Local and regional founder effects in lake zooplankton persist after thousands of years despite high dispersal potential

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### Abstract

We reconstructed the genetic structure of a planktonic crustacean *Daphnia longispina* living in high mountain lakes and ponds in the Pyrenees to investigate whether it was shaped by persistent founder effects originating shortly after the last glacial maximum or by ongoing dispersal and effective migration (gene flow). We found that the genetic structure can largely be explained by a single colonization event following gradual deglaciation of the Pyrenees ~10 000–15 000 years ago. Nuclear genetic diversity declined steeply from southeast to northwest, suggestive of serial colonization of avail- able habitats with advancing deglaciation. The spatial genetic structure suggests that founder effects were major determinants of the present-day diversity, both at the catch- ment level and at the level of individual water bodies, further supporting extremely low effective migration rates. This study reveals a prime example of a founder effect that is both long lasting and maintained at small spatial scales. Our data suggest a process of isolation by colonization as a result of strong priority effects and monopoli- zation. We found evidence for the spread of haplotypes with Pyrenean ancestry across the Palaearctic over distances up to 5500 km, although the local genetic structure after colonization was hardly influenced by contemporary dispersal. Finally, our data also suggest that mitochondrial mutation rates in the studied populations were seven times higher than typically assumed. Overall, we show that founder effects can persist for centuries even at small spatial scales at which the potential for dispersal is high.

*Keywords*: alpine lakes, *Daphnia*, dispersal–geneflow paradox, founder effects, monopolization hypothesis, Pyrenees

### Introduction

The relative importance of neutral vs. selective forces in determining local genetic differentiation in natural popu- lations is a major question in contemporary evolutionary ecology, which is subject to much debate (Hey 1999).

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Understanding species distributions and the relevance of environmental factors affecting them, relative to spatial variation, requires an understanding how local environmental factors affect the dynamics of local populations and structure of metapopulations in rela- tion to regional spatial constraints. Local adaptation can result from immigration of preadapted individuals and further spread of the traits in the rest of the population, that is, lineage sorting (Wade 2000), or from standing

genetic variation present in the founding population (Barrett & Schluter 2008; Orsini *et al.* 2013a). Whereas the first mechanism is expected to cause a pattern of genetic isolation by environment for neutral markers [or environmental genetic structuring, see Nosil *et al.* (2008)], the latter is expected to reflect colonization patterns, which may result in a pattern of isolation by distance (cf. concept of isolation by colonization pro- posed by Orsini *et al.* 2013a). These two mechanisms provide additional explanations to population genetic differentiation at the landscape level to the traditional neutralist view of the isolation-by-dispersal limitation, which typically leads to a pattern of isolation by dis- tance (see Orsini *et al.* 2013a). Which of these mecha- nisms is most likely to act in natural populations depends on the rate of effective gene flow into the pop- ulation, the amount of standing genetic variation for the traits under selection and the increase in genetic varia- tion through mutations.

In many species, assumptions about dispersal rates based on ecological insights strongly contrast with geneflow estimates. A good example is freshwater zooplankton, for which studies on the rapid coloniza- tion of new habitats and spread of invasive species suggest a high potential for dispersal (Havel *et al.* 2000; Louette & De Meester 2004; Mergeay *et al.* 2006), but genetic studies show that the realized gene flow is often much lower (Boileau *et al.* 1992; De Meester 1996). This dispersal–geneflow paradox can be explained by foun- der effects (Boileau *et al.* 1992), further enhanced by local adaptation, thereby causing monopolization of resources by local populations (De Meester *et al.* 2002). This process leads to a pattern in which population genetic variation at the landscape level is correlated with the pattern of colonization (isolation by coloniza- tion; Orsini *et al.* 2013a). Insight into the importance of realized gene flow among populations is central to answering whether adaptation occurs through gene flow and lineage sorting, or through standing genetic variation and local adaptation. This information can partly be acquired by studying the distribution of neu- tral genetic variation among populations on different temporal and spatial scales, for example, by combining phylogeography with population genetics at hierarchi- cal spatial scales.

Here, we focus on the genetic diversity and distribu- tion of *Daphnia longispina* (O.F. Mu€ller, 1776), a wide- spread and ecologically plastic Palaearctic species of

freshwater zooplankton (see Petrusek *et al.* 2008), which we studied across lakes and ponds of the eastern Pyre- nees. *Daphnia* are keystone species in freshwater habitats, being grazers of suspended algae and prey for vertebrate and invertebrate zooplanktivores. Although this is certainly not the first study on the genetic

diversity and distribution of *Daphnia* in high mountain ranges (e.g. Aguilera *et al.* 2007; Petrusek *et al.* 2007; Mergeay *et al.* 2008; Hamrov'a *et al.* 2012), it is notewor- thy that comparatively little attention has been paid to

the ecology and evolution of high mountain zooplank- ton compared to lowland populations (Wetzel 2001). This is despite the fact that high mountain regions can be considered important reservoirs of genetic variation, given the often complex response of biota to past climate changes, steep ecological gradients found over small to large spatial scales and resulting complex patterns of glacial refugia (Hewitt 2000).

Like most high mountain ranges, the Pyrenees have a varied spatial structure (Catalan *et al.* 2009). These moun- tains are dotted with ~4000 lakes and ponds of glacial ori- gin, dating from the late Pleistocene (Catalan *et al.* 2006). Deglaciation during the last glacial period occurred markedly earlier in the Pyrenees (15 000–10 000 years ago, in the altitudes where glacial lakes are situated) than in the Alps and northern Europe (Delmas *et al.* 2008), making Pyrenean glacial lakes slightly older than others in Europe. The whole mountain range of the Pyrenees has been defined, based on lake species, as a single ecoregion (Catalan *et al.* 2009), which makes it a suitable area for studying species distribution at the metapopulation level. In addition, the island-like nature of limnetic habitats, and the particular nonhomoge- neous distribution of Pyrenean lakes with various groups of lakes distributed along the mountain range, is expected to create higher opportunities for local genetic differentiation and adaptation to develop. More- over, *D. longispina* occurs throughout the Pyrenees both in very small shallow ponds and in larger deep lakes across different catchments. Such habitats differ enor- mously (e.g. ponds freeze entirely during winter and many dry over the summer), and likely also require very different adaptations, to the point that many speciation events in *Daphnia* have been attributed to shifts from lakes to ponds and vice versa (Lynch 1985; Lynch & Spitze 1994). This study provides an opportu- nity to investigate whether or not shifts from lakes to ponds in the Pyrenees have occurred only once (with subsequent environmental lineage sorting across the region) or whether it has occurred independently at multiple occasions across catchments.

Our general aim was to study the relative importance of environmental vs. spatial structure in determining local genetic structure in Pyrenean high mountain lake populations of *D. longispina*, and to assess which is the most likely mechanism causing population genetic differentiation: isolation-by-dispersal limitation, isolation by adaptation or isolation by colonization. Specifically, we determined genetic diversity and differentiation at a mitochondrial marker (12S rDNA) and at nine nuclear

microsatellite loci. Whereas microsatellites provide information on relatively recent demographic processes, mitochondrial DNA is more suited to reconstruct more ancient patterns of colonization and dispersal (phyloge- ography). From the spatial distribution of haplotypes among waters within and among catchments, we can make inferences about the importance of dispersal as an ecological process at three spatial scales: the whole sampled region (eastern Pyrenees), at the catchment level and at the individual lake level. We then com- bined this genetic information with lake characteristics to analyse whether adaptation to local selection factors occurred and whether they were most likely driven by gene flow (lineage sorting) or by parallel local adaptation. In this way, we can differentiate among isolation-by-dispersal limitation, isolation by adaptation or isolation by colonization (Orsini *et al.* 2013a).

### Materials and methods

#### Study area and sample collection

Zooplankton samples were collected from 25 alpine lakes and ponds in the eastern (Catalan) Pyrenees (Fig. 1 and Table S1, Supporting Information) during the lake ice-free periods from 2005 to 2008. Samples were obtained by vertical net hauls from the surface with a 200-lm net and preserved in absolute ethanol for genetic analysis. We sampled nine different catch- ments and attempted to sample multiple lakes (2–6) per catchment. In two cases, the catchment consisted of a single lake. Often, water bodies within a catchment

were interconnected or were located close to each other (see Table S1, Supporting Information for further details). Such connectivity levels allowed us to calculate expected passive dispersal rates of adults across inter- connected lakes.

For each sampled lake, the following environmental variables were recorded: area, maximum depth, pres- ence of potential predators (fish, *Gammarus lacustris*, *Cyclops abyssorum*), total nitrogen content (TN), total phosphorus content (TP), pH, alkalinity, conductivity, concentrations of major cations (K, N, Mg, Ca), dis- solved organic carbon (DOC) and dissolved inorganic carbon (DIC). The sampling procedures and analytical methods are explained in detail elsewhere (Ventura *et al.* 2000).

#### Genetic analysis

DNA was extracted from 30 to 60 *Daphnia longispina* individuals per population by proteinase K digestion (Schwenk *et al.* 1998) in 100 lL volumes. One microlitre of DNA extract from these samples was used in subse- quent polymerase chain reactions (PCR).

Per population, DNA from ~10 individuals was subjected to DNA sequencing to provide a reasonable coverage of haplotype diversity within and among lakes. We amplified ~540 nucleotides (nt), a long fragment of the mitochondrial 12S rRNA gene (12S) following stan- dard protocols (e.g. Petrusek *et al.* 2008; Appendix S1, Supporting Information). Purified PCR products were sequenced using forward primers on an ABI PRISM 3130 capillary DNA sequencer (Applied Biosystems).

