995; Keller *et al.*, 2008). This complex includes at least six species and several cryptic lineages (Petrusek *et al.*, 2008a, 2012), and several of its members are highly abundant in European lakes (Schwenk & Spaak, 1995; Keller *et al.*, 2008). Species in this complex show various degrees of reproductive isolation (Keller *et al.*, 2007) and significant inter- and intraspecific genetic subdivision (Thielsch *et al.*, 2009). Analyses conducted on mitochondrial and allozyme markers suggest that all naturally hybridizing taxa in this complex occur within one clade that has diverged relatively recently, that is less than 6 million years ago (Schwenk, 1993; Taylor *et al.*, 1996). These observations, together with studies on taxa-specific ecological preferences [food quantity (e.g. Weider & Wolf, 1991; Boersma & Vijverberg, 1994), food quality (e.g. Repka, 1996; Seidendorf *et al.*, 2007) and vulnerability to vertebrate and invertebrate predation (e.g. Spaak & Hoekstra, 1995; Spaak & Boersma, 2006)], suggest that current barriers to gene flow between lake populations in this complex are largely prezygotic. These involve ecological-based barriers such as habitat (Petrusek *et al.*, 2008b) and temporal (allochronic) isolation (Jankowski & Straile, 2004). However, post-zygotic barriers, for example differences in the success of sexual reproduction between species and hybrids, have been reported as well (Jankowski & Straile, 2003; Keller & Spaak, 2004; Keller *et al.*, 2007).

Beyond these considerations, this genus has been successfully used to retrace changes in the genetic structure of populations during periods of environmental disturbance (Petrusek *et al.*, 2008b; Brede *et al.*, 2009; Weider *et al.*, 2010). For instance, a field survey conducted on 43 peri-alpine lakes along a longitudinal transect revealed that among members of the *D. longispina* species complex, *D. longispina* (including the form previously known as *D. hyalina*, see Petrusek *et al.*, 2008a) dominated in Swiss oligotrophic lakes north of the Alps whereas *D. galeata* mainly occurred in warm and productive Italian lakes south of the Alps. Hybrids were most abundant in lakes that had experienced a history of high eutrophication, particularly north of the Alps (Keller *et al.*, 2008). It has therefore been proposed (Keller *et al.*, 2008; Brede *et al.*, 2009; Rellstab *et al.*, 2011) and experimentally supported (Spaak *et al.*, 2012) that the presence of *D. galeata* and the occurrence of hybrids in many peri-alpine lakes are mostly driven by human activity and a consequence of the invasion of this species into anthropogenically eutrophied lakes, formerly dominated by *D. longispina*. So far, there are no available data for lakes south-west of the Alps. These lakes are of special interest, however, as they have not only been subjected to eutrophication but are also situated along a migratory corridor used by birds (Muséum National d'Histoire Naturelle, 2003–2013), which likely represents an important route of dispersal for zooplankton resting eggs (Havel & Shurin, 2004).

In most cases, evidence for hybridization is provided using nuclear markers only. Due to contrasting modes of inheritance of biparentally inherited nuclear genes and uniparentally inherited cytoplasmic genes, the joint consideration of nuclear and cytoplasmic data can be useful to characterize hybrid zones in more detail, that is to determine the directionality of hybridization and introgression and underlying evolutionary mechanisms

(e.g. Lamb & Avise, 1986; Toews & Brelsford, 2012). However, the actual factors affecting patterns of hybridization, directionality and introgression over many generations have rarely been included in such studies.

Therefore, a palaeogenetic analysis of the resting egg banks of two members of the *D. longispina* species complex (i.e. *D. galeata* and *D. longispina*), over the last century was performed for three large and deep temperate lakes located south-west of the Alps, that have experienced varying intensities of eutrophication (Alric *et al.*, 2013). This approach provides the opportunity to empirically examine long-term hybridization trends between divergent lineages that occur in syntopy as a consequence of anthropogenic habitat disturbance. The suitability of resting egg banks for our study has been established by previous work showing that although egg banks do not necessarily always reflect the extant pelagic population in the short term (Jankowski & Straile, 2003; Keller & Spaak, 2004), they provide a useful archive to investigate the taxonomic (e.g. Weider *et al.*, 1997; Duffy *et al.*, 2000) and evolutionary changes over the long term (e.g. Hairston *et al.*, 1999; Cousyn *et al.*, 2001; Frisch *et al.*, 2014). The overarching aim of our study was to reconstruct taxa composition and patterns of hybridization and to test whether and to what extent anthropogenic perturbations can affect the genetic structure of populations, the chances of establishment of invaders, and patterns of hybridization in syntopic systems. Temporal population structure and directionality as well as extent of hybridization and introgression are characterized here to provide insights into the evolutionary trajectories of populations.

Material and methods

Study sites

Lakes Geneva, Bourget and Annecy are monomictic lakes situated south-west of the Alps at or close to the border of France and Switzerland (Fig. 1). These three lakes are large (27–582 km^2) and deep (69–310 m) temperate lakes. Due to their geographic vicinity, they have a similar climatic history characterized by an increase in mean annual air temperature by more than 2 °C over the last 100 years (Auer *et al.*, 2007). Human activities in the watershed of these lakes have increased from the early 20th century, triggering dramatic changes in total phosphorus input and total phosphorus concentrations (TP) in the lakes as revealed by previous palaeolimnological reconstructions (Berthon *et al.*, 2013). All three lakes had been oligotrophic at the end of the 19th century but started to receive increasing amounts of phosphorus from the 1940s on. The three lakes reached different levels of maximum eutrophication (Fig. 1). TP abatement measures were undertaken early in Lake Annecy, which therefore never exceeded an oligo-mesotrophic status (diatom-inferred TP (DI-TP) = 13 μg/L in 1969). In Lakes Geneva and Bourget, remediation measures were not undertaken before the late 1970s and an eutrophic status (DI-TP = 74–80 μg/L) had been reached until then. TP abatement measures, comprising the building of a sewage collector (in 1967 around the shore of Lake Annecy), the installation of water treatment plants (early 1970s around Lake Geneva and late 1970s in Lake Bourget), the diversion of treated sewage waters into the Rhône rivers (in 1981 in Lake Bourget) and the ban of P-containing detergents (1986 in Switzerland and 2007 in France), resulted in TP reductions in the lakes. Based on phosphorus concentrations, Lake Annecy is today categorized as oligotrophic (TP < 6 μg/L) whereas the other two lakes are oligo-mesotrophic (TP < 20 μg/L). During this time period, and in accordance with the top-down/bottom-up coupling, fish predation pressure was low under oligotrophic conditions but increased during eutrophication. A reversal of this pattern, that is a decline in fish predation pressure during re-oligotrophication, was only observed in Lake Bourget, whereas fish predation pressure has remained high, or even increased, in the other two lakes (Alric *et al.*, 2013). Different strategies regarding stocking age stages of whitefish (*Coregonus lavaretus*) as well as different fishing size limits for perch (*Perca fluviatilis*) juveniles (YOY) have created varying fish predation pressures between these lakes. In Lake Bourget, local fish management practices (whitefish stocking with juvenile stages and fishing of perch YOY) preserved the top-down/bottom-up coupling. In contrast, in the other two lakes, whitefish stocking with early stages and a perch YOY fishing interdiction disrupted top-down/bottom-up coupling and increased fish predation pressure on zooplankton (Alric *et al.*, 2013).

