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ORIGINAL ARTICLE

Spatiotemporal genetic structure in the *Daphnia pulex* complex from Sierra Nevada lakes (Spain): reproductive mode and first record of North American *D.* cf. *pulex* in European alpine lakes

JOSÉ M. CONDE-PORCUNA[1,2,*], JESÚS VEIGA[1,2,†], EMILIO MORENO[1,2], LAURA JIMÉNEZ[1,2], ELOÍSA RAMOS-RODRÍGUEZ[1,2] AND CARMEN PÉREZ-MARTÍNEZ[1,2]

[1]UNIVERSITY INSTITUTE OF WATER RESEARCH, UNIVERSITY OF GRANADA, RAMÓN Y CAJAL 4, GRANADA E-18071, SPAIN AND [2]DEPARTMENT OF ECOLOGY, FACULTY OF SCIENCES UNIVERSITY OF GRANADA, FUENTENUEVA S/N, GRANADA E-18071, SPAIN

*CORRESPONDING AUTHOR: jmconde@ugr.es.

†PRESENT ADDRESS: DEPARTMENT OF FUNCTIONAL ECOLOGY AND EVOLUTION, ARID ZONE EXPERIMENTAL STATION (EEZA-CSIC), CTRA. DE SACRAMENTO S/N, LA CAÑADA DE SAN URBANO, ALMERÍA E-04120, SPAIN

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Daphnia is a good model organism for studying factors affecting dispersal and patterns of genetic diversity. Within this genus, the *Daphnia pulex* species complex includes lineages from North America and Europe, with *s*ome considered invaders in various continents, although their colonization history is poorly known. We used mitochondrial DNA and microsatellite markers to identify the *D. pulex* complex lineages in Sierra Nevada, determine their reproductive mode and reconstruct their genetic history (over the past ~25 to 65 years). We present the first recording of North American (NA) *D.* cf. *pulex* in a European high-mountain lake, showing its arrival ~65 years ago in lake Borreguil without temporal changes in its genetic structure. European (Eu) *D.* cf. *pulicaria* is the only lineage present in other Sierra Nevada lakes and also showed no genetic change over time. The results for both species are congruent with obligate parthenogenetic reproduction mode. Moreover, water mineralization may influence the clonal distribution of the *D. pulex* complex in Sierra Nevada, without ruling out dispersal limitation and/or founder effects. Although NA *D.* cf. *pulex* had not spread to other Sierra Nevada lakes, it could threaten Eu *D.* cf. *pulicaria* in Sierra Nevada and other European alpine lakes.

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KEYWORDS: *Daphnia*; diapausing egg bank; DNA barcoding; population genetic structure; ancient DNA

INTRODUCTION

Numerous freshwater taxa are distributed over wide geographical areas through dispersal by diapausing propagules. Diapausing propagules of zooplankton can be dispersed by abiotic and biotic vectors such as wind (Moreno *et al.*, 2016; Vanschoenwinkel *et al.*, 2008), waterbirds (Green *et al.*, 2013; Moreno *et al.*, 2019) and even human activities (Havel and Medley, 2006), contributing to the gene flow (Slusarczyk *et al.*, 2019) and favoring colonization and the spreading of invasive species (Havel *et al.*, 2000; Mergeay *et al.*, 2006).

Daphnia is a good model organism for studying aquatic dispersal via diapausing propagules because it cannot be dispersed in active form and has been described in recent invasion and colonization studies (Slusarczyk *et al.*, 2019), among other reasons. Within the genus *Daphnia*, numerous evolutionary and taxonomy studies have been performed on the widely distributed *Daphnia pulex* complex (e.g. Dufresne *et al.*, 2011; Lynch, 1983; Ma *et al.*, 2019; Mergeay *et al.*, 2008). However, species delimitation within this complex species is extremely difficult because of the negligible morphological differences among many taxa (Burillo *et al.*, 2019; Marková *et al.*, 2007).