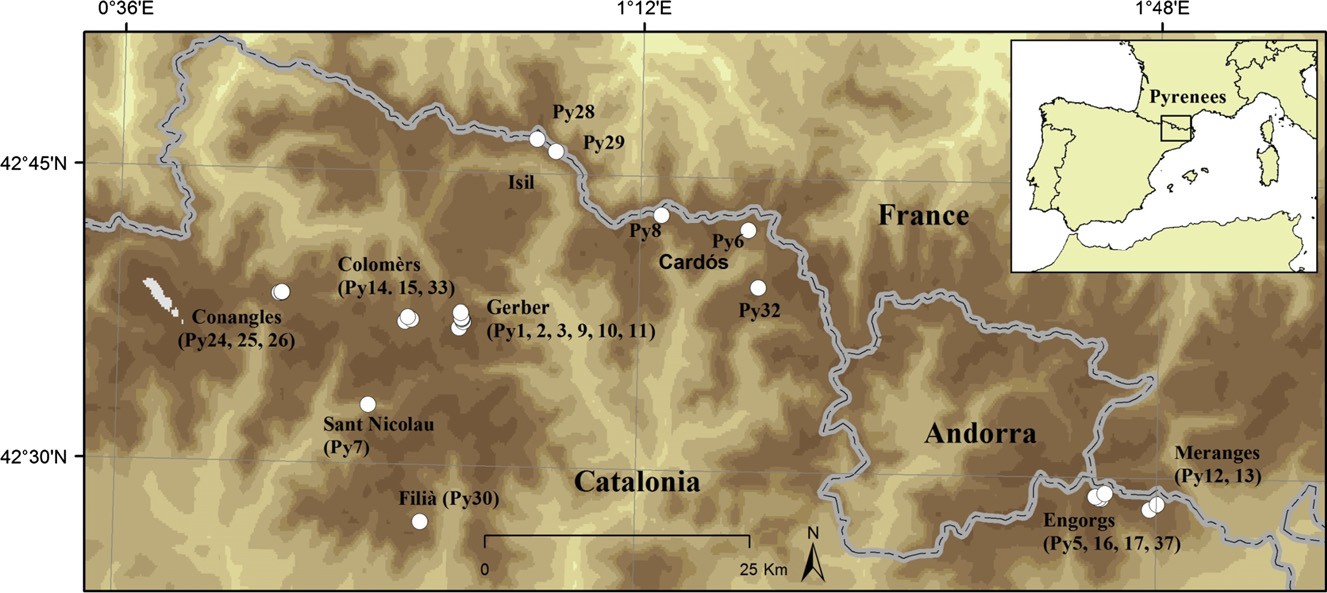


Fig. 1 Distribution of sampling sites along the eastern (Catalan) Pyrenees. White circles represent each of the 25 water bodies sampled; names indicate the different catchments. See Table 1 for the basic lake characteristics.

We failed to amplify sequences from six water bodies due to low template DNA quality and the sequences could not be obtained due to unavailability of other samples at the time of analysis. The resulting 127 sequences of 528 nt length were aligned with 417 sequences of other available *D. longispina* populations from the known species distribution range (Table S2, Supporting Information) using the CLUSTALW algorithm in MEGA 5 (Tamura *et al.* 2011).

Nine microsatellite loci were amplified in a single mul- tiplex PCR in 10 lL volume consisting of 5 lL HotStar *Taq* DNA polymerase buffer (Qiagen, Hilder, Germany),

0.4 lM of each primer for locus SwiD15, 0.3 lM for loci SwiD1, SwiD2, SwiD12, SwiD14, Dpu6 and Dgm109,

0.2 lM for loci SwiD10 and Dp196NB and 0.05 lM for Dp281NB (Brede *et al.* 2006). Cycling conditions were 15 min at 95 °C followed by 30 cycles of 30 s at 94 °C, 90 s at 54 °C and 60 s at 72 °C and a final elongation step at 60 °C for 30 min. Polymorphism was assessed on an ABI PRISM 3130 capillary DNA sequencer, using an internal Liz Gene-scan size standard (Applied Biosys- tems). All primer pairs amplified unambiguous PCR products between 69 and 266 nucleotides long. Tests of data quality were performed using MICRO-CHECKER (Van Oosterhout *et al.* 2004), and all markers passed the qual- ity test with absence of null alleles at all loci.

#### Genetic variation of mitochondrial and nuclear markers and demography inferences

Haplotype and nucleotide diversity levels per lake for mtDNA were calculated using DNASP 5.0 (Rozas *et al.* 2003). We performed a network analysis to estimate gene genealogies using HAPLOVIEWER, which turns trees built from traditional phylogenetic methods into haplo- type genealogies (Salzburger *et al.* 2011). We estimated the phylogeny using a maximum-likelihood method with RaxML Blackbox (Stamatakis 2006), with gamma model of rate heterogeneity (GTR + G) and no invariant sites as suggested by the model. Input data were 12S sequences from each individual, subsequently collapsed into haplotypes. Sequences with ambiguous bases were not included in the analysis. The best tree (using the log-likelihood criterion) was selected for network construction using HAPLOVIEWER.

For microsatellite loci, population genetic parameters such as deviations from Hardy–Weinberg and linkage equilibria, inbreeding coefficients and the number of private alleles were calculated using programs GENEPOP

4.2 (Raymond & Rousset 1995) and GENALEX 6.5 (Peakall

& Smouse 2006). Estimation of clonal diversity and genetic differentiation was based on several population genetic parameters, such as the number and relative abundances of distinct multilocus genotypes (MLG),

Simpson’s diversity index and a standardized measure of allelic richness to a constant sample size (Szpiech *et al.* 2008). We took the minimum number of individu- als analysed in a population as the sample size for all populations (29), to standardize the richness measure.

For mtDNA, we tested the hypothesis of a population expansion event by calculating Tajima’s *D* (Tajima 1989) with 10 000 permutations in ARLEQUIN 3.5 (Excoffier & Lischer 2010). Negative values in neutrally evolving genes suggest deviations from mutation–drift equilib- rium due to population expansion events. Next, we calculated the mismatch distribution among Pyrenean haplotypes (Rogers & Harpending 1992), under the spatial expansion model. The sudden expansion model was not considered for our data set because it is less likely to represent the real situation than a spatial expansion model, given the spatially structured nature of lake populations and the gradual deglaciation of the Pyrenees from SE to NW. The mismatch distribution characterizes the expected number of pairwise differ- ences among sequences given a certain model of popu- lation change. ARLEQUIN 3.5 thus simulates, given the data, the expected pattern under a spatial expansion model and estimates whether or not the actual data sig- nificantly differ from the simulated distributions. This analysis gives the demographic parameter tau, which was used to estimate the timing of the clade expansion (*t*), using the equation s = 2*m*Tl*t*, with *m*T being the number of nucleotides (528) and l the mutation rate of the 12S gene per generation. For high mountain ultraoli- gotrophic lake *Daphnia pulicaria*, one generation per year has been described (Ventura & Catalan 2005), although in north temperate lakes other authors have inferred five generations per year (Costanzo & Taylor 2010). In order to confirm the number of generations per year in our study species, we sampled one of the studied lakes (Lake Llarg; Fig. S2, Supporting Information) with a relatively higher nutrient content (thus being at the upper range within our study sites) during two consec- utive ice-free seasons (2010–2011). The lake was sampled at monthly intervals at the deepest point of the lake by vertical hauls, and lake *Daphnia* were counted and measured following standard procedures (Ventura & Catalan 2005) in order to obtain an estimate of the number of generations.

The experimental quantification of mtDNA mutation rate of *Daphnia* has recently been quantified to be among the highest in eukaryotes [ranging from 14% to 17% per million (M) generations; Xu *et al.* 2012]. This mutation rate is similar to those of other invertebrates such as *Caenorhabditis elegans* (16% M generations; Denver *et al.* 2000), but substantially higher than those estimated for the mitochondrial gene cytochrome *c* oxi- dase subunit I (COI), and used in many studies (2.3%

M generations; Brower 1994) or those used for north temperate *Daphnia* (6.6% M generations; Costanzo & Taylor 2010). 12S could be assumed to be at the lower end of the mutation rates found in mtDNA. This is justified by the comparison with COI, for which a direct comparison of its variation with the 12S rRNA gene in *Daphnia* reveals that at shallow levels of genetic diver- gence, the 12S gene is ~1.7 times less variable than COI (J. Mergeay, unpublished). In *Daphnia* living in environ- ments with high UV stress, mutation rates have been found to be ~2.9 times higher than normal (Hebert *et al.* 2002). All *Daphnia* in the study lakes and ponds are melanized, supporting that assumption that the UV stress is high at these high-altitude habitats (Laurion *et al.* 2000). Since the date of melting of the upper glaciers of the Pyrenees (where the high mountain lakes are located) is known (ranged between 15 000 and 10 000 years ago; Delmas *et al.* 2008), we used this advantage of our study system to test which mutation rate was needed to explain the measured diversification rates, assuming that muta- tions occurred *in situ* (see below). Therefore, we simu- lated different colonization times based on the timing of clade expansion for a range of mutation rates from 1.5% to 24% per million generations and different number of generations (from 1 to 5).

We estimated the number of independent coloniza- tion or dispersal events at three spatial scales (based on the sharing of observed haplotypes or the most parsi- monious ancestral ones deduced from the haplotype network). This was done at the regional level (Pyrenees vs. rest of the species range), at the catchment level within the Pyrenees and at the water body level within a catchment by performing analysis of molecular vari- ance (AMOVA) at these three levels using ARLEQUIN 3.5, separately for mtDNA and nDNA data. For mtDNA, we used the Kimura 2-parameter distance among haplotypes to infer genetic differentiation (*F*ST).