Coring, chronology and sampling of Daphnia diapausing eggs

Eight sediment cores (4 of 90 mm diameter and 4 of 63 mm diameter) were collected for each lake between 2010 (Lakes Geneva and Bourget) and 2011 (Lake Annecy) from the deepest area of the respective lake (Geneva: N46 26.844°/E6 34.554, ca. 310 m water depth; Bourget: N45 45.334°/E5 51.332°, ca. 145 m water depth; Annecy: N45 52.210°/E6 09.549°, ca. 69 m water depth; Fig. 1) using a quadruple gravity corer (UWITEC, Mondsee, Austria). These coring locations were suitable to collect resting eggs because of known constant sedimentation rates and the great depth protecting ephippia from exposure to hatching stimuli. Sediment cores were stored at 4 °C in the dark until processing. For each lake, dating of sediment cores from which ephippia were extracted was achieved by

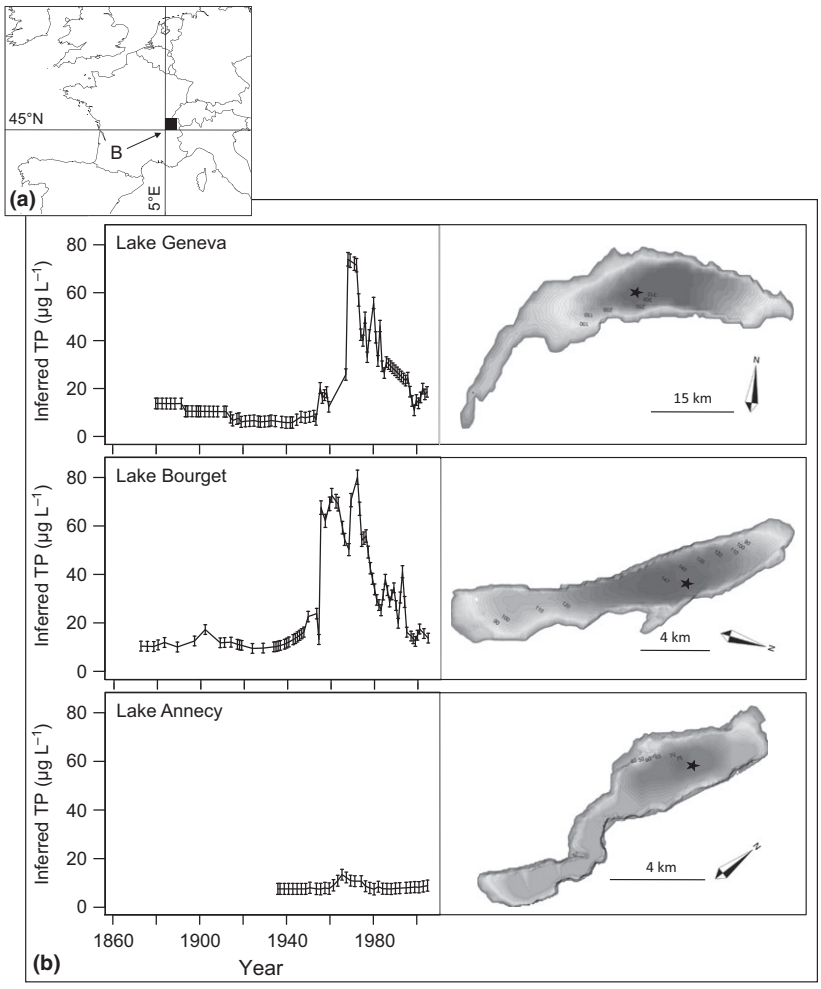

Fig. 1 (a) Location map of Lakes Geneva, Bourget and Annecy. (b) Total phosphorus concentrations (TP) over the last 150 years and bathymetric map of Lake Geneva, Lake Bourget and Lake Annecy. Asterisks indicate coring sites. TP graphs are modified from Alric *et al.* (2013).

correlation to a reference core using lithological tie points. Details on the dating of the reference cores can be found in Alric *et al.* (2013). Briefly, sediment dating was performed using gamma spectrometry (measurement of ^{210}Pb, ^{226}Ra, ^{137}Cs and ^{241}Am activities) at the Modane underground laboratory environmental radioactivity facility. Because of the low density of ephippia in the sediment, several cores (up to 16 half cores for the oldest periods in the three lakes) were sampled simultaneously and different adjacent sediment layers were pooled (covering on average 11–22 years over the three lakes; range: 10–50 years for Lake Geneva, range: 5–23 years for Lake Bourget and range: 8–41 years for lake Annecy). Ephippia were isolated by stirring sediment in distilled water and sieving through a 100-μm mesh size filter.

Genetic analysis

Ephippia were manually flipped open with sterilized dissection needles under a stereo microscope, and eggs were isolated and placed individually into 0.2-mL tubes for DNA extraction using the hot sodium hydroxide and Tris method (HOTShot) according to Montero-Pau *et al.* (2008). Mitochondrial 16S rDNA (mtDNA) and nuclear microsatellite markers were used for genotyping each resting egg. Polymerase chain reactions (PCR) amplification of mtDNA was based on a protocol by Brede *et al.* (2009) using conserved primers (Schwenk *et al.*, 1998). The final synthesis step at 72 °C from the original protocol was excluded. Subsequently, mtDNA PCR products were used for a restriction fragment length polymorphism (RFLP) assay with restriction enzymes recommended by Schwenk *et al.* (1998) and following the technical notes of Brede *et al.* (2009). The mtDNA sequences and restriction patterns differ significantly between *D. galeata* and *D. longispina* (Schwenk *et al.*, 1998), and the mtDNA RFLP assay has successfully been applied in previous studies (Schwenk *et al.*, 1998; Brede *et al.*, 2009). Separation and size identification of digested products were performed using 2.5% NuSieve® 3-1 Agarose gels (Lonza, Basel, Switzerland) and a ϕX 174 DNA/HinfI (Promega, Madison, WI, USA) size marker.

Microsatellite analyses were based on eight polymorphic loci that were developed for the *D. longispina* species complex (Brede *et al.*, 2006): DaB10/14, Dp512, SwiD1, SwiD5, SwiD10, SwiD12, SwiD14, SwiD15. All markers were combined into a single Multiplex PCR performed in 0.2-mL tubes in a total reaction volume of 11.5 μL containing 1.5 mM $MgCl_2$, 1 × PCR reaction buffer, 0.2 mM dNTP, 1 U of HotStart *Taq* DNA Polymerase (Qiagen, Hilden, Germany) and 1 μL of DNA template. The respective forward and reverse primer concentrations were 0.3 μM for SwiD1, SwiD10, Swid15 and DaB10/14, 0.4 μM for Swid5 and SwiD14, 0.5 μM for Dp512, and 0.6 μM for SwiD12. The thermal cycling conditions consisted of 15 min initial denaturation of the DNA template and HotStart *Taq* DNA Polymerase activation at 95 °C, followed by 32 cycles of 0.5 min at 94 °C, 1.5 min at 54 °C and 1 min at 72 °C and a final extension step of 30 min at 60 °C. PCR products were analysed on an ABI 3700 capillary sequencer using a GeneScan500 LIZ size standard (Applied Biosystems, Zug, Switzerland). Allele scoring was performed using GeneMapper 3.7 software (Applied Biosystems). In order to assign genotypes to certain taxa, an additional set of 24 *D. galeata* and 15 *D. longispina* laboratory clones originating from 19 European lakes was used as reference data set (Table S1).