Genetic techniques are highly useful for the differentiation of species within complexes such as *D. pulex* (Marková *et al.*, 2013), but the study of this complex is also complicated by nomenclature issues (Ma *et al.*, 2019; Mergeay *et al.*, 2008), with the same names being used for different evolutionary lineages from distinct biogeographical regions. According to Mergeay *et al.* (2008), we can distinguish the American lineage *Daphnia pulicaria* Forbes 1893, the European lineage *D.* cf. *pulicaria* (sensu Alonso, 1996), the European species *D. pulex* Leydig 1860 and the American–Panarctic lineage *D.* cf. *pulex* (sensu Hebert, 1995). Henceforth, we refer to them as North American (NA) *D. pulicaria*, European (Eu) *D.* "*pulicaria*," Eu *D. pulex* and NA *D.* "*pulex*," respectively. The study of mitochondrial DNA (mtDNA) by Crease *et al.* (2012) confirmed the existence of at least 12 lineages in the *D. pulex* complex, including NA *D. pulicaria* and NA *D.* "*pulex*," which are sister lineages in the same mtDNA clade ("*pulicaria* group"); Eu *D.* "*pulicaria*" (member of the "tenebrosa group"); and Eu *D. pulex*, which forms a clade on its own (Marková *et al.*, 2013). Previously, Mergeay *et al.* (2008) also described three endemic South American lineages of the *D. pulex* complex.

Researchers have demonstrated the presence of clones of NA *D. pulicaria* in New Zealand lakes (Duggan *et al.*, 2012; Z. Ye *et al.*, submitted for publication) and the presence, in Europe, of a hybrid lineage of NA *D.* "*pulex*" and NA *D. pulicaria* (Fadda *et al.*, 2011; Mergeay *et al.*, 2006; Vergilino *et al.*, 2011), named hereafter hNA *D.* "*pulex*". All *D. pulex* found in Africa are descendent of a single asexual hybrid clone (hNA *D.* "*pulex*") that invaded from North America (Mergeay *et al.*, 2006), and hybrids have also artificially and/or naturally colonized New Zealand (Z. Ye *et al.*, submitted for publication) and Japan (So *et al.*, 2015). Given this propensity of NA *D.* "*pulex*" hybrids to invade, it might pose a risk to native European *Daphnia* populations (Fadda *et al.*, 2011). In fact, hNA *D.* "*pulex*" has been recorded in Europe in medium- to low-land bodies of water (altitude <714 m.a.s.l.) in eastern Spain, northern Italy and Sardinia (Fadda *et al.*, 2011; Schwenk *et al.*, 2000; Vergilino *et al.*, 2011). Mergeay *et al.* (2006) reconstructed the invasion history of hNA *D.* "*pulex*" in Africa, and Möst *et al.* (2015) reconstructed the invasion by Eu *D.* "*pulicaria*" of a European peri-alpine lake. However, there have been no historical reconstructions of the invasions of NA *D. pulicaria* or NA *D.* "*pulex*" into Europe, and these could assist our understanding of these processes and their possible consequences. Moreover, historical reconstructions are useful to identify not only phases of colonization/invasion but also changes in genetic diversity or possible colonization by pre-adapted obligate parthenogens of *Daphnia* in the absence of historical genetic changes.

The investigation of sedimentary egg banks is useful to detect historic changes in the genetic structure of *Daphnia* populations (Monchamp *et al.*, 2017; Möst *et al.*, 2015). Various studies have observed genetic changes over time in relation to eutrophication (Brede *et al.*, 2009; Monchamp *et al.*, 2017; Weider *et al.*, 1997), fish predation (Cousyn *et al.*, 2001) and invasions (Mergeay *et al.*, 2006; Möst *et al.*, 2015). Kerfoot and Weider (2004) found that microevolutionary changes in *Daphnia* can take place over a short time interval of ~60 years. In general, few data are available on the initial phases of *Daphnia* species colonization or invasion (Brede *et al.*, 2009; Möst *et al.*, 2015; Rellstab *et al.*, 2011).

Mergeay *et al.* (2006) showed that an obligate asexual hNA *D.* "*pulex*" was able to colonize rapidly the entire African continent by its wide ecological amplitude, while an indigenous sibling species that combined sexual and asexual reproduction was not detected in recent decades. Given that asexual reproduction avoids the costs associated with the production of males, sexual populations may be replaced by invading obligate asexual clones

(Innes and Ginn, 2014). Hence, knowledge of the reproductive mode of populations of the *D. pulex* complex may also indicate their potential risk of replacement by obligate asexual invaders.