#### Disentangling the drivers of population genetic differentiation

To examine genetic patterns caused by spatial structure that would reflect of isolation by distance, we per- formed a redundancy analysis (RDA) using distance- based Moran eigenvector maps (MEM) as independent variables and the nuclear genetic data as dependent variables (Borcard *et al.* 2004; Dray *et al.* 2006). This approach is much more powerful to detect spatial struc- ture and the scale of it than more commonly used tests such as Mantel tests between pairwise geographical and genetic distances (Legendre & Fortin 2010). We calcu- lated Nei’s genetic distance determined at nine micro- satellite loci in GENALEX between all populations, and this distance matrix was used to calculate principal

coordinates (PCo) of the genetic data in the R package Vegan (Oksanen *et al.* 2010). This approach is preferable to using the allelic frequencies directly as dependent variables, because of the intrinsic dependency of alleles within a locus, but not among loci. The MEM were constructed using the spatial coordinates of each sampled lake or pond. In addition, three evenly spaced dummy points were added between the lake PY32 and the pond PY5 because of a relatively large unsampled area between them. Adding a small number of dummy samples can help to construct better spatial models when sampling designs are irregular (Borcard & Legendre 2002). The MEM were calculated by determining the min- imum-spanning Euclidean distances among all sampling sites and by truncating distances larger than the largest minimum-spanning distance to four times this distance (Borcard *et al.* 2011). This truncated matrix was then sub- jected to a PCo analysis, which yielded five positive axes (MEM variables). They are ordered to represent a gradi- ent from large-scale spatial structure (approximately among catchments and between regions) to small-scale structure (approximately within catchments). From such data, the relative importance of small and large spatial scales in the genetic data can be determined during a for- ward selection procedure in RDA (Borcard *et al.* 2004), by estimating which variables contribute most to the overall explained variance. Adjusted *R*2 values were cal- culated to account for random correlations when the number of explanatory variables is large compared to the number of dependent variables. During the forward selection procedure, we used Blanchet’s procedure to avoid the problem of inflation of the overall type I error and to reduce the risk to incorporate too many variables in the model (Blanchet *et al.* 2008).

In addition, we determined the contribution of envi- ronmental structure to the genetic variation in a similar way to verify to what extent there was isolation by environment. We used a subset of the measured envi- ronmental variables, selected by a principal components analysis of all measured environmental variables. Whenever correlated variables clearly overlapped in the analysis (e.g. Mg-Ca conductivity, lake area and lake depth), a single variable was retained. Hence, 12 vari- ables were kept in the full environmental model: the presence of three predators (*Gammarus*, *Cyclops* and fish), lake size (log-transformed), pH, TP, TN, DIC, DOC, Na, K and conductivity. To disentangle the contributions of space and environment, we partitioned the genetic variance into purely environmental (*E*) and spatial (*S*) components, following the procedure explained in Peres-Neto and Legendre (2010). We first tested whether the overall model of each matrix (*S* or

*E*) was significant. If so, we used forward selection of variables to produce a more parsimonious model. Only

significant variables (*P* < 0.05) were retained in further analyses and for variance partitioning. RDA and variance partitioning were performed according to the R package Vegan. We estimated the proportions of spatially structured genetic variation (*S*), environmen- tally structured variation (*E*), overall structure (*S* + *E*), exclusively spatial (*S*|*E*) and environmental (*E*|*S*) components and their overlap (shared spatial and environmental variance).

Isolation by distance is typically interpreted as the result of additive effects of continuous gene flow, but can also result from serial founder effects (Orsini *et al.* 2013a). Such priority effects along a linear path of colo- nization have been observed in a number of organisms (Ramachandran *et al.* 2005; e.g. Clegg *et al.* 2002; Duvernell *et al.* 2008), but typically on large spatial scales. In the Pyrenees, we hypothesized that a decrease in genetic diversity following the retreat of the Late Pleistocene glaciers (~10 000–15 000 years ago) from ESE to WNW (a surrogate of the age gradient of the lakes) would be in agreement with expectations under a scenario of serial founder effects and thus reflect a case of isolation by colonization. To test for such relation- ship, we performed a linear multiple regression analysis between nuclear gene diversity (*H*e) as dependent variable and longitude and latitude as independent variables in the R package Vegan. We performed a regression analysis with sequential forward variable selection. Lake size is expected to affect gene diversity, as it is a proxy for population size, which in turn affects the equilibrium gene diversity. We therefore included lake size (log-transformed) as a covariable when regressing the geographical variables against *H*e, to esti- mate the contribution of geography to genetic diversity irrespective of lake size.

### Results

#### Genetic diversity

The alignment of 127 Pyrenean sequences of mtDNA contained 64 variable nucleotides of a total of 531. In total, we found 61 haplotypes in the Pyrenean lakes and ponds, all belonging to *Daphnia longispina*. No other *Daphnia* species or cryptic lineages were found in the sampled lakes. The remaining 417 sequences, mostly European but including also samples from Siberia, the Middle East and Africa, had 97 variable nucleotides, with 118 haplotypes. The average nucleotide diversity from the Pyrenees (Pi) was 0.0099, very similar to those from the Old World (Pi = 0.01062; Appendix S1, Sup- porting Information).

With regard to the variation in nuclear DNA, we recorded a total of 132 alleles at nine microsatellite loci,

yielding an average of 14.7 alleles per locus overall (min: 5 at Dp196NB; max: 24 at Dgm109). At the popu- lation level, the average number of alleles per locus was much lower, from 2.3 in Py26 to 6.9 in Py8 (see Table S3 and Appendix S1, Supporting Information for detailed results).

#### Timing of colonization and expansion

The network analysis of the 12S mtDNA (Fig. 2a) shows that no single haplotype was shared between the Pyre- nees and populations analysed elsewhere, although sev- eral European or African haplotypes differed only by a single point mutation from Pyrenean haplotypes. More- over, the haplotype variation indicates that almost all sequences from the Pyrenees likely had a single local ancestor (Fig. 2a). The exception were two related sequences from Py12 for which most closely related haplotypes were found in the Carpathians (Tatra Moun- tains, ~1500 km away) and Russia (2900–5500 km). In reverse, we found non-Pyrenean haplotypes that have the most parsimonious ancestor from within the Pyre-

nees. These include temporary ponds in southern Spain (Zahillo, Sopeto'n and Taraje; all in Don~ana National Park at ~800 km), lakes and ponds in Switzerland

(Arosa, 760 km and St-Bernard, ~560 km), a reservoir in Czechia (Vranov, ~1300 km) and lakes in Slovakia (Dankovo, ~1500 km) and Russia (Glubokoje, ~2900 km and Chany Lake basin, ~5500 km).

Tajima’s *D* yielded a significantly negative value of

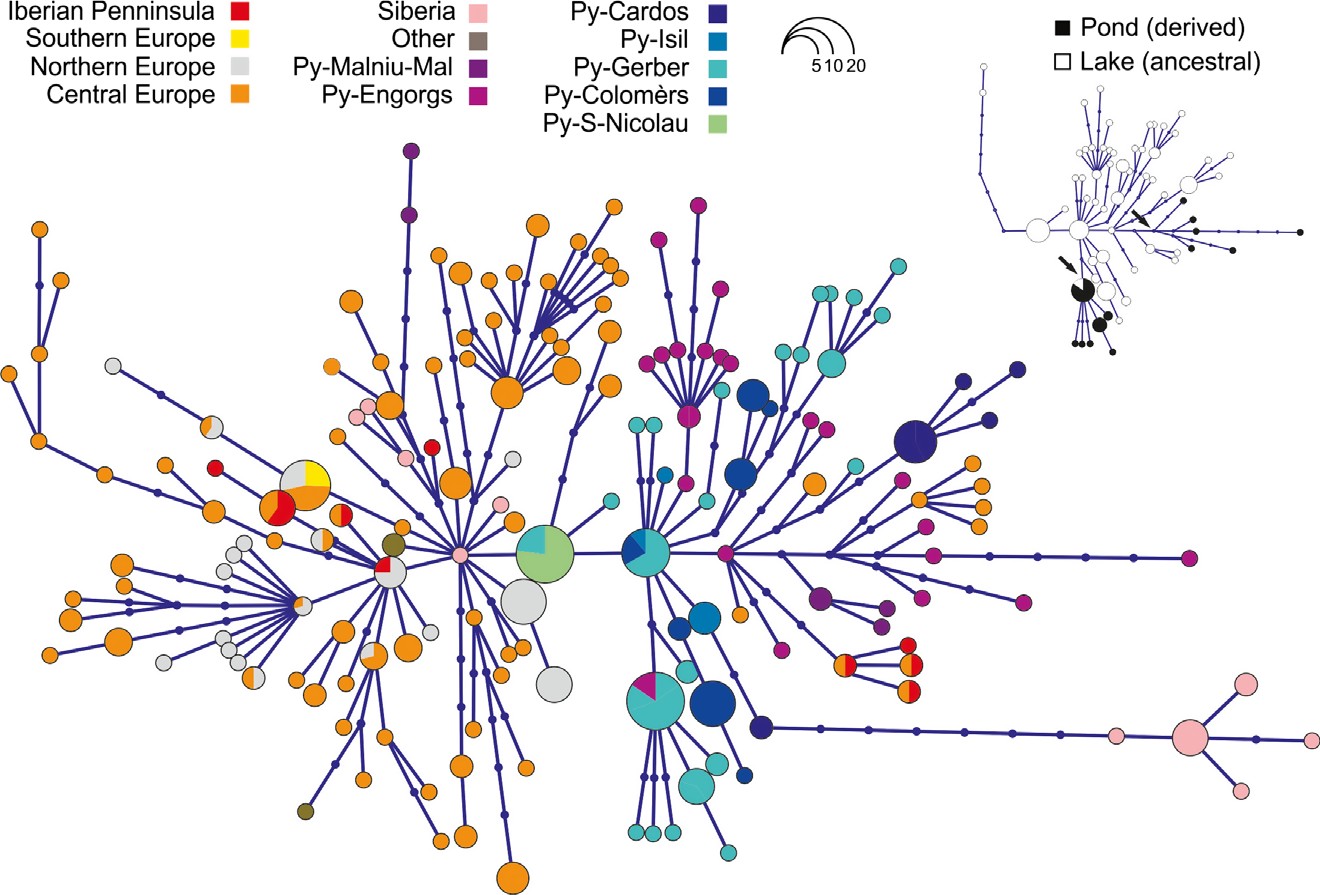
-1.81 (Table 1), suggesting a deviation from mutation– drift equilibrium indicative of a historical popula- tion expansion event. The mismatch distribution was unimodal, evidencing an expansion from a single ances- tral source (Fig. S1, Supporting Information), showing an excellent fit of the observed data to the spatial expansion model. Parameters of the mismatch distribu- tion and the estimated time of divergence are given for all samples, and for the eastern and western halves of the sampling area, in Table 1.