Population genetics analysis

The genetic structure of the populations in each lake was investigated using factorial correspondence analysis (FCA) implemented in Genetix 4.05 (Belkhir *et al.*, 1996–2004) and a nonmodel based clustering method, that is discriminant analysis of principal components (DAPC; Jombart *et al.*, 2010) available in the adegenet package (Jombart, 2008; Jombart & Ahmed, 2011) for R 3.2.2 (R Core Team 2015). As DAPC requires prior groups, we ran the sequential *K*-means clustering algorithm for $K = 1$ to $K = 10$ determined with the implemented *find.clusters* function in the adegenet package. Following the Bayesian information criterion (BIC) approach, *K* for which BIC does not decrease significantly any more is the number of genetic clusters that summarize the data set best (in our case, we found $K = 6$ for Lakes Geneva and Bourget). Prior information (i.e. historical data and FCA results) showed that there are only two parental species (*D. galeata* and *D. longispina*) in these two lakes. Therefore, we focused on the detection of the relative contribution of each parental species to hybridization rather than the detailed population substructure. Only $K = 2$ was further considered for Lakes Geneva and Bourget (but see Figs S1 and S2 for details of the DAPC analysis for $K = 2$ to $K = 6$). DAPC for $K = 2$ was performed, retaining three (41.6% of the total variance, Lake Geneva) and two (36.2% of the total variance, Lake Bourget) principal components (PC) for prior data transformation. These retained PCs, determined following the α-score optimization procedure implemented in the adegenet package, correspond to the number of PCs optimizing the trade-off between power of discrimination and overfitting of the discriminant function based on too many PCs. For Lake Annecy, all resting eggs clustered with *D. longispina* reference clones in the FCA and carried the mitochondrial haplotype of *D. longispina,* and therefore, these data cannot be analysed in the same way as for the other two lakes. However, DAPC ($K = 2$) on a data set including resting eggs plus reference clones was performed to verify that all resting eggs clustered well with *D. longispina* reference clones.

For comparison, a Bayesian model-based clustering method implemented in STRUCTURE 2.3 (Pritchard *et al.*, 2000) was used. According to the ad hoc procedure of Evanno *et al.* (2005) and technical notes of Gilbert *et al.* (2012), the most likely number of genetic clusters was determined following STRUCTURE analyses with 20 runs for each *K* ranging from 1 to 10, using an admixture model, without prior population information and with a burn-in period of 10^5 iterations and 10^5 Markov-Chain-Monte-Carlo iterations for each run. STRUCTURE analyses were also performed for $K = 2$ to $K = 6$ (see Figs S1 and S2 for details of the STRUCTURE analysis). For the same reason as outlined above for the DAPC approach, the STRUCTURE analysis was run for $K = 2$ only for a data set including resting eggs and reference clones for Lake Annecy.

The accurate identification of parental and admixed (i.e. hybrids, backcrosses) individuals in a given sample is sensitive to threshold *q*-values used to assign each individual to a given cluster (Vähä & Primmer, 2006). Therefore, we assessed the power of admixture analyses on simulated genotypes to avoid false classification of parental or admixed individuals. For the simulation analyses, data sets including predefined parental (*D. galeata* and *D. longispina*) and admixed individuals were selected for Lakes Geneva and Bourget. Resting eggs defined as parental individuals had to meet the following requirements, that is cluster with one group of reference clones (*D. galeata* or *D. longispina*) in FCA, STRUCTURE ($K = 2$) and DAPC ($K = 2$) analyses, and carry the corresponding mitochondrial haplotype, whereas individuals defined as admixed had to occupy an intermediate position in the FCA space between parental species as well as have intermediate posterior probability in admixture analyses. DAPC and STRUCTURE analyses for $K = 2$ were performed on these new data sets. Two subsamples of resting eggs of 12 individuals for Lake Geneva and 30 individuals for Lake Bourget with the highest *q*-values for the *D. galeata* cluster (i.e. STRUCTURE analysis, Lake Geneva: q-value > 0.99, Lake Bourget: q-value > 0.98; DAPC, Lake Geneva: q-value > 0.9999, Lake Bourget: q-value > 0.9998) and *D. longispina* cluster (i.e. STRUCTURE analysis, Lake

Geneva: q-value > 0.96, Lake Bourget: q-value > 0.95; DAPC, Lake Geneva: q-value > 0.9996, Lake Bourget: q-value > 0.9999) were used to generate samples of 100 simulated genotypes replicated 10 times for three genotype classes (parental, F1 and F2 hybrids) using the implemented *hybridize* function in the adegenet package. Then, we ran DAPC and STRUCTURE analyses (see above for settings) with the simulated genotypes to estimate the power of admixture analysis and to determine the threshold for genotype class assignments.

Multilocus genotype data including all eight microsatellite loci were analysed to characterize genetic variability within and among populations. Number of alleles, allelic richness and private alleles were estimated for each population using GENALEX v.6.5 (Peakall & Smouse, 2012). To determine how genetic variance was partitioned at various levels of organization a hierarchical analysis of molecular variance (AMOVA) was performed in GENODIVE v.2.0b23 (Meirmans & Van Tienderen, 2004). Pairwise F_{ST} values were calculated as part of the AMOVA output, using the method of Weir & Cockerham (1984), and significance was tested by 9999 permutations. The genetic differentiation measure of Jost, D (Jost, 2008), was also calculated to check the extent to which different and small population sample sizes and low number of microsatellite loci may lead to an underestimation of F_{ST} values (Jost, 2008; Heller & Siegismund, 2009; Meirmans & Hedrick, 2011) using the *diffCalc* function implemented in diveRsity package for R and significance was tested by 9999 bootstrap replicates.

The relatedness among the different populations was assessed using the unweighted pair group method with arithmetic mean (UPGMA) based on Nei's genetic distance (sample size bias corrected; Nei, 1978) calculated by 1000 bootstrap replicates with POPTREE2 (Takezaki *et al.*, 2010). Directionality of hybridization and mitochondrial introgression were assessed through joint consideration of nuclear and cytoplasmic data. Three categories can be distinguished: (i) pure parental species (P_{gal}...*D. galeata* and P_{long}...*D. longispina*; with mitochondrial and microsatellite markers indicating the same species), (ii) hybrids (microsatellite markers indicate admixed ancestry and mitochondrial markers indicate one of each species: H_{gal}...hybrids with mitochondrial haplotype of *D. galeata*; H_{long} ...hybrids with mitochondrial haplotype of *D. longispina*), and (iii) mitochondrially introgressed parental species, that is mitochondrial backcross (microsatellite markers indicate one species but mitochondrial markers the other species: $BP_{l\ (mt-g)}$...*D. longispina*, defined by microsatellite analysis, carrying mtDNA haplotype of *D. galeata*; $BP_{g\ (mt-l)}$...*D. galeata*, defined by microsatellite analysis, carrying mtDNA haplotype of *D. longispina*). The maternal contribution of the parental species (P_{gal} and P_{long}) to hybridization was tested under the null hypothesis that their mitochondrial haplotypes are expected to be represented in the same proportions (50:50) in the hybrid populations, using a Fisher's exact test with the *fisher.test* function implemented in R software. The mixing of genetically divergent populations can generate nonrandom genetic associations (i.e. linkage disequilibria), even between physically unlinked regions (e.g. nuclear and cytoplasmic genes). Therefore, the departure from random cytonuclear associations was tested using the program CNDm (Asmussen & Basten, 1994, 1996; Basten & Asmussen, 1997). Normalized cytonuclear disequilibrium was calculated for allelic and genotypic associations, and statistical significance of deviations was tested by a Monte Carlo approach that approximates Fisher's exact test. The number of batches and observations per batch were set to 100 and 1000, respectively. Nuclear loci were encoded as bi-allelic by pooling all alleles according to their degree of association with one parental species (Fields *et al.*, 2014). For this analysis, alleles defined as being specific for one of the two species had to be diagnostic for one parental species or show a high association with one species and a low association with the other species (< 5% of the total number of alleles for a locus). Four loci (SwiD5, SwiD10, Swid12 and Swid15) fulfilled these criteria and were used for the CNDm analysis. Cytonuclear disequilibrium (CND) has four estimators, D, D_1, D_2 and D_3, where significant positive $D = D_G^A$ indicates a positive association between nuclear alleles (A) and mtDNA (G) from the same parental species, $D_1 = D_G^{AA}$ describes the association between a parental mtDNA and its corresponding nuclear genotype (AA), $D_2 = D_G^{Aa}$ represents the association between a parental mtDNA and an admixed nuclear genotype (Aa), and $D_3 = D_G^{aa}$ is a measure of the association between the mtDNA of a parental species and the nuclear genotype of the other parental species (aa). A significant D_2 value is of special interest because it indicates nonrandom interspecific mating and directionality of interspecific mating, whereas significant positive D_1 together with negative D_3 estimates and nonsignificant D_2 values suggest assortative mating of species and a random contribution to the hybrid pool.