The *D. pulex* species complex shows a substantial variation in the reproductive mode. This complex is the only species complex within *Daphnia* genus in which obligate parthenogenesis has been described (Colbourne *et al.*, 1998; Dufresne *et al.*, 2011). Geographical parthenogenesis in *D. pulex* complex, i.e. the prevalence of asexual populations at high latitudes and altitudes and in extreme environments (Vandel, 1928; cited by Dufresne *et al.*, 2011), has been reported in North American arctic and subarctic lakes (Beaton and Hebert, 1988) and in South American high-mountain lakes (Mergeay *et al.*, 2008). However, obligate parthenogens have been found in a wide altitudinal range in China, from 1050 m.a.s.l. (Eu *D.* "*pulicaria*" and NA *D.* "*pulex*") to almost 4000 m.a.s.l. (NA *D. pulicaria*) (Ma *et al.*, 2019). In Europe, a mixed breeding system has been suggested for Eu *D.* "*pulicaria*" in lowland lakes (Dufresne *et al.*, 2011). In European alpine lakes, this species reproduces by obligate parthenogenesis in the High Tatra Mountains and mainly by cyclic parthenogenesis in the Pyrenees (Dufresne *et al.*, 2011). Moreover, there is no information on the reproductive mode of the *D. pulex* species complex in the southernmost alpine mountain range in Europe (Sierra Nevada, Spain). Obligate parthenogenesis reproduction can be expected in Sierra Nevada lakes, where *Daphnia* males are extremely scarce (Pérez-Martínez *et al.*, 2007). Moreover, the presence of males does not rule out the possibility of obligate parthenogenesis (Innes and Hebert, 1988).

According to De Meester *et al.* (2002), in obligate parthenogens, the distribution of genotypes should be interpreted as the consequence of clonal selection according to their fitness to a given habitat, and the correlation of environmental conditions with genotypes results from the colonization of pre-adapted genotypes. These authors hypothesized that the first immigrants grow faster, thereby forming a population and egg bank able to reduce the effect of new immigrants, which would therefore have little impact on the genetic structure of species, with no change in genetic diversity over time (Haileselasie *et al.*, 2018).

In the present study, our objectives were (i) to identify the *Daphnia* lineages and possible invaders in Sierra Nevada lakes (Spain) using mitochondrial molecular markers; (ii) to reconstruct the genetic variability of *Daphnia* within a lake over time and among different lakes, based on the study of ephippia in sediments from the past ~6 decades; (iii) to determine the reproductive mode of *Daphnia* in these lakes by comparing present and past genotypes and (iv) to examine clonal distribution in relation to environmental parameters by studying the relationship between genetic differentiation, using microsatellite markers, and lake environment characteristics.

No study has found NA *D. pulex* in any European high-altitude lake. In Sierra Nevada lakes, previous studies have morphologically identified *Daphnia* as *D. pulicaria* (sensu lato) in all lakes in which this genus is present (e.g. Morales-Baquero *et al.*, 2019). Marková *et al.* (2013) identified Eu *D.* "*pulicaria*" individuals in two Sierra Nevada lakes (Río Seco and La Caldera according to their geographical coordinates) by mtDNA sequencing; however, no information is available for other Sierra Nevada lakes.

Jiménez *et al.* (2018) observed that *Daphnia* has been present for at least two centuries in some Sierra Nevada lakes, although it had only been present for around 65 years in one lake (Borreguil). Now, in the present study, genetic analyses of sediment resting egg banks will reveal whether the significant increase in *Daphnia* populations in Sierra Nevada lakes since ~1970 (Jiménez *et al.*, 2018) is associated with changes in their genetic structure. This study may reveal not only genetic changes over time but also possible colonization or invasion processes from their commencement, given the recent appearance of *Daphnia* in lake Borreguil.

METHOD

Study site

Sierra Nevada contains ~50 small lakes of glacial origin, all above the tree line (2800–3100 m.a.s.l.) on siliceous bedrock. All of these lakes are low primary production and alkalinity lakes, fish-free and snow-covered during 5–8 months of the year. Additional meteorological and land use information are available from Morales-Baquero *et al.*, 2006; Jiménez *et al.*, 2018 and Pérez-Martínez *et al.*, 2020a, among others. We selected eight Sierra Nevada lakes, which comprise most of the lakes inhabited by *Daphnia* in the Sierra Nevada alpine region (Fig. 1): Borreguil (BG), Caballo (CB), Cuadrada (CD), La Caldera (CL), Las Yeguas (YE), Río Seco (RS), Río Seco Superior (RSS) and Virgen Media (VM). These lakes are located in different valleys of Sierra Nevada (Fig. 1).