The seasonal study in Py17 showed that most of the individuals of *D. longispina* spend the ice-cover period as resting stages, whereas the number of generations per year did not exceed three (one sexual generation associated with the production of the ephippia, and one or two asexual generations) in the two studied consecu- tive years (Fig. S2, Supporting Information). The simula- tion of the different colonization times as a function of mutation rate at various generations per year revealed that only at mutation rates higher than 8% per million generations the results fit the known postglacial ice-melting period (Fig. 3) and a scenario of a single founding event for most Pyrenean haplotypes (as indi- cated by the haplotype network). Assuming that three

generations per year are typical for the studied lakes, a mutation rate of 14% per million generations (the mini- mum mutation rate measured by Xu *et al.* 2012) yields an estimated time since divergence (and hence of colo- nization of the eastern Pyrenees) of 8000 years ago (4000–15 000 years ago), and the difference of coloniza- tion time between the eastern and western areas was 3400 years ago (Table 1).

#### Dispersal within the Pyrenees inferred from mtDNA

The haplotype network revealed a high degree of endemicity of Pyrenean haplotypes and haplotype groups, and very remarkable patterns of genetic iso- lation despite direct connectivity of some habitats (Fig. S3, Supporting Information). Only six haplotypes were shared between two or more sampled water



**(b)**

**(a)**

Fig. 2 Network of haplotypes of the mitochondrial gene for 12S rRNA. Each circle represents a unique haplotype, and its size is pro- portional to the number of individuals sharing that specific haplotype. Each branch with more than one mutational step is labelled.

1. Pyrenean catchments are indicated by different colours, as well as haplotypes from the rest of the species’ range. The small insert
2. is a simplified network showing whether the haplotype was found in a lake (white circle) or a pond (black circle) or both (mixed); related haplotypes from the same water body or catchment were collapsed into an ancestral haplotype. Shifts from lake to pond or vice versa have occurred at least two times in the Pyrenees (black arrows).

Table 1 Estimated parameters of the mismatch distribution (spatial model for the Pyrenees, demographic expansion model for the individual clades). The mutation rate is set at 11% per million generations, and the number of generations per year is set at 2. CI, confidence interval at the 95% level

|  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| Clade | Tajima’s *D* | *P* | Fu | *P* | Tau est. | *P* | Tau 95% CI | Age (years ago) | Age CI |
| Pyrenees | -1.81 | 0.008 | -25.40 | <0.001 | 3.56 | 0.952 | 1.85–6.48 | 8027 | 4171–14 610 |
| Engorgs–Meranges lakes | -1.53 | 0.05 | -21.41 | <0.001 | 4.24 | 0.117 | 2.87–6.49 | 9560 | 6471–14 543 |
| Colom'ers–Gerber lakes | -1.45 | 0.05 | -16.86 | <0.001 | 2.71 | 0.878 | 1.48–5.20 | 6110 | 3089–11 905 |

Brower no.

Colonisation time (kY)

250

200

Colonisation time (kY)

150

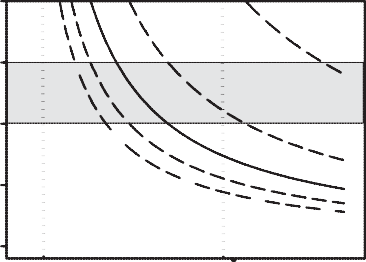
100

50

0

Fig. 3 Modelling of *Daphnia longispina* colonization time as a function of differ- ent mutation rates and number of gener- ations per year given the observed diversity in haplotypes. Each line repre- sents the colonization time calculated for 1–5 generations per year (numbers in the graph); three generations per year are highlighted by a solid line. Grey area marks the period when upper glaciers melted within this area of the Pyrenees (Delmas *et al.* 2008); lines above this area are thus erroneous estimates. Vertical dotted lines indicate the Brower number (Brower 1994) and the experimentally measured *Daphnia* mutation rate (Xu *et al*. 2012). The small panel is a closer view to the period when the ice melt started in the Pyrenees.

0 2 4 6 8 10 12 14 16 18 20 22 24



20

1 15

1

10

2

5 3

4

5

2 0

0 4 8 12 16 20 24

Mutation rate (% M generations)

3

4

5

*Daphnia*

Mutation rate (% M generations)

bodies. In three cases, these were shared between interconnected waters, whereas on the remaining eight occasions these were shared among nonconnected waters (three within catchments, five among different catchments; see Appendix S1, Supporting Information for more details). We found only sparse evidence for multiple colonization/dispersal events per catchment or per lake. The average number of independent colo- nization/dispersal events at the catchment level was

estimated at 1.71 (Gerber: 2; Colom'ers: 2; Cardo's: 2;

Engorgs: 2; Meranges: 2; Isil: 1; Sant Nicolau: 1). At the pond or lake level, this trend was further confirmed, with an average of only 1.1 independent ancestral haplotypes per water body (Table S1, Supporting Infor- mation).

#### Spatial vs. environmental structure

The patterns of nuclear genetic variation revealed by PCo (Fig. 4B) showed a clear separation into two main geographical groups: the most eastern lakes (Meranges, Engorgs and Cardo's) from the rest. Moreover, popula- tions were generally clustered according to their catch- ment. Ponds and lakes from the same catchment were

grouped together, not showing genetic segregation according to habitat type, indicative of independent habitat shifts through local adaptation instead of lineage sorting.

The analysis of molecular variance (AMOVA) revealed a strong genetic structure among catchments, with nearly 30% of the total genetic variation explained by catchment for the microsatellite loci and 21% for the mitochondrial DNA, compared to 10% and 46%, respec-

tively, among populations within catchments; all values were highly significant (*P* < 0.0001; Table 2). When this analysis was repeated with populations grouped according to habitat type (lake vs. pond), only 2.15% (*P* = 0.134) of the diversity was explained by this grouping, compared to 35.6% explained by among-pop- ulation variation within lake/pond groups.

In the redundancy analyses (Table 3), four of five spatial MEM variables were selected in the spatial model (*S*), explaining 67.1% (adjusted *R*2) of the genetic variation, and only the intermediate MEM3 was not retained. After taking covariation with envi- ronmental variables into account, purely spatial varia- tion (*S*|*E*) still explained 47.2% of the genetic variance. Similar to the AMOVA results, small-scale spa- tial variation (MEM4-5) explained a smaller propor- tion of the genetic structure than large-scale spatial variation (MEM1-2). In the environmental RDA matrix, only *Gammarus* and lake area were retained after forward selection, explaining 20.1% of the genetic structure. When space was included as a co- variable matrix (*E*|*S*), no residual variation was explained. Combined (*S* + *E*), the forward selected spatial and environmental variables explained 70.1% of the genetic structure. The spatially structured envi- ronmental variance (shared) amounted to 22.9%, lar- gely reflecting the fact that *Gammarus* only occurs in certain eastern lakes.

In a sequential multiple regression analysis, longitude conditionally explained 60.7% (adjusted *R*2) of the varia- tion in gene diversity (*P* = 0.002), lake size explained 5.0% of the remaining variation (*P* = 0.014) and latitude did not have any significant additional explanatory

**(a)**

*Area*

Py11

*Depth max*

Py8

*Fish*

Py7

*Cyclops*

Py30

1.0

*Conductivity*

*Ca*

*Alcalinity Mg*

Fig. 4 Genetic similarity of *Daphnia* populations and environmental variation of their habitats. (a) A visual representa- tion of the first two PCA axes summariz- ing environmental variables and the position of each water body relative to these variables. Ponds are shown as

squares, and lakes as circles. (b) Position

Py14 Py15

*pH*

of each population characterized by the

Py6

PCA2 (0.242)

Py33 Py10

Py13

*K*

Py12

Py9

Py29

*Na*

Py17

Py37

*DIC*

*Gammarus*

first two axes of a PCo on the genetic

structure based on the nine microsatellite loci.

Py32 Py24-26

Py16

Py28

*Altitude*

Py5

*TN TP*

Py1-3

–1.0

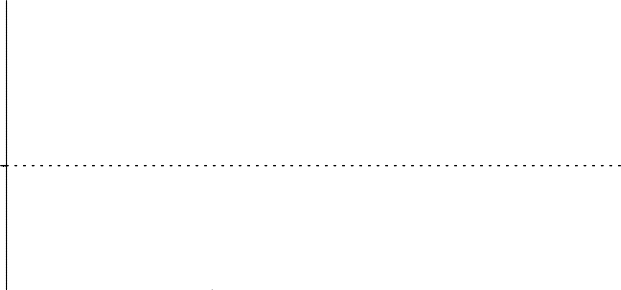
*DOC*

–1.0 1.0

PCA1 (0.287)

**(b)**

Py26 Py25



0.8

Py7

Py30

Py16

Py5

Gerber Colomèrs Filià



Isil

Py11 Py24

PCoA2 (0.093)

Py33 Py15

Py10

Py14

Py17 Py12 Py37

Py6

Py8

Py32

Llebreta Engorgs Meranges

Py1 Py9

Py3 Py2

–0.6

Py29

Py28

Py13

Conangles Cardos

–1.0 2.0

PCoA1 (0.631)

Table 2 Results of the analysis of molecular variance (AMOVA), partitioning the overall genetic variation at nuclear (microsatellites) and mitochondrial DNA (12S) within and among different catchments

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| Source of variation d.f. Sum of squares | | | | Variance components | Percentage of variation | *P* |
| Microsatellites | Among catchments | 6 | 1511.7 | 0.84 | 29.4 | <0.001 |
|  | Among populations | 23 | 480.3 | 0.29 | 10.2 | <0.001 |
|  | within catchments  Within populations | 1962 | 3400.5 | 1.73 | 60.4 | <0.001 |
|  | Total | 1991 | 5392.5 | 2.87 |  |  |
| 12S | Among catchments | 6 | 112.8 | 0.56 | 20.5 | <0.001 |
|  | Among populations | 12 | 109.0 | 1.27 | 46.4 | <0.001 |
|  | within catchments  Within populations | 108 | 97.3 | 0.90 | 33.0 | <0.001 |
|  | Total | 126 | 319.2 | 2.72 |  |  |

power (*P* = 0.552). When accounting for lake size as a covariable, longitude uniquely explained 53.4 % of the variation in gene diversity (*P* = 0.002). Lake size alone

marginally explained 7.8% of gene diversity (*P* = 0.048). A correlation analysis showed that genetic diversity strongly decreased from east to west (Pearson’s