Results

Taxa composition of resting egg banks

The presence of population structure was obvious in the three lakes from the FCA results. The examination of reference clones in the FCA revealed that genotypes of the two parental species (P_{gal} and P_{long}) were well resolved by the first two axes and hybrid genotypes occupied an intermediate position in the FCA space between parental species (Fig. 2). For the STRUCTURE analysis, the simulation study revealed that none of the simulated F1 and F2 hybrids showed a membership probability q-value greater than those of predefined

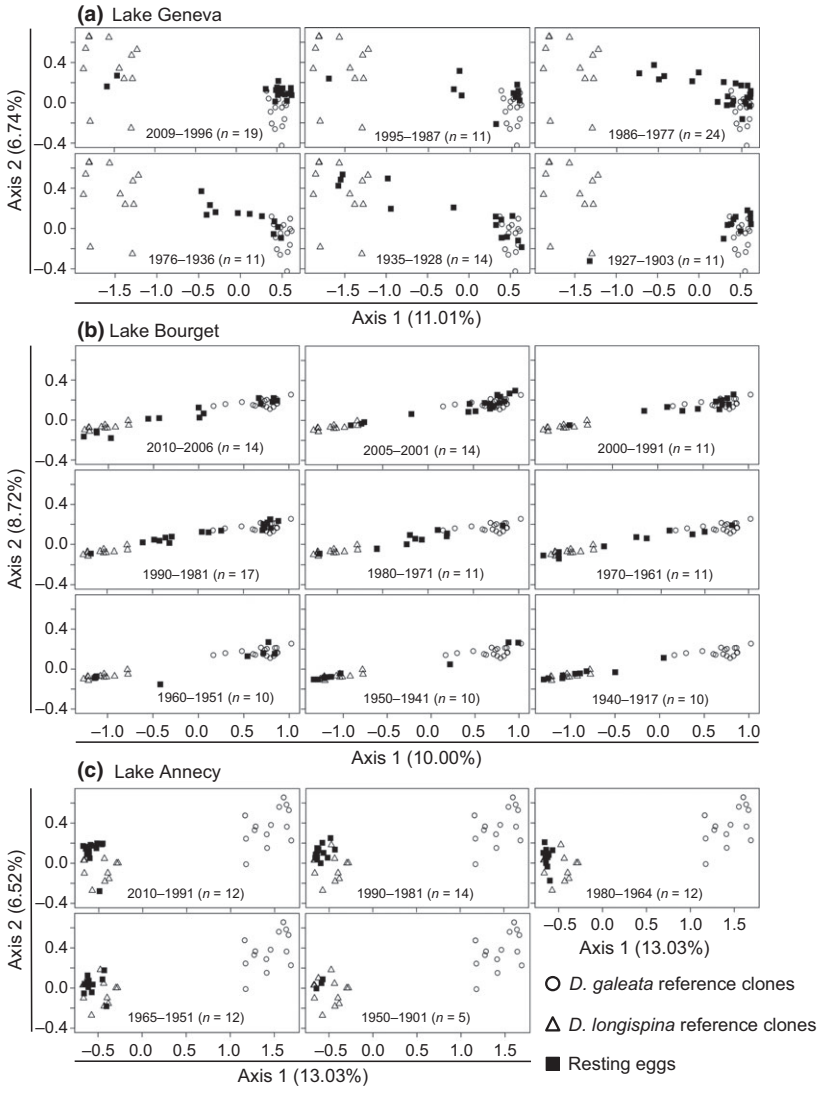

Fig. 2 Factorial correspondence analysis showing the temporal changes in the genotype composition of resting egg banks in (a) Lake Geneva, (b) Lake Bourget and (c) Lake Annecy based on microsatellite analysis. Sample sizes are given in parentheses. Reference clones for *D. galeata* and *D. longispina* are included in the analysis. The first two axes account for 18%, 19% and 20% of the total variation in the genetic composition of the egg banks in Lakes Geneva, Bourget and Annecy, respectively. *D. galeata* and *D. longispina* are separated along axis 1 while axis 2 accounts for the variation within taxa.

parental individuals used to simulate genotypes classes (see Material and methods section for the *q*-value of predefined parental *D. galeata* and *D. longispina* individuals). Consequently, the absence of overlap in *q*-values for the three genotype classes allows to classify each resting egg as parental or hybrid individuals in both Lakes Geneva and Bourget (Table 1). Also for the DAPC approach, reliable ranges of membership probabilities for three genotype classes could be determined in Lakes Geneva and Bourget (Table 1). According to the ranges of membership probability determined by the simulation study, DAPC ($K = 2$) clusters the two parental species regardless of the time period and location of sampling and admixed individuals exhibiting intermediate genotypes represent hybrid genotypes (Figs 3a,b, S1 and S2). Bayesian model-based clustering ($K = 2$) provided congruent results (Figs 3a,b, S1 and S2) with a clear distinction between P_{gal} and P_{long} and admixed individuals. More precisely, 98% and 100% of resting eggs assigned as P_{gal} and P_{long} by DAPC were also assigned to the corresponding groups by STRUCTURE in Lake Geneva. The assignment agreement was 94% for both P_{gal} and P_{long} in Lake Bourget and in Lake Annecy, all resting eggs were assigned as P_{long} by DAPC and STRUCTURE analysis.

Daphnia longispina, identified by microsatellite analysis, exhibited two mitochondrial haplotypes (referred to as *L1* and *L2*) that were detected in all three lakes (Fig. 3). Both parental species, their hybrids and mitochondrial backcrosses, were found in Lakes Geneva and Bourget. Contribution of different taxa to the egg banks during the time yet differed between the two lakes (Pearson's Chi-square test, $\chi^2 = 309.48$, $P = 4.998*10^{-4}$, *P* computed by Monte Carlo test with

Table 1 Mean and range for 10 replicates of the maximum membership probability *q*-values for simulated genotypes classes in both *Daphnia galeata* and *Daphnia longispina* cluster.

		Admixture analysis			
		DAPC		STRUCTURE	
Lake	Class	*D. galeata* cluster	*D. longispina* cluster	*D. galeata* cluster	*D. longispina* cluster
Geneva	F1	0.946 [0.939–0.958]	0.913 [0.903–0.925]	0.703 [0.694–0.712]	0.667 [0.648–0.688]
	F2	0.997 [0.996–0.998]	0.996 [0.995–0.998]	0.853 [0.849–0.857]	0.808 [0.802–0.816]
	Membership probability (*q*-value)*	Parental species: > 0.998 F1, F2 hybrids: < 0.998	Parental species: > 0.998 F1, F2 hybrids: < 0.998	Parental species: > 0.857 F1, F2 hybrids: < 0.857	Parental species: > 0.816 F1, F2 hybrids: < 0.816
Bourget	F1	0.961 [0.954–0.971]	0.962 [0.952–0.972]	0.646 [0.642–0.650]	0.612 [0.605–0.613]
	F2	0.996 [0.995–0.997]	0.9993 [0.9991–0.9997]	0.805 [0.801–0.809]	0.717 [0.693–0.724]
	Membership probability (*q*-value)*	Parental species: > 0.997 F1, F2 hybrids: < 0.997	Parental species: > 0.9997 F1, F2 hybrids: < 0.9997	Parental species: > 0.809 F1, F2 hybrids: < 0.809	Parental species: > 0.724 F1, F2 hybrids: < 0.724

F1 and F2, hybrids; *range of membership probability used to estimate the threshold determination of genotype class assignment in the final DAPC and STRUCTURE analysis.