Field samples and environmental data

We collected *Daphnia* individuals from the water column and/or *Daphnia* ephippia from sediment samples in the selected lakes over the ice-free period in 2008 (RS), 2011 (BG, CD and RSS), 2013 (CB, VM and YE) and 2014 (CL). *Daphnia* individuals were obtained in successive

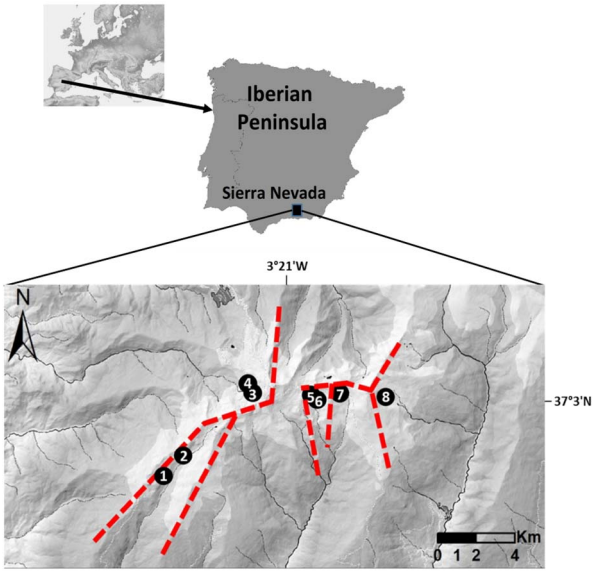

Fig. 1. Location of the Sierra Nevada lakes (Spain). A dashed line indicates the line of peaks of Sierra Nevada mountains. 1: Caballo (CB); 2: Cuadrada (CD); 3: Virgen Media (VM); 4: Las Yeguas (YE); 5: Río Seco Superior (RSS); 6: Río Seco (RS); 7: La Caldera (CL) and 8: Borreguil (BG).

horizontal and vertical hauls (250 μm mesh), immediately fixed with 95% ethanol and preserved at 4°C until laboratory analyses. Sediment samples were collected from the deepest part of these lakes using a hand-operated sediment core sampler composed of a 2–4 m long shaft and 50 cm long acrylic tube (see Vandekerkhove *et al.*, 2005). Sediment cores (10 cm long) were extracted in a methacrylate cylinder, extruded on site at ∼1-cm interval for the upper 4–7 cm of sediment, immediately sealed in sterile bags and stored at 4°C in the dark. Sedimentary *Daphnia* ephippia were isolated using the sugar flotation method developed by Onbe (1978) and modified by Marcus (1990). Similar or related sugar flotation methods have previously been used for isolating ephippia from different core sediment depths (e.g. Cáceres, 1998; De Stasio, 1990; Vandekerkhove *et al.*, 2004). Sediment cores could not be obtained from lakes YE and CL because of their rocky substrate; therefore, *Daphnia* samples were collected from the water column alone for YE and CL. Sediment samples alone were gathered in lakes RSS, BG and VM, because *Daphnia* was not present in the water column on the sampling day.

Sediment cores were dated using gamma spectroscopy in all lakes except for VM and CB (Jiménez *et al.*, 2018), preventing study of the relationship of findings with sediment chronology in these two lakes. In the other lakes, sediment layers from 0 to 5–6 cm depth correspond to different dates according to Jiménez *et al.* (2018) (see Fig. S1 and Table S1 in Supplementary Information), and the average sediment accumulation rate for the past ∼65 years in Sierra Nevada lakes shows a narrow range of variation (Jiménez *et al.*, 2018).

Data on the altitude, maximum depth, catchment area and surface area of each lake were obtained from measurements conducted in this study and those previously published by Morales-Baquero *et al.* (1999). Each lake was physicochemically characterized by measuring the conductivity and pH on-site with an Oakton PC300 multiparameter probe and by gathering the following chemical variables from the analysis of water column samples taken on each core sampling day and at later time points (Pérez–Martínez *et al.*, 2020a): alkalinity and concentrations of calcium, total nitrogen (TN), total phosphorus (TP) and chlorophyll-*a*. For each lake, physicochemical data from other years were also obtained from Sánchez-Castillo *et al.* (1989); Reche *et al.* (2005), Morales-Baquero *et al.* (1999, 2006), Linares-Cuesta *et al.* (2007), González-Olalla *et al.* (2018) and Guerrero-Jiménez (2020). Overall mean values are displayed in Table I.