Table 3 Results of the redundancy analysis (RDA) to test for isolation by environment and isolation by distance and for their unique contributions (partial RDA) to the overall genetic structure. Each model first shows the overall model perfor- mance (global model) and next the sum of the performance of the forward selected variables (FS total) and the contribution of each selected variable to the model when significant. *S*, spatial variables; *E*, environmental variables. *S* + *E* summarizes contri- bution of all variables, which can be split to the unique contri- bution of space (*S*|*E*), the unique contribution of environmental variables (*E*|*S*), explained variance that cannot be attributed uniquely to *S* or *E* (shared). The variance not explained by variables included in the model (complement to *S* + *E*) is indicated as unexplained

#### Habitat shifts

Based on the haplotype network (Fig. 2b), we can iden- tify at least two independent habitat shifts from lake to pond in the Pyrenees. They correspond to the two catchments where both lakes and ponds were sampled (Fig. 2b): Py17 (lake) to Py5 (pond), Py9 and Py15 (lakes) to Py1, Py2 and Py3 (ponds). In addition, a dispersal event from pond to lake occurred from Py1-Py3 to Py17. An additional shift also occurred from Py17

(lake) to Zahillo, Sopeto'n and Taraje in southern Spain

(ponds in the Don~ana National Park). Further circum- stantial evidence for independent habitat shifts from lakes to ponds is apparent from Fig. 4. Ecological differ-

RDA model *R*2 *R*2

adj:

*S*

|  |  |  |  |
| --- | --- | --- | --- |
| Global model | 0.748 | 0.681 | 0.0010 |
| FS total | 0.726 | 0.671 | 0.0001 |
| dbMEM1 | 0.470 |  | 0.0001 |
| dbMEM5 | 0.119 |  | 0.0005 |
| dbMEM2 | 0.080 |  | 0.0006 |
| dbMEM4 | 0.057 |  | 0.0005 |
| *E* |  |  |  |
| Global model | 0.650 | 0.30 | 0.0405 |
| FS total | 0.268 | 0.201 | 0.0057 |
| Gammarus | 0.167 |  | 0.0117 |
| Lake area | 0.101 |  | 0.0430 |
| *S* + *E* | 0.776 | 0.701 | 0.0001 |
| *S*|*E* | 0.560 | 0.470 | 0.0001 |
| *E*|*S* | 0.060 | 0 | 0.0014 |
| Shared | 0.156 | 0.229 |  |
| Unexplained | 0.224 | 0.355 |  |

MEM, Moran eigenvector maps.

*r* = 0.802, *P* < 0.0001

0.6

0.4

He

0.2

0.0

*P*

ences among spatially close lakes and ponds are typi-

cally large (Fig. 4b), whereas genetic distances among the same lake–pond pairs are typically small (Fig. 4b).

### Discussion

#### Genetic diversity

The mitochondrial DNA diversity found in *Daphnia longispina* from 19 water bodies of the Catalan Pyrenees (2700 km2) is remarkably high, comparable to the diver- sity found so far in the rest of the studied species’ range. The nucleotide diversity in our samples was similar to that found in other parts of Europe and Sibe- ria (Petrusek *et al.* 2008; Giessler & Englbrecht 2009;

Thielsch *et al.* 2009; Hamrov'a *et al.* 2012; Zuykova *et al.*

2013), and the haplotype diversity in our samples (61 haplotypes of 127 sequences from 19 Pyrenean lakes and ponds) was also of the same order of magnitude as the 118 haplotypes from the 417 sequences from the Old World (Fig. 2a). Moreover, nearly all Pyrenean haplotypes likely originated from a single ancestral hap- lotype and were genetically distinct from most other European haplotypes, except for some lineages appar- ently derived from the Pyrenean clade. This indicates that the diversification that happened within the Pyre- nees is relatively old, likely going back to the early stages of the formation of these lakes (see below). Our results also agree with recent findings of notable differ- entiation of *D. longispina* in Eastern European mountain

lakes (Hamrov'a *et al.* 2012).

0.8 1.0 1.2 1.4 1.6 1.8

Longitude (decimal degrees)

Fig. 5 Plot of eastern Pyrenean *Daphnia longispina* nuclear gene diversity (*H*e) vs. lake or pond longitude. Pearson’s correlation coefficient is given in the figure.

*r* = 0.802, *P* < 0001), as expected under a model of sequential colonization and persistent founder effects (Fig. 5).

#### Colonization history and mutation rates

Colonization of the glacial lakes may have occurred directly from external sources to the eastern side of the studied area after the start of the deglaciation of the Pyrenean cirque lakes (ranging between 15 000 and 10 000 years ago; Delmas *et al.* 2008), with subsequent dispersal of *Daphnia* among the newly available glacial

lakes. Alternatively, the colonization might have been from populations with longer history of regional presence in the Pyrenees at altitudes that had remained unglaciated throughout the Late Pleistocene (Gonzalez- Samperiz *et al.* 2006). However, if the diversification had occurred earlier but at lower elevations, with grad- ual colonization of ponds and lakes higher up the slopes, we would not expect such extreme levels of haplotype endemicity combined with high diversity. It is thus more likely that colonization started only after deglaciation of the upper glacial cirques. Unlike in the Alps, all lowland glacial lakes of the Pyrenees were

likely filled with sediment at least *~*10 000 years ago (Pall'as *et al.* 2006), which likely favoured the subse-

quent dispersal of zooplankton mainly through the upper glacial areas.

Establishing a timing of Pyrenean colonization based on the timing of clade expansion requires a reliable esti- mate of the number of generations per year and of a population mutation rate. The results of the phenology

of *D. longispina* coincide closely with those of the same species from the Tatra Mountains (Hamrov'a *et al.* 2011)

and from other observations in the Pyrenees (M. Ven- tura, personal observation) in that this species has one sexual (resulting in dormant eggs) and one or two asex- ual generations per year (taking place in the plankton). This species spends the ice-covered period as dormant eggs (encased in ephippia), unlike the other typical *Daphnia* species of the Pyrenees, *Daphnia pulicaria*, which stays in the plankton (Ventura & Catalan 2005). Spending the ice-covered period as dormant eggs implies that each ice-free season the population may be inoculated through hatching of a large number of eggs, resulting in a higher potential for genotype coexis- tence. On the contrary, in those species or popula- tions overwintering in plankton, it is more likely that clonal selection combined with asexual reproduction results in a lower genetic diversity over the

years (Hamrov'a *et al.* 2011). The observed high

levels of genetic diversity at nuclear microsatellite DNA loci match those observed in the same species

in other studies (Thielsch *et al.* 2009; Hamrov'a *et al.*

2011) and are likely a result of overwintering as dormant eggs and the low number of generations per year.

All *Daphnia* in the study lakes and ponds are melan- ized, supporting the assumption that the UV stress is high at these high-altitude habitats (Laurion *et al.* 2000). Therefore, we expected that the estimated minimal mutation rate would be similar to the one inferred from other UV-stressed *Daphnia* populations (Hebert *et al.* 2002). Given that we have insufficient knowledge on the actual mutation rate of mtDNA in these UV-stressed systems, but that we have knowledge of the number of

generations per year and the maximum age of the lakes, we estimated the mutation rates needed to have a colo- nization of the lakes later than 15 000 years ago (Fig. 3). The figure shows that, for two to three generations per year, there should have been a mutation rate at least higher than 8–12% per million generations. This value is notably higher than previously used for different types of invertebrates including other *Daphnia* (Brower 1994; Costanzo & Taylor 2010), but is close to those experimentally determined for *Daphnia pulex* (Xu *et al.* 2012). Overall, this adds to the evidence that the use of universal molecular clocks and universal mutation rates over wide ranges of organisms or conditions is not war- ranted (Lynch 2010).

#### Mechanism explaining the genetic differentiation among populations

Our analyses demonstrate a very strong effect of spatial structure on the genetic data, both at small and at large spatial scales, resulting in a pattern of isolation by distance. After taking environmental covariation into account, the spatial model still explained nearly 50% of the total genetic structure in our nuclear genetic data; the contribution of environmental variation to the genetic data was, on the other hand, negligible (Table 3). The main environmental variable contributing to the model was the presence of *Gammarus*, which was strongly spatially structured: only eastern lakes and ponds host this predator. We thus found no support for isolation by environment and thus for lineage sorting. The very strong spatial structure, even across contrast- ing environments, indicates that priority effects are long lasting. Given the age of studied habitats, it seems likely that mechanisms further stabilizing initial priority effects played a role, such as local adaptation and build-up of dormant egg bank suggested by the monop- olization hypothesis (De Meester *et al.* 2002). Combined with the evidence at the mtDNA of a single founder event for the eastern Pyrenees, a subsequent serial foun- der effect in a westward direction and an absence of observed gene flow among water bodies (as indicated by the overall paucity of haplotype sharing among lakes and ponds, even within catchments), this strongly suggests that new habitats are typically colonized from the closest neighbouring ones, after which the popula- tion may adapt to the local environmental conditions. Therefore, the genetic structure of studied populations strongly suggests that the pattern of isolation by distance is the result of a mechanism of isolation by colo- nization combined with a serial colonization (Orsini *et al.* 2013a), rather than of isolation-by-dispersal limitation.

Earlier studies have shown the persistent nature of some founder effects (Boileau *et al.* 1992; Orsini *et al.*

2013b). In our study, we can provide an assessment of their scale. We presume the stepwise colonization of catchments within the Pyrenees by *D. longispina* started

*~*8000 years ago, shortly after deglaciation of the Pyrenees, in a westerly direction, starting from the southernmost parts of the Pyrenees and closest to the climate-buffering influence of the Mediterranean Sea. We thus provide a clear example of priority effects lasting in time (>8000 years old) and occurring at a regional scale (<90 km, the spatial extent of our study) and provide empirical evidence for the strength of the dispersal–geneflow paradox in zooplankton.