2000 replicates). The contribution of *L1* to the egg banks decreased steadily in Lake Bourget (from 78% before the early 1940s to 0% in the early 2000s) whereas it disappeared completely from Lake Geneva already in the early 1940s. Mitochondrial haplotype *L2* was mainly recorded during the recent time period in Lake Geneva and only in the early 2000s in Lake Bourget. P_{gal} was present and the most abundant taxon (representing 45–91% of resting eggs) throughout the studied time period in Lake Geneva, whereas it did not appear before the 1940s in Lake Bourget, where it then increased up to a maximum proportion of 73% during the 1990s. Increased numbers of hybrid genotypes in the egg banks were recorded during the eutrophication period with a maximum value of 27% and 54% for Lakes Geneva (range 1935–1976) and Bourget (range 1970–1980) (Fig. 3a,b). Mitochondrial backcrosses were identified even before the eutrophication phase in Lake Bourget and only from the 1930s in Lake Geneva. In contrast, in Lake Annecy, only one taxon (P_{long}) was found for the entire investigated time period. More precisely, haplotypes *L1* and *L2* were found for all studied time periods except for the time period of maximum eutrophication (1960–1970) when only *L1* was present (Fig. 3c).

Population genetic analysis of microsatellite loci

All eight microsatellite loci were polymorphic, with 6 to 11 alleles per locus overall and a range from 3 to 10 alleles per population. Over the three lakes, a total of 67 alleles were detected across eight microsatellite loci, of which 60 alleles were scored in P_{gal} genotypes (three private alleles), 59 alleles in P_{long} genotypes (three private alleles), 56 alleles in hybrid genotypes and 41 alleles in mitochondrial backcross genotypes. Distribution of private alleles in P_{gal} and P_{long} populations yet differed among lakes. One of three private alleles in P_{gal} genotypes was shared between P_{gal} populations of both Lakes Geneva and Bourget (DaB10/14 allele '229') whereas the other two were specific to one lake population (SwiD12 allele '120', SwiD14 allele '190' in Lake Bourget). Two of three private alleles of the P_{long} genotypes were exclusively found in Lake Annecy (SwiD 15 allele '74', SwiD15 allele '94') and the third one in Lake Bourget (SwiD10 allele '200'). Besides, P_{gal} populations in Lakes Geneva and Bourget exhibited temporal changes in their allelic structure with 10 and 18 new alleles, respectively, appearing at low frequencies, during the early and intense eutrophication (Fig. S3). In both Lakes Geneva and Bourget, six of 10 new alleles and 10 of 18 new alleles, respectively, disappeared in the following populations whereas the other four and eight alleles were found in the most recent post-eutrophication egg bank (Fig. S3).

AMOVA revealed that a higher net proportion of the genetic variance was found among populations within lakes than among lakes (16.3% and 11.7%, respectively, Table S2), supporting the genetic structure found in DAPC and STRUCTURE analysis. Pairwise F_{ST} comparisons revealed also genetic differentiation between the different genotype classes (parental species, hybrids and mitochondrial backcrosses) under the three trophic states, with F_{ST} values ranging from 0.089 to 0.416 in Lake Geneva and from 0.070 to 0.436 in Lake Bourget (Table 2). Parental species exhibited higher pairwise F_{ST} values whereas hybrids and mitochondrial backcrosses presented a lower genetic differentiation relative to both parental species. Pairwise Jost's *D* comparisons showed higher values (ranging from 0.041 to 0.713 in Lake Geneva and from 0.049 to 0.761 in Lake Bourget) than F_{ST}, but the pattern of pairwise differentiation was similar between the two estimates (Table 2). Consistent with these observations, the UPGMA dendrogram based on Nei's genetic distance clusters individual genotypes

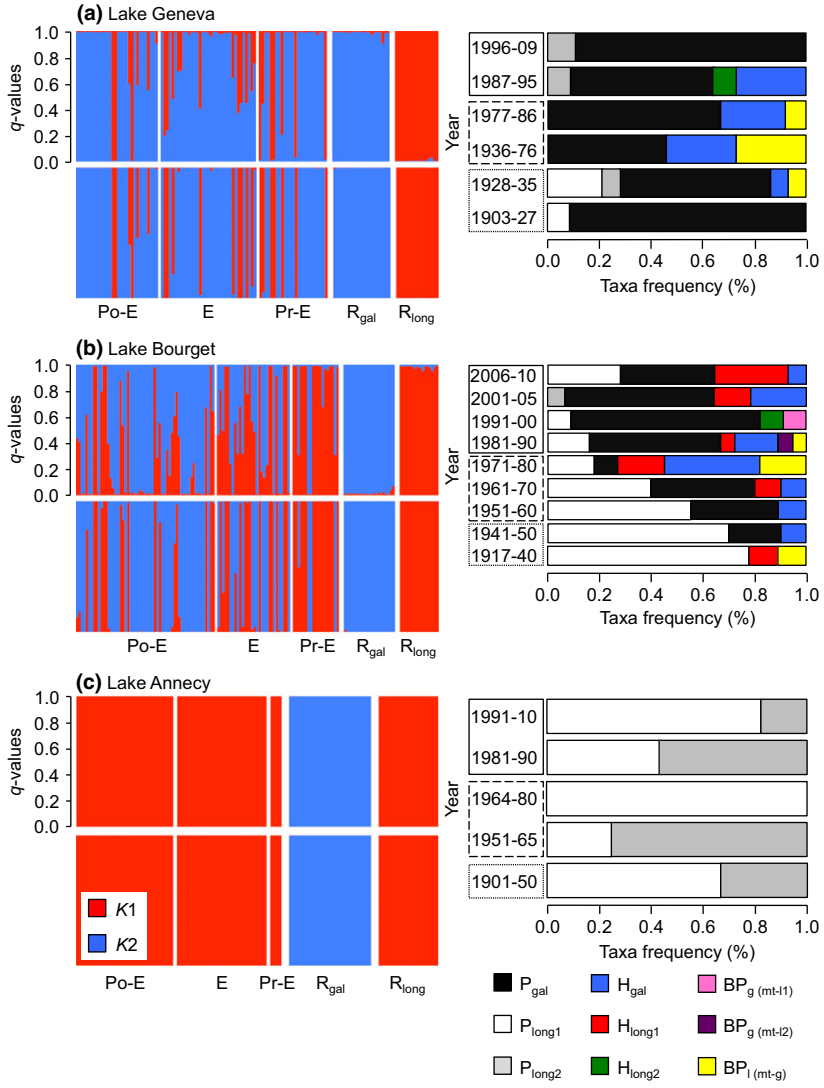

Fig. 3 Genetic structure of resting egg banks identified by RFLP analysis of mtDNA and DAPC and STRUCTURE analysis of eight microsatellite loci in (a) Lake Geneva, (b) Lake Bourget and (c) Lake Annecy. For each lake, the left panel shows bar plots of individual assignment probabilities (*q*-values) to clusters *K*1 and *K*2 as determined, respectively, by STRUCTURE (top graph), and DAPC analysis (bottom graph). Each cluster represents a parental species (*K*1 = *D. longispina*, red; *K*2 = *D. galeata*, blue), and each resting egg or reference clone is represented by a vertical line partitioned into *K* shaded segments corresponding to the probability of belonging to either one of the two clusters. Pr-E. . .pre-eutrophication, E. . .eutrophication; Po-E. . .post-eutrophication. Right panels show the taxonomic composition and directionality of hybridization determined by comparison of microsatellite and mitochondrial markers in relation to trophic status. For microsatellite markers, assignment of resting eggs as parental species or hybrids was based on cut-off values derived from simulations (see Table 1). Parental species according to microsatellite markers and mtDNA: P_{gal} . . .*D. galeata*; $P_{long\ 1}$ and $P_{long\ 2}$. . .*D. longispina* (mitochondrial haplotype *L1* or *L2*); Hybrids partitioned by mitochondrial haplotypes (first name corresponding to mitochondrial/maternal haplotype): H_{gal}. . .*D. galeata* × *D. longispina*; $H_{long\ 1}$ and $H_{long\ 2}$ . . .*D. longispina* (mitochondrial haplotype *L1* or *L2*) × *D. galeata*; Mitochondrial backcrosses: $BP_{l\ (mt-g)}$. . .assigned to *D. longispina* by microsatellite markers with mtDNA from *D. galeata*; $BP_{g\ (mt-l1)}$ and $BP_{g\ (mt-l2)}$. . .assigned to *D. galeata* by microsatellite markers and mtDNA from *D. longispina*; RC_{gal}. . .*D. galeata* reference clones; RC_{long}. . .*D. longispina* reference clones. Boxes correspond to the periods of pre-eutrophication (dotted line), eutrophication (dashed line) and post-eutrophication (solid line).

into two distinct groups that clearly segregate the parental genotypes separated by a genetic distance of more than 0.8 while hybrid and mitochondrial backcross genotypes were distributed over these two clusters (Fig. 4).