Genetic samples and DNA extraction

Genetic analyses were performed in *Daphnia* individuals from the water column and in resting eggs from randomly selected sedimentary ephippia, isolating one resting egg from each sedimentary *Daphnia* ephippium after decapsulation. A total of 55 individuals from the water column and 131 sedimentary resting eggs were analyzed (Table S1 in Supplementary Information). DNA was extracted from the individuals and resting eggs by the HotShot method (Montero-Pau *et al.*, 2008).

Phylogenetic analyses

Phylogenetic analyses of the mitochondrial NADH dehydrogenase 5 (ND5) gene were conducted to identify the mtDNA lineages of the *D. pulex* complex. We used only a few individuals or resting eggs from each lake except one, sufficient to identify the most similar lineages to our Sierra Nevada populations. Specifically, we examine one ND5 sequence from VM (resting egg), two from CD (individual and resting egg), two from RS (individual and resting egg), one from RSS (resting egg), two from CB (individual and resting egg), one from YE (individual) and a much higher number (39) from BG (all resting eggs) because of the distinct lineage found in this lake (see results). In the case of CL, we used data published by Marková *et al.* (2013) on three sequences from *Daphnia* individuals.

Polymerase chain reaction (PCR) reaction for ND5 sequencing used DpuND5b (GGGGTGTATCTATTAA-

Table I: Location and environmental characteristics of the eight study lakes in the Sierra Nevada

	Latitude	Longitude	Altitude (m.a.s.l.)	Lake area (ha)	Catchment area (ha)	Maximum depth (m)	pH	Alkalinity (meq/L)	Conductivity (μS/cm)	TP (μM)	TN (μM)	Ca (mM)	Chla (μg/L)
Borreguil (BG)	37.052	-3.300	3020	0.18	50.9	2.5	6.95 (6.25–8.3)	0.09 (0.09–0.10)	13.64 (12.7–14.6)	0.58 (0.43–0.85)	19.99 (12.85–27.13)	0.02 (0.02–0.03)	2.00 (0.7–4.3)
Caballo (CB)	37.015	-3.428	2850	0.48	10.5	4.0	7.05 (6.6–7.5)	0.09 (0.07–0.11)	9.59 (8.0–11.2)	0.38 (0.22–0.60)	9.08 (6.66–11.49)	0.02 (0.02–0.02)	0.55 (0.3–0.7)
La Caldera (CL)	37.055	-3.329	3050	2.10	23.5	12	7.34 (6.4–8.4)	0.32 (0.23–0.43)	48.74 (18.7–146.5)	0.17 (0.04–0.35)	23.62 (7.20–40.97)	0.10 (0.08–0.12)	2.51 (0.2–5.5)
Cuadrada (CD)	37.027	-3.419	2910	0.24	4	5.0	7.98 (7.7–8.3)	0.12 (0.09–0.14)	7.39 (6.2–8.8)	0.30 (0.26–0.34)	5.95 (2.89–9.00)	0.02 (0.01–0.03)	1.13 (6.2–8.8)
Virgen Media (VM)	37.052	-3.380	2945	0.01	21.2	0.8	6.72 (6.0–7.2)	0.20 (0.14–0.26)	37.60 (29.2–46.0)	0.77 (0.21–1.10)	36.98 (*)	0.12 (0.06–0.18)	1.18 (0.2–2.2)
Río Seco (RS)	37.052	-3.346	3020	0.46	9.9	2.9	6.97 (6.0–8.4)	0.11 (0.05–0.20)	23.80 (10.5–77.4)	0.54 (0.17–0.87)	28.74 (13.69–52.23)	0.03 (0.01–0.05)	1.94 (0.5–6.5)
Río Seco Superior (RSS)	37.052	-3.348	3040	0.06	4.7	2.6	6.97 (6.2–7.8)	0.16 (0.14–0.19)	15.39 (14.0–16.8)	0.49 (0.42–0.55)	20.28 (9.50–31.06)	0.05 (0.03–0.10)	2.84 (0.4–10.1)
Las Yeguas (YE)	37.056	-3.381	2883	0.33	50	10.5	7.25 (6.7–7.7)	0.50 (0.21–0.96)	27.90 (*)	0.24 (0.04–0.65)	25.24 (8.07–42.41)	0.12 (0.09–0.14)	5.39 (1.8–12.0)

Mean values of physicochemical and biological parameters for water column (minimum and maximum values are displayed in brackets). Environmental data are from Sánchez-Castillo *et al.* (1989), Reche *et al.* (2005), Morales-Baquero *et al.* (1999, 2006), Linares-Cuesta *et al.* (2007), González-Olalla *et al.* (2