#### Long-distance dispersal

Dispersal is a crucial process for high mountain lake zooplankters, but the previous section indicates that it is most relevant for the colonization phase of water bodies. We found some evidence for dispersal among catchments within the Pyrenees probably representing true gene flow instead of colonization (e.g. a haplo- type of Py10 rooted in haplotypes endemic to the Engorgs catchment and a haplotype from the Gerber catchment that dispersed to the Engorgs catchment; Fig. S3, Supporting Information), but these are rela- tively rare events. In addition, we found evidence of multiple ‘ancient’ dispersal events in *D. longispina* over thousands of kilometres (which may have occurred in a stepping stone pattern), ending up to almost 5000 km. This contrasts with the much more limited inferred dispersal distances of Ishida and Taylor (2007) on this species (called European *Daphnia rosea*

s.l. in their study).

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M.V. and J.M. designed the research. M.V., D.B. and

A.M. performed the sampling. M.V., E.H. and J.M. per- formed the genetic analyses. M.V. and J.M. analysed the data. All authors contributed to writing and revising the paper.

### Data accessibility

New mtDNA 12S sequences obtained or used in this study are deposited in GenBank, AN: KF977622– KF977696; KJ024375.

Genotypes for microsatellite, as well as a fasta file containing the alignment of the new 12S mtDNA sequences are deposited in the DRYAD databank: doi: 10.5061/dryad.2dh5n

Location and environmental characteristics of studied habitats, descriptive statistics of genetic diversity of microsatellites loci and complementary data analyses and additional results are uploaded as online supple- mentary material.

### Supporting information

Additional supporting information may be found in the online version of this article.

Table S1 Overview of the sampled water bodies, their mor- phological characteristics, location and connectivity to others in the catchment.

Table S2 List of Accession nos from this study and those from the western Palaearctic retrieved from GenBank.

Table S3 Descriptive statistics of genetic diversity at nDNA, measured at nine microsatellite loci.

Fig. S1 Evidence for a spatially structured demographic expan- sion.

Fig. S2 Changes in *Daphnia longispina* abundance along the ice-free periods of 2010 and 2011 in Lake Llarg (upper panels) and their body size distribution.

Fig. S3 Haplotype network for variation at the mitochondrial 12S rDNA gene.

Appendix S1 Extended methods on genetic analysis and extended results on genetic diversity and dispersal inferred from mtDNA.

## Local and regional founder effects in lake zooplankton persist after thousands of years despite high dispersal potential

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5. Research Institute for Nature and Forest, Gaverstraat 4, B-9500 Geraardsbergen, Belgium Keywords: Founder effects, *Daphnia*, alpine lakes, Pyrenees, monopolisation hypothesis, dispersal- gene flow paradox

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Running Title: Persistent founder effects in zooplankton Type of Article: Original Articles

**Supporting information**

**Table S1.** Overview of the sampled water bodies, their morphological characteristics, location and connectivity to others in the catchment. The number of external haplotypes is a proxy for the number of independent colonization events for each water body.

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| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| Code | Name | Catchment/region | Sampling | Lake (L)/ Pond (P) | Fish | Maximum depth | Lake area | Altitude | Longitude | Latitude | Group | N° external haplotypes | Connectivity |
|  |  |  | year |  |  | m | ha | m | Dec deg. | Dec deg. |  |  |  |
| Py1 Bassa Llong Gerber 3 Gerber 2008 P N 0.6 0.48 2319 0.9966 42.6253  Py2 Bassa Llong Gerber 5 Gerber 2008 P N 0.1 0.01 2325 0.9959 42.6263  Py3 Bassa Llong Gerber 6 Gerber 2008 P N 0.2 0.02 2321 0.9965 42.6261  Py5 Bassa engorgs Engorgs 2007, 2008 P N 1 0.07 2547 1.7358 42.4813  Py6 Mariola Cardós 2008 L N 46 17.80 2276 1.2243 42.7174  Py7 Llebreta St. Nicolau 2008 L Y 11.5 8.00 1620 0.8903 42.5508  Py8 Romedo de dalt Cardós 2008 L Y 40 11.88 2110 1.3247 42.7060  Py9 Redo de Gerber Gerber 2008 L N 17.9 2.18 2339 0.9980 42.6231  Py10 Illa Gerber 2007, 2008 L N 18 2.07 2452 0.9935 42.6184  Py11 Gerber Gerber 2008 L Y 63 14.88 2170 0.9947 42.6307  Py12 Malniu Meranges 2008 L Y 13.3 5.46 2250 1.7924 42.4738  Py13 Mal Meranges 2008 L Y 3.4 3.69 2260 1.8012 42.4782  Py14 Plan Colomèrs 2006, 2008 L Y 13.5 4.95 2188 0.9307 42.6225  Py15 Manhera Colomèrs 2006, 2008 L Y 9.7 1.92 2188 0.9379 42.6243  Py16 Llarg Engorgs 2007, 2008 L N 2 1.81 2490 1.7412 42.4870  Py17 Aparellats de dalt Engorgs 2007, 2008 L N 4.4 0.77 2550 1.7342 42.4826 | | | | | | | | | | | NW | 1 | <150 m from Py2, Py3 |
| NW | 1 | Headwater |
| NW | 2 | Water from Py2 |
| SE | 1 | Water from Py17 |
| Central | 1 | c. 8 km from Py8 |
| SW | 1 | c. 6 km from Py33 |
| Central | 1 | c. 8 km from Py6 |
| NW | 1 | Water from Py10 |
| NW | 1 | Headwater |
| NW | 1 | Water from Py9, Py3 and Py1 |
| SE  SE | 2  1 | May have a connection to Py13, 700 m distance, no ridge  May have a connection to Py12, 700 m |
| NW | 1 | distance, no ridge  500 m from Py15, ridge in between |
| NW | 1 | 500 m from Py14, ridge in between |
| SE | 1 | Water from Py17 and occasionally Py5 |
| SE | 1 | Water from Py37 |

SW NA water from Py25

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|  |  |  |  |  |  |  |  |  |  |  |
| Py24 | Bassa baix Redon | Conangles | 2005 | P | N | 2 | 0.16 | 2345 | 0.7851 | 42.6439 |
| Py25 | Bassa mig Redon | Conangles | 2005 | P | N | 0.8 | 0.07 | 2380 | 0.7876 | 42.6442 |
| Py26 | Bassa petita Redon | Conangles | 2005 | P | N | 0.4 | 0.01 | 2380 | 0.7880 | 42.6441 |
| Py28 | Port d'Aulà | Isil | 2006 | L | N | 3 | 0.56 | 2130 | 1.1005 | 42.7695 |
| Py29 | Clavera | Isil | 2006 | L | N | 4 | 0.48 | 2230 | 1.0783 | 42.7769 |
| Py30 | Filia | Filià | 2006 | L | Y | 5.5 | 1.37 | 2140 | 0.9533 | 42.4512 |
| Py32 | Becero | Cardós | 2006 | L | N | 5 | 0.62 | 2270 | 1.3374 | 42.6539 |
| Py33 | Clòto de Naut | Colomèrs | 2006 | L | Y | 8 | 0.91 | 2330 | 0.9372 | 42.5045 |
| Py37 | Minyons | Engorgs | 2008 | L | N | 1.9 | 0.93 | 2580 | 1.7305 | 42.4838 |

SW NA 20 m from Py26

SW NA 20 m from Py25

NW 1 Separated from Py29 by a small ridge NW NA Separated from Py28 by a small ridge SW NA c. 6 km from Py33

Central 1 c. 5.3 km from Py8

NW 1 Water from Py15 and Py14 SE NA Water to Py17

**Table S2.** List of accession numbers from this study and those from other parts of *D. longispina* range retrieved from GeneBank. Sources abbreviations are T96: (Taylor *et al.* 1996) ; P07: (Petrusek *et al.* 2007) ;P08: (Petrusek et al. 2008) ; T09: (Thielsch *et al.* 2009) ; GE09: (Giessler & Englbrecht 2009)

;H12: (Hamrová *et al.* 2012); Z13: (Zuykova *et al.* 2013).

|  |  |  |  |
| --- | --- | --- | --- |
| **Name**  Bassa Llong Gerber 3 | **country**  Spain | **Source**  This study | **Accession No.**  KF977622, KF977623, KF977624 |
| Bassa Llong Gerber 5 Bassa Llong Gerber 6 Bassa engorgs Mariola  Llebreta Romedo de dalt Redo de Gerber  Illa Gerber Malniu Mal Plan  Manhera  Llarg d'Engorgs  Aparellats de dalt Port d'Aulà Becero | Spain Spain Spain Spain Spain Spain Spain Spain  Spain Spain Spain Spain Spain Spain Spain  Spain Spain | This study This study This study This study This study This study This study This study  This study This study This study This study This study This study This study  This study This study | KF977622, KF977623, KF977625  KF977622, KF977623, KF977626, KF977627, KF977628, KF977629 KF977638, KF977639, KF977640, KF977641, KF977642, KF977643 KF977644, KF977645, KF977646  KF977647  KF977644, KF977648  KF977649, KF977650, KF977651  KF977652, KF977653, KF977654, KF977655, KF977656, KF977657, KF977658, KF977659, KF977660  KF977647, KF977661, KF977662, KF977663 KF977664, KF977665, KF977666  KF977667, KF977668  KF977669, KF977670, KF977671 KF977649, KF977672, KF977673  KF977633, KF977674, KF977675, KF977676  KF977622, KF977630, KF977631, KF977632, KF977633, KF977634, KF977635, KF977636, KF977637, KF977677, KF977678  KF977649, KF977679, KF977680 KF977681 |
| Jezerce "7", Prokletije Mountains | Albania | H12 | JX134347 |
| Ligeni i Dashit, Prokletije Mountains  Mondsee | Albania  Austria | H12  P08, T09 | JX134344  EF375827, FJ178322 |
| Jugovo, Zelengora Mountains Veliko, Treskavica Mountains  Ribno Banderishko, Pirin Moutains | Bosnia and Herzegovina Bosnia and Herzegovina Bulgaria | H12 H12  H12 | JX134328 JX134325  JX134339 |
| Sulzata, Rila | Bulgaria | H12 | JX134322 |
| Horní Polka, Bohemian Forest | Czech Republic | P08 | EF375837 |