Patterns of hybridization and nuclear and mitochondrial introgression

Bidirectional hybridization was detected in the egg banks of both Lakes Geneva and Bourget (Fig. 3a,b).

Table 2 Pairwise genetic differentiation from eight polymorphic microsatellite loci between the four-genotype classes (i.e. the two parental species; their hybrids and mitochondrial backcrosses) for Lakes Geneva and Bourget. F_{ST} (Weir & Cockerham, 1984) and *D* (Jost, 2008) values are given below and above the diagonal, respectively.

Lake	Taxon	P_{gal}	P_{long}	H	BP_{mt}
Geneva	P_{gal}		0.713 (*n* = 3, 23)	$0.032^{n.s}$ (*n* = 4, 23)	NA
			NA	0.095 (*n* = 9, 20)	0.338 (*n* = 5, 20)
			0.551 (*n* = 5, 17)	NA	NA
	P_{long}	0.416 (*n* = 23, 3)		0.289 (*n* = 4, 3)	NA
		NA		NA	NA
		0.363 (*n* = 15, 5)		NA	NA
	H	0.164 (*n* = 23, 4)	0.262 (*n* = 3, 4)		NA
		0.089 (*n* = 20, 9)	NA		0.041 (*n* = 5, 9)
		NA	NA		NA
	BP_{mt}	NA	NA	NA	
		0.255 (*n* = 20, 5)	NA	$0.025^{n.s}$ (*n* = 9, 5)	
		NA	NA	NA	
Bourget	P_{gal}		0.655 (*n* = 9, 30)	0.156 (*n* = 15, 30)	$0.040^{n.s}$ (*n* = 3, 30)
			0.761 (*n* = 8, 11)	0.106 (*n* = 9, 8)	NA
			NA	NA	NA
	P_{long}	0.367 (*n* = 30, 9)		0.199 (*n* = 15, 9)	0.150 (*n* = 3, 9)
		0.436 (*n* = 8, 11)		0.274 (*n* = 9, 11)	NA
		NA		NA	NA
	H	0.168 (*n* = 30, 15)	0.109 (*n* = 9, 15)		0.049 (*n* = 3, 15)
		0.070 (*n* = 8, 9)	0.287 (*n* = 11, 9)		NA
		NA	NA		NA
	BP_{mt}	0.152 (*n* = 30, 3)	0.184 (*n* = 9, 3)	$0.071^{n.s}$ (*n* = 15, 3)	
		NA	NA	NA	
		NA	NA	NA	

For each lake, pairwise F_{ST} and *D* values were calculated for the three time periods (i.e. white, post-eutrophication; light grey, eutrophication; dark grey, pre-eutrophication). Statistical significance was tested for F_{ST} values, using 9999 permutations test and for *D* values, using 9999 bootstrap replicates. All pairwise were statistically significant ($P < 0.05$) except those indicated with $^{n.s}$. P_{gal}...*D. galeata* parental species; P_{long}...*D. longispina* parental species; H...hybrids; BP_{mt}...mitochondrial backcross. *n*...number of resting eggs for taxa in column and row, respectively. NA...nonavailable.

D. galeata was identified as the main maternal species of hybrids in Lake Geneva (94%) whereas the other hybrids exhibited the mitochondrial haplotype *L2* of *D. longispina* (Fisher's exact test, $P = 0.014$, *P* computed by Monte Carlo test with 2,000 replicates). Weaker and nonsignificant asymmetry was found in Lake Bourget, where 54% of hybrids carried the mitochondrial haplotype of *D. galeata* whereas 46% showed the mitochondrial haplotype *L1* or *L2* of *D. longispina* (Fisher's exact test, $P > 0.05$, *P* computed by Monte Carlo test with 2000 replicates). Tests for CND in the two lakes showed significant positive associations between the mitochondrial haplotype of P_{gal} and its corresponding nuclear alleles as well as its corresponding nuclear genotypes (Table 3; coefficient *D* and D_1) and significant negative associations with nuclear genotypes of P_{long} (Table 3; coefficient D_3). In contrast, associations between the mitochondrial haplotype of P_{gal} and admixed nuclear genotypes were nonsignificant (Table 3; coefficient D_2). Mitochondrial introgression was also recorded in the egg banks of Lakes Geneva and Bourget with an asymmetric contribution of parental species to the mitochondrial gene pool of introgressed individuals (Fig. 3a,b). In Lake Geneva, all mitochondrial backcrosses carried the *D. galeata* mitochondrial haplotype. In Lake Bourget, the three mitochondrial haplotypes were present in backcrossed individuals at 66%, 17% and 17%, respectively, for *D. galeata* haplotype, *D. longispina L1* haplotype and *D. longispina L2* haplotype.

Discussion

Temporal patterns of taxonomic composition of Daphnia spp. populations in the three lakes

According to previous studies, the taxonomic composition of *Daphnia* spp. communities in lakes north of the Alps, formerly dominated by *D. longispina*, has changed over time in the course of invasion and successful establishment of *D. galeata* accompanied by hybridization as a consequence of eutrophication (Jankowski & Straile, 2003; Brede *et al.*, 2009; Spaak *et al.*, 2012). Consistent with the hypothesis of successful establishment of *D. galeata* being facilitated by eutrophication, historical data indicated an absence of *D. galeata* in Lake

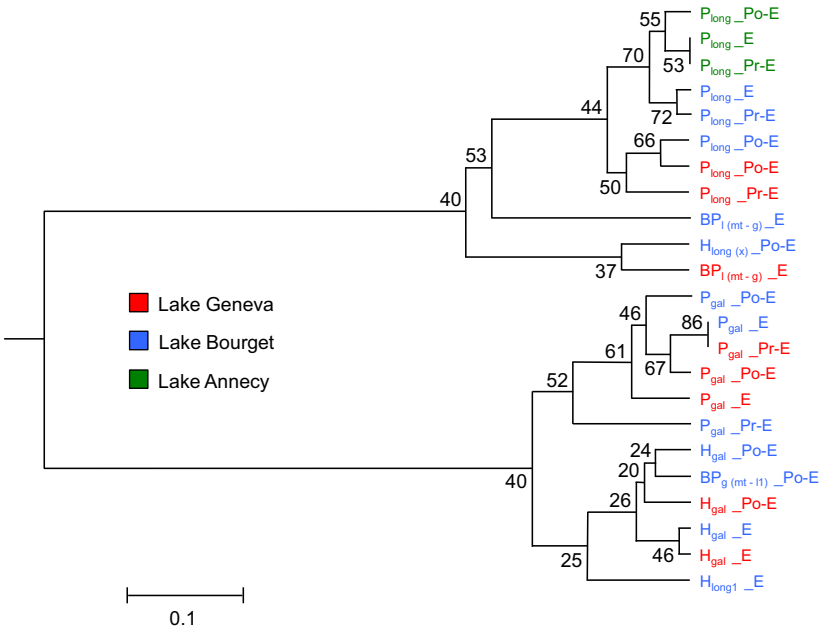

Fig. 4 UPGMA clustering of Nei's genetic distance (sample size bias corrected) calculated from 8 microsatellite loci for the different taxa of *Daphnia* identified by combining DAPC and mtDNA analyses. Taxa abbreviations are given in Fig. 3. The different taxa were pooled for each lake: Lake Geneva (red); Lake Bourget (blue); Lake Annecy (green) in three time periods according to the evolution of TP (Pr-E...pre-eutrophication; E...eutrophication; Po-E...post-eutrophication). Bootstrap support values are indicated for the node where support was greater than 20%.