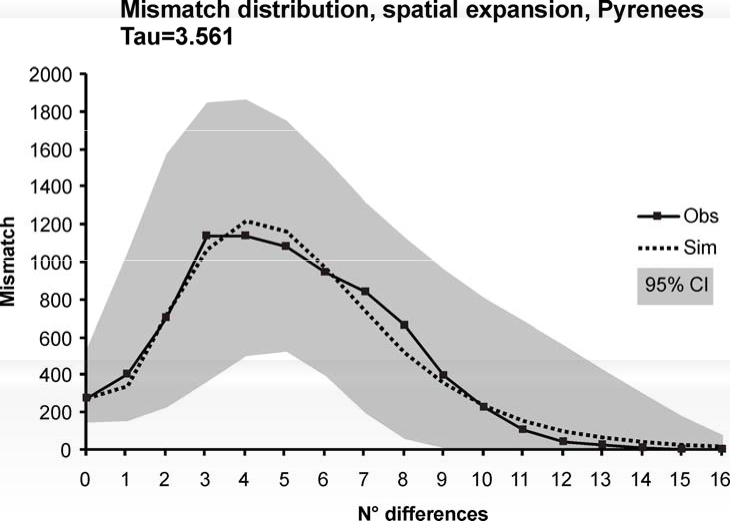
|  |  |  |  |
| --- | --- | --- | --- |
| Jelení, Bohemian Forest  pond near Dobrá, Bohemian Forest Prášilské Lake, Bohemian Forest Říjiště, Bohemian Forest | Czech Republic Czech Republic Czech Republic Czech Republic | This study This study This study This study | KF977683 KF977683 KF977684 KF977685 |
| Slapy reservoir | Czech Republic | This study | KF977686 |
| Vranov reservoir | Czech Republic | T09 | FJ178341 |
| Žďárské, Bohemian Forest | Czech Republic | P08 | EF375835 |
| Brededam | Denmark | P08 | EF375836 |
| Erikadam | Denmark | This study | KF977687 |
| Pernillesø | Denmark | P08 | EF375837 |
| Store Kobberdam | Denmark | P08 | DQ536400 |
| Tana | Ethiopia | P08 | EF375828 |
| Bodensee | Germany | P08 | EF375829 |
| castle fountain in Heidelberg | Germany | This study | KJ024375 |
| Frankfurt am Main - botanical garden  Zidak pond, Drouzkovice | Germany  Czech Republic | P08  P08 | EF375839  EF375834 |
| Hartsee | Germany | GE09 | FJ943792 |
| Helgoland | Germany | This study | KF977688 |
| Ismaning | Germany | P08,GE09 | EF375838, FJ943787 |
| Klostersee | Germany | GE09 | FJ943793 |
| Stechlinsee | Germany | P08 | EF375831 |
| Hula | Israel | P08 | EF375840 |
| Lago di Campo IV | Italy | T96 | U34643 |
| Hridsko, Prokletije Mountains Malo Šiško, Bjelasica mountains, | MHornitenegProrokletije Montenegro | H12ME H12 | JX134332  JX134334 |
| Modro, Durmitor | Montenegro | H12 | JX134336 |
| Valovito, Durmitor | Montenegro | H12 | JX134327 |
| D’Aova | Morocco | This study | KF977689 |
| Goksjø | Norway | P08,T09 | EF375832, FJ178313 |
| Hurdalsvatn | Norway | This study | KF977690 |
| Mildevatn | Norway | P08 | EF375841 |
| Molandsvann | Norway | This study | KF977691 |
| Nordfjordvatn | Norway | T09 | FJ178330 |
| Storveavatn | Norway | T09 | FJ178339 |
| Trollvann | Norway | P08 | EF375842 |
| Bucura, Retezat Mountains | Romania | H12 | JX134330 |
| Glubokoje | Russia | P08 | EF375833 |

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| --- | --- | --- | --- |
| Barabinsk pond 1, Chany Lake basin  Barabinsk pond 2, Chany Lake basin  Inflow of Hargat to Chany Lake | Siberia Siberia  Siberia | Z13 Z13  Z13 | JN903675 JN903680  JN903664 |
| Zdvinsk pond, Chany Lake basin | Siberia | Z13 | JN903669 |
| Dankovo, Spišská Magura | Slovakia | P07 | DQ337938 |
| Jamské, High Tatra Mountains | Slovakia | P07 | DQ337932 |
| Malé Čierne Pliesko, High Tatra Mountains  Nižné Jamnické, West Tatra Mountains  Nižné Rakytovské, High Tatra Mountains  Prvé (Dolné) Roháčske, West Tatra Mountains  Štvrté (Horné) Roháčske, West Tatra Mountains  Tretí Roháčske, West Tatra Mountains  Vyšné Furkotské, High Tatra Mountains  Vyšné Jamnícke, West Tatra Mountains  Vyšné Račkové, West Tatra Mountains  Vyšné Rakytovské, High Tatra Mountains  Vyšné Satanie, High Tatra Mountains  Laguna del Sopetón, Doñana | Slovakia Slovakia Slovakia Slovakia Slovakia Slovakia Slovakia Slovakia Slovakia Slovakia Slovakia  Spain | P07  P07, T09 P07  P07, H12 P07, H12 P07, H12 P07  H12  H12, P07 P07  P07, T09  This study | DQ337933 DQ337937, FJ178327 DQ337931 DQ337935, DQ337936, JX134352  DQ337934, DQ337935, JX134353, JX134354, DQ337929  JX134349  JX134350, JX134351, DQ337934 DQ337930  DQ337939  KF977692 |
| pond Dulce, Doñana | Spain | This study | KF977693 |
| pond Taraje, Doñana | Spain | This study | KF977694 |
| Villar del Rey reservoir, Badajoz | Spain | P08,T09 | EF375844, FJ178310 |
| Zahillo pond, Doñana | Spain | P08,T09 | EF375843, FJ178344 |
| Göteborg, pond in Laerjeholm | Sweden | P08, T09 | EF375845, FJ178320 |
| Koarp | Sweden | This study | KF977695, KF977696 |
| Kellersee | Switzerland | P08, T09 | EF375827, FJ178322 |
| ponds above Great St. Bernard pass | Switzerland | P08, T09 | EF375847, FJ178336, FJ178335 |
| Unterer Arosasee | Switzerland | P08 | EF375846 |

## **Table S3.** Descriptive statistics of genetic diversity at nDNA, measured at 9 microsatellite loci. Abbreviations: N, sample size averaged over loci; P, number of polymorphic loci (out of 9); MLG, clonal richness (number of multilocus genotypes); MLG/N, clonal richness divided by sample size; Div, Simpson’s Index of diversity; A, average number of alleles; PA, average number of private alleles; AR29, standardised allelic richness to 29 samples; He, expected heterozygosity; Ho, observed heterozygosity; HWE, Hardy-Weinberg equilibrium; LD, proportion of locus pairs which deviate significantly from linkage equilibrium; Fis, average inbreeding coefficient.

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| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| **Population** | **N** | **P** | **MLG** | **MLG/N** | **Div** | **A** | **PA** | **A** | **AR29** | **He** | **Ho** | HWE | LD | **Fis** |
| Py01 | 32 | 8 | 32 | 1 | 0.97 | 2.8 | 0.00 | 2.8 | 2.51 | 0.313 | 0.330 | 0.81 | 0.06 | -0.054 |
| Py02 | 33 | 8 | 33 | 1 | 0.97 | 2.6 | 0.00 | 2.6 | 2.30 | 0.346 | 0.350 | 0.39 | 0.17 | -0.011 |
| Py03 | 32 | 8 | 31 | 0.97 | 0.97 | 2.4 | 0.00 | 2.4 | 2.29 | 0.324 | 0.372 | 0.02 | 0.09 | -0.146 |
| Py05 | 31 | 9 | 31 | 1 | 0.97 | 5.2 | 0.00 | 5.2 | 2.30 | 0.464 | 0.434 | 1.00 | 0.26 | 0.065 |
| Py06 | 32 | 9 | 32 | 1 | 0.97 | 4.7 | 0.22 | 4.7 | 4.24 | 0.490 | 0.486 | 0.89 | 0.14 | 0.007 |
| Py07 | 32 | 6 | 32 | 1 | 0.97 | 2.6 | 0.00 | 2.6 | 3.77 | 0.341 | 0.350 | 0.20 | 0.03 | -0.027 |
| Py08 | 31 | 9 | 31 | 1 | 0.97 | 6.9 | 0.78 | 6.9 | 2.31 | 0.599 | 0.470 | 1.00 | 0.69 | 0.216 |
| Py09 | 30 | 8 | 30 | 1 | 0.97 | 4.2 | 0.00 | 4.2 | 5.59 | 0.346 | 0.370 | 0.55 | 0.11 | -0.071 |
| Py10 | 31 | 8 | 31 | 1 | 0.97 | 5.8 | 0.22 | 5.8 | 3.43 | 0.366 | 0.271 | 1.00 | 0.63 | 0.260 |
| Py11 | 30 | 5 | 29 | 0.97 | 0.96 | 3.0 | 0.00 | 3.0 | 4.28 | 0.285 | 0.267 | 1.00 | 0.03 | 0.066 |
| Py12 | 29 | 8 | 14 | 0.48 | 0.86 | 3.0 | 0.11 | 3.0 | 2.62 | 0.504 | 0.629 | 0.00 | 0.37 | -0.247 |
| Py13 | 30 | 7 | 30 | 1 | 0.97 | 3.0 | 0.00 | 3.0 | 2.82 | 0.424 | 0.463 | 0.04 | 0.06 | -0.092 |
| Py14 | 32 | 9 | 32 | 1 | 0.97 | 5.6 | 0.22 | 5.6 | 2.81 | 0.410 | 0.283 | 1.00 | 0.83 | 0.309 |
| Py15 | 30 | 7 | 30 | 1 | 0.97 | 2.9 | 0.00 | 2.9 | 4.29 | 0.292 | 0.263 | 0.98 | 0.06 | 0.098 |
| Py16 | 32 | 9 | 32 | 1 | 0.97 | 5.7 | 0.00 | 5.7 | 2.50 | 0.537 | 0.515 | 0.93 | 0.17 | 0.040 |
| Py17 | 32 | 9 | 32 | 1 | 0.97 | 5.6 | 0.00 | 5.6 | 4.55 | 0.496 | 0.429 | 1.00 | 0.31 | 0.135 |
| Py24 | 32 | 8 | 32 | 1 | 0.97 | 3.2 | 0.11 | 3.2 | 3.67 | 0.327 | 0.321 | 0.94 | 0.06 | 0.019 |
| Py25 | 60 | 6 | 43 | 0.72 | 0.97 | 2.6 | 0.00 | 2.6 | 2.73 | 0.216 | 0.211 | 0.76 | 0.03 | 0.025 |
| Py26 | 40 | 8 | 30 | 0.75 | 0.95 | 2.3 | 0.00 | 2.3 | 1.96 | 0.225 | 0.212 | 0.72 | 0.49 | 0.058 |
| Py28 | 32 | 6 | 31 | 0.97 | 0.97 | 3.0 | 0.22 | 3.0 | 1.98 | 0.311 | 0.302 | 0.77 | 0.09 | 0.028 |
| Py29 | 31 | 8 | 31 | 1 | 0.97 | 4.4 | 0.22 | 4.4 | 2.55 | 0.318 | 0.290 | 1.00 | 0.26 | 0.090 |
| Py30 | 32 | 7 | 31 | 0.97 | 0.97 | 2.4 | 0.00 | 2.4 | 3.39 | 0.336 | 0.281 | 1.00 | 0.17 | 0.164 |
| Py32 | 32 | 8 | 32 | 1 | 0.97 | 4.0 | 0.22 | 4.0 | 3.17 | 0.412 | 0.347 | 1.00 | 0.14 | 0.157 |
| Py33 | 32 | 9 | 32 | 1 | 0.97 | 4.0 | 0.11 | 4.0 | 3.17 | 0.328 | 0.340 | 0.73 | 0.11 | -0.038 |
| Py37 | 40 | 9 | 39 | 0.98 | 0.97 | 5.3 | 0.11 | 5.3 | 4.16 | 0.543 | 0.517 | 0.99 | 0.29 | 0.047 |