Table 3 Normalized cytonuclear disequilibria between four microsatellite loci and mitochondrial haplotype from resting egg bank in Lakes Geneva and Bourget. A pooled data set with parental species, hybrids and mitochondrial backcrosses was used for these analyses. CND...cytonuclear disequilibrium. All CND were statistically significant ($P < 0.05$) except those indicated with$^{n.s.}$

		Locus			
Lake	CND	SwiD5	SwiD10	SwiD12	SwiD15
Geneva	D_1	1.0000	1.0000	0.8541	0.7253
	D_2	0.5146$^{n.s}$	−0.0538$^{n.s}$	1.0000$^{n.s}$	1.0000$^{n.s}$
	D_3	−0.8737	−0.8602	−0.8746	−0.7528
	D	0.9274	0.9267	0.8651	0.7398
Bourget	D_1	0.8359	0.8057	0.8948	0.7370
	D_2	0.2471$^{n.s}$	−0.1048$^{n.s}$	0.0580$^{n.s}$	−0.2004$^{n.s}$
	D_3	−0.8118	−0.8401	−0.8546	−0.9200
	D	0.8229	0.8221	0.8739	0.8096

The estimates are shown for *D. galeata*, that is nuclear alleles (A) and mitochondrial haplotype (G) of *D. galeata*. Symmetrical results with opposite sign were obtained for *D. longispina*. Positive D shows a positive association between nuclear alleles and mtDNA from the same species, D_1 is the association between parental mtDNA and its nuclear genotype, D_2 is the association between parental mtDNA and the admixed nuclear genotype, and D_3 is the association between mtDNA of one parental species and the nuclear genotype of the other parental species.

Bourget in the 1920s (Pelosse, 1926) whereas our study revealed the appearance of *D. galeata* from the early 1940s, that is eutrophication period, with subsequent drastic shifts in the taxonomic composition of *Daphnia* spp. as reconstructed from the egg bank (Fig. 3b). In contrast to many lakes north of the Alps (e.g. Jankowski and Straile, 2003; Brede *et al.*, 2009; Rellstab *et al.*, 2011), however, *D. galeata* has been present and even dominant in the egg bank of Lake Geneva even before the onset of eutrophication, suggesting either an earlier invasion or native status (Fig. 3a). Consistently, historical data stated that *D. galeata* has been present in Lake Geneva in the early 1900s (Stingelin, 1908). These results also show that in both lakes increased eutrophication coincided with dominance of *D. galeata* and the presence of hybrids and mitochondrial backcrosses in the egg banks, whereas *D. longispina* genotypes were present in the pre-eutrophication period but became rarer or even absent from the egg banks during the peak of eutrophication.

The observed changes in the contribution of *D. galeata* and *D. longispina* to egg banks have in part been related to changes in the population size of species (Jankowski & Straile, 2003). While *D. galeata* requires more eutrophic conditions to persist, *D. longispina* survives and grows under oligotrophic conditions but performs even better under eutrophic conditions (Spaak *et al.*, 2012) suggesting that factors, other than the direct effects of TP, affect the changes in the taxa's contributions. A more suitable explanation to these changes, in particular the decrease and virtual absence of *D. longispina* during eutrophication period, could instead result from the higher sensitivity of *D. longispina* to fish predation pressure compared to *D. galeata* (Nilsson & Pejler, 1973; Petrusek *et al.*, 2007, 2008b). Indeed, a high top-down control exerted by zooplanktivorous fish on the cladoceran community was

observed during maximum eutrophication (Alric *et al.*, 2013). Our results support such a scenario, because the proportion of *D. longispina* in the egg banks has been increasing again during the re-oligotrophication period, when a reduction of fish predation pressure on *Daphnia* spp. was observed (Alric *et al.*, 2013). Furthermore, a higher proportion of *D. longispina* during re-oligotrophication period was observed in Lake Bourget that exhibited a greater decrease of fish predation pressure than Lake Geneva (Alric *et al.*, 2013).

Another striking pattern was the absence of *D. galeata* genotypes from the egg bank of Lake Annecy (Figs 2c and 3c), which experienced only weak eutrophication (max DI-TP = 13 μg/L in 1969). This observation is in line with studies suggesting that invasion and successful establishment of *D. galeata* is depending on the magnitude of eutrophication, that is in lakes that never exceed the oligo-mesotrophic status, *D. galeata* does not successfully establish, or at least does not persist for long enough to significantly modify the genetic structure of the population (Rellstab *et al.*, 2011; Spaak *et al.*, 2012). Despite the lack of *D. galeata* or hybrid genotypes in the egg bank of Lake Annecy throughout the investigated time period, *D. galeata* mitochondrial haplotypes were recently detected in the pelagic parthenogenetic *Daphnia* spp. population (results not shown). These results highlight the possibility for *D. galeata* gene flow into unproductive lakes such as Lake Annecy. Further work is needed to reveal whether these *D. galeata* haplotypes represent pure *D. galeata*, hybrids or mitochondrial backcrosses and if they persist over the time. Moreover, in contrast to ultra-oligotrophic Lake Brienz where *Daphnia* spp. could not permanently establish before eutrophication (Rellstab *et al.*, 2011), resting eggs were found in sediment samples of Lake Annecy before 1850 despite similar trophic conditions. Therefore, other factors than trophic state, for example food quality, glacial influence, temperature, and fish predation pressure, may modify the establishment probability for *Daphnia* spp. in unproductive lakes. The presence and successful establishment of *Daphnia* spp. at low trophic levels were also supported by post-abdominal claws of *Daphnia* spp. found in sediments from the mid-19th century when all three lakes were still oligotrophic (Alric *et al.*, 2013).

Directionality of hybridization and introgression in the D. longispina species complex

The hybrids detected in Lakes Geneva and Bourget could originate from an invasion of hybrids into both lakes, for example via dispersal of ephippia (Bilton *et al.*, 2001). However, several studies indicated that maintained presence of hybrids in a specific habitat is explained by local production rather than by external production followed by invasion of new habitats (Keller *et al.*, 2008). In addition, hybrids of this species complex show reduced sexual fitness (Keller & Spaak, 2004; Keller *et al.*, 2007), indicating reduced dispersal capacity. The hybrids did not group into a single cluster in the UPGMA tree but were distributed over two distinct clusters that separate the parental species (Fig. 4) supporting the hypothesis of multiple hybridization events and local hybrid production.

The patterns of hybridization also revealed bidirectional maternal contribution in both Lakes Geneva and Bourget, which contrasts with previous studies reporting predominantly cases of unidirectional maternal contribution, that is hybrids between *D. galeata* and *D. longispina* carried *D. galeata* mitochondrial haplotypes (Schwenk, 1993; Brede *et al.*, 2009). The observed bidirectionality of hybridization should, however, be interpreted with caution. Sediment sampling integrates few years up to several decades, and the reported patterns could also be the result of a succession of unidirectional hybridization in different directions rather than simultaneous contribution of both parental species to introgressive hybridization as maternal genotype.