**Fig. S1.** Evidence for a spatially structured demographic expansion. Mismatch distribution plot from mitochondrial data (12S) for populations of the Eastern Pyrenees. Observed frequencies are solid lines, predicted frequencies (spatial expansion model) are dashed lines. The 95% confidence interval (CI) is shown in grey. Tau = 3.561. The sudden expansion model provided a similar fit.



**Fig. S2** Changes in *Daphnia longispina* abundance along the ice free periods of 2010 and 2011 in Lake Llarg (upper panels) and their body size distribution. Black circles and bars correspond to reproductive females and white circles and bars to juveniles. Horizontal black bars in the upper panels show the ice-covered period.

15

adult females juvenile females males

*Daphnia longispina*

Abundance (Ind L-1)

10

5

0

05 06 07 08 09 10 11

15

10

5

0

05 06 07 08 09 10 11

2010 2011

80 80

19/06/2011

15/07/2011

10/08/2011

13/09/2011

17/10/2011

28/07/2010

26/08/2010

23/09/2010

28/10/2010

*Daphnia longispina* female percentage size distribution

50

50

20

20

80

80 50

50 20

20 80

50

80 20

50 80

20 50

20

80

80

50

50

20 20

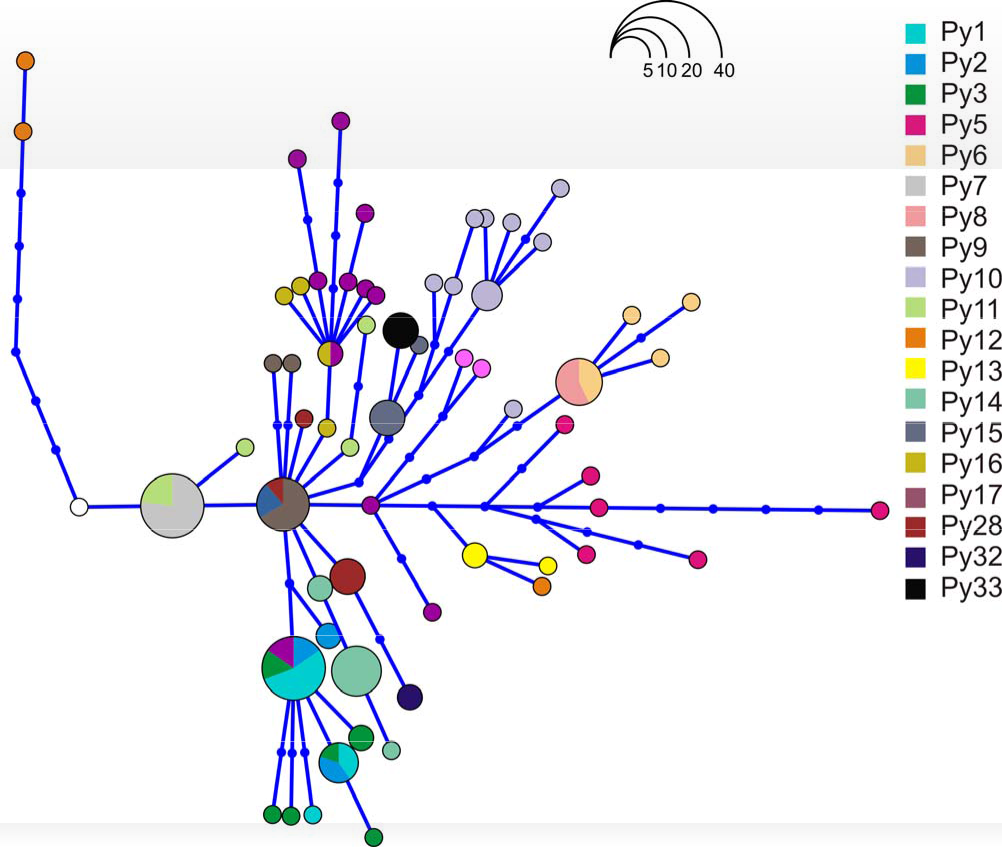
750 1250 1750 2250

Size class (µm)

750 1250 1750 2250

## **Fig. S3.** Haplotype network depicting variation at the mitochondrial gene for 12S rRNA in studied

*D. longispina* populations in the Pyrenees. Each circle represents a unique haplotype and its size is proportional to the number of individuals sharing that specific haplotype. Each branch with more than one mutational step is labelled. Studied localities are indicated by a different colour. The locality codes refer to Table S1.



**Appendix S1 Extended methods**

*Extended genetic analysis*

We amplified and sequenced *ca*. 540 nucleotides (nt), a long fragment of the mitochondrial 12S rRNA gene (12S) using primers 12S-F (5′-ATGCACTTTCCAGTACATCTAC- 3′) and 12S-R (5′- AAATCGTGCCAGCCGTCGC-3′) (Taylor *et al.* 1996). The reaction mix of the total volume 25 μL contained 1× PCR buffer (Silverstar, Eurogentec), 1.5 mM MgCl2, 200 µM of each dNTP, 0.2

µM of each primer, 1 µL of template DNA and 1–2 U *Taq* polymerase. PCR amplifications for 12S

involved a denaturing step of 5 min at 95 °C, followed by 30–40 cycles of 45 s at 95 °C, 45 s at 53

°C, 45 s at 72 °C, and a final elongation of 7 min at 72 °C. Purified amplification reactions were sequenced using forward primers on an ABI PRISM 3130 capillary DNA sequencer (Applied Biosystems).

**Extended results**

*Extended genetic diversity*

Fourty-five of the 61 haplotypes found in the Pyrenees were singletons; 10 of the remaining 16 sequences occurred in just a single lake. The average number of nucleotide differences between sequences (k) was 4.96, yielding an overall nucleotide diversity (Pi) of 0.0099. The remaining 417 sequences, mostly European but including also samples from Russia, the Middle East and Africa, had 97 variable nucleotides, with 118 haplotypes. One hundred and one of these haplotypes were singletons, 29 of the remaining 52 haplotypes were present in just a single lake and only 4 haplotypes had sequences from lakes belonging to different countries. The average nucleotide difference was 5.334, and nucleotide diversity was 0.01062, indicating that the genetic diversity

within the Pyrenees alone is similar to the so far known genetic diversity across the rest of the species range.

With regards to the molecular variability in nuclear DNA, only three populations (Py3, Py12 and Py13) significantly deviated from Hardy-Weinberg equilibrium with Py3 and Py13 only

slightly divergent. Observed clonal (i.e., multi-locus genotype, MLG) richness levels, expressed as MLG/N and Simpson diversity, reached maximal possible values in samples in most populations, i.e., all or all but one multilocus genotypes were different (with MLG/N exceeding 0.9 and usually reaching 1), but marked reductions of clonal richness were observed in Py12, Py25 and Py26. Other measures of genetic diversity (including heterozygosity parameters, standardised allelic richness

and inbreeding coefficients) are presented in Table S3. The proportion of private alleles was generally low (0 in 14 of the lakes) and had their maximum in Lake Py08. The proportion of locus pairs deviating significantly from linkage equilibrium was relatively low, with the exception of lakes Py8, Py10 and Py14 that had more than half of the locus pairs with significant values.

*Dispersal inferred from mtDNA*

Several examples from different catchments may demonstrate these patterns. Within the Gerber catchment, the headwater lake Py10 hosts a diverse but endemic array of related haplotypes, despite discharging directly into Py9, and indirectly into Py11. Similarly, no haplotype sharing was found between the connected Py9 and Py11, whereas both lakes shared haplotypes with lakes or ponds from other catchments (Table S1; Figure S3). Py33 in the Colomèrs catchment receives water from both Py14 and Py15, but we did not find any shared haplotypes with either of them. The sole haplotype found in Py33 is, nevertheless, most related to haplotypes from Py15 (Table S1; Figure S3). Finally, Py5 in the Engorgs catchment is a small pond (c. 600 m2) that receives water from Py17, 60 m away. Although its haplotypes are more related to those from Py17 and Py16 than to

any other water, it is genetically distinct and very diverse, with only endemic haplotypes recorded (Table S1). Py17 also discharges into Py16, and yet it shared only one out of fourteen haplotypes

detected in 20 analysed specimens. Most haplotypes recorded in Py17 have a single ancestor within the Engorgs catchment; however, one haplotype from this lake was shared with the Gerber catchment

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