The characterization of cytonuclear disequilibria can provide insights into processes explaining the directionality of hybridization. For example, asymmetric interspecific behaviour, for example females of one species are more attractive for males of the other species than in the opposite case, can affect the directionality of hybridization (Asmussen *et al.*, 1989). Likewise, environmental sex determination in *Daphnia* (Taylor & Hebert, 1993), that is chemical cues during crowding successfully induce male but not female production even among distantly related species leading to disproportionately high male production in the rarer species (Hobæk & Larsson, 1990), could cause such disequilibria. For such instances, significant CNDs involving admixed genotypes (coefficient D_2) are expected, which was, however, not observed for the populations in Lakes Geneva and Bourget (Table 3). The lack of significant D_2 estimates, indicating random hybrid production, is not surprising for Lake Bourget, where mitochondrial haplotypes of both species pooled over time are found in roughly the same proportion in the hybrids (Fig. 3b). This does, however, not exclude the possibility that there was alternating directionality of hybridization over time. In fact, the changing dominance of H_{long} and H_{gal} over time suggests such a scenario but sample sizes were too small to test this hypothesis. For Lake Geneva, we found a strong and significant asymmetry with the vast majority of hybrids carrying the mitochondrial haplotype of P_{gal} (Fig. 3a) and, still, D_2 estimates were, although very high in some cases, statistically nonsignificant. This discrepancy could result from relatively small sample sizes, our pooling of individuals over several decades ignoring changes in allele frequencies over time, or the fact that alleles were not completely fixed for the parental species. Consequently, we cannot rule out directionality for hybridization in Lake Geneva.

The fact that we found significant CNDs for the parental genotypes (Table 3, coefficient D_1 and D_3) indicates that species integrity is maintained in the face of the gene flow. Furthermore, the clear segregation of the two parental species in the FCA, STRUCTURE and DAPC analyses (Figs 2a,b, 3a,b) and the significant differences in pairwise Jost's D and F_{ST} values (Table 2) suggest that in these *Daphnia* spp. populations, mechanisms of reproductive isolation between parental species have been sufficient to prevent complete admixture so far. However, the evidence for historic mitochondrial introgression (Fig. 3a,b) suggests that fertile hybrids are produced, reproduce successfully and contribute to future generations. Patterns of mitochondrial introgression differ between Lakes Geneva and Bourget. The general pattern emerging from recent studies suggests that mitochondrial introgression is generally unidirectional (e.g. Brede *et al.*, 2009; Nevado *et al.*, 2011; Lack *et al.*, 2012). In accordance with this view, unidirectional introgression was found for Lake Geneva; however, bidirectional mitochondrial introgression was recorded in the egg bank of Lake Bourget. It has recently been reported that the directionality of introgression can, in addition to natural selection and post-zygotic barriers, also arise from merely neutral processes (i.e. allele surfing) that are driven by a stochastic demographic events (Currat *et al.*, 2008; Excoffier & Ray, 2008). In the neutral case, asymmetric mitochondrial introgression would be expected from *D. longispina* (native species) into *D. galeata* (invading species), but no such pattern was observed. Alternatively, selective processes, for example selection of a well-adapted mitochondrial haplotype or differences in the fitness of hybrids and their ability to backcross with parental species, could be potential mechanisms driving the directionality of mitochondrial introgression (Borge *et al.*, 2005). Finally, as mitochondrial introgression is a consequence of hybridization, its directionality is in the first place linked to the directionality of hybridization and the underlying driving forces. We found that the patterns of hybridization and introgression are consistent because hybrids carrying the *D. galeata* haplotype are dominating, in particular in Lake Geneva, and mitochondrial introgression from *D. galeata* into *D. longispina* predominates (Fig. 3a,b).

Based on the changes in taxonomic composition of *Daphnia* spp. populations and the patterns of hybridization and introgression, we hypothesize the following scenario for the evolutionary history of *Daphnia* communities of lakes south-west of the Alps. Our results suggest a gradual increase of *D. galeata* in Lake Bourget following a range expansion. At the beginning of the invasion process, *D. galeata* represented the rare species and the rare invaders were more likely to reproduce with the native and abundant *D. longispina* resulting in the presence of hybrid rather than pure *D. galeata* resting eggs. In accordance with a scenario of biased male production in the rare species described above, H_{long} (here the haplotype *L1*) was dominant in the hybrids. The following increase of *D. galeata* abundance and decrease of *D. longispina* favoured conspecific mating as evidenced by the presence of *D. galeata* resting eggs and as a consequence of the dominance of *D. galeata*, H_{gal} increased in the egg bank. The reverse pattern holds for the re-oligotrophication period when the proportion of *D. longispina* in the egg bank increased again. Certainly, further studies will be necessary to corroborate this scenario of biased male production. In Lake Geneva, the directionality of hybridization (with mainly H_{gal}, 94%) is likely best explained by the original taxonomic composition (i.e. a dominance of *D. galeata*) and the sensitivity of *D. longispina* to fish predation pressure. *D. longispina* is presumably more sensitive to fish predation compared with *D. galeata* (Nilsson & Pejler, 1973; Petrusek *et al.*, 2007, 2008b). In particular the *D. longispina* females are, according to the size-selective hypothesis (Brooks & Dodson, 1965), expected to be more strongly affected by predation than the smaller males that, in addition also show a different swimming behaviour reducing the risk of predation (Brewer, 1998; Pietrzak *et al.*, 2010). Together, this may help to maintain the dominance of *D. galeata* females in the population.

Conclusion

Consistent with other studies in the field of community genetics (Vellend & Geber, 2005), our empirical data on Lakes Geneva, Bourget and Annecy showed drastic shifts in the genetic and taxonomic structure of the *D. longispina* species complex as a consequence of environmental-mediated changes in taxonomic composition during eutrophication. The effect of changes in lake trophic status on the genetic trajectories of *Daphnia* spp. populations was, however, different among lakes and depending on the original taxonomic composition. Moreover, indirect effects of eutrophication on taxonomic shifts cannot be excluded, especially in the light of high fish predation pressure sustained by elevated P-levels. Given that environmental perturbation persisted over many generations, modification of genetic structure at the community scale is expected to be irreversible (Gilman & Behm, 2011), because it is highly unlikely that assortative mating, genetic drift, or selection may completely re-establish the original genotypic structure. Therefore, the restoration of habitats may not be sufficient to conserve or restore genetic structure. Due to the high impact of environmental perturbations on the genetic structure of the *Daphnia* spp. community as well as the different ecological niches of parental species and hybrids, introgressive hybridization may affect the role of *Daphnia* spp. in the ecosystem. In order to quantify the effects of these changes on the adaptive landscape, such

palaeogenetic approaches should be combined with experimental work and quantitative trait loci (QTL) studies to identify the ecologically relevant traits and their underlying genetic basis in order to gain a better understanding of the consequences of introgressive hybridization and for the eco-evolutionary dynamics of populations and communities.

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Supporting information

Additional Supporting Information may be found online in the supporting information tab for this article:
Table S1 List of 39 references clones (24 *D. galeata* and 15 *D. longisp*ina) originating from 19 European lakes around the Alps including genotypes based on eight microsatellite markers.
Table S2 Analysis of molecular variance (AMOVA), based on eight polymorphic microsatellite loci, partitioning genetic variation among individuals and populations (see Fig. 3 for populations) of *D. longispina* species complex and among lakes.
Figure S1 Estimated genetic structure of the *Daphnia* populations in Lake Geneva from DAPC and STRUCTURE analysis for $K = 2$ to $K = 6$, based on the eight microsatellite loci.
Figure S2 Estimated genetic structure of the *Daphnia* populations in Lake Bourget from DAPC and STRUCTURE analysis for $K = 2$ to $K = 6$, based on the eight microsatellite loci.
Figure S3 Allele frequencies at eight microsatellite loci through time in *D. galeata* population of Lakes Geneva and Bourget.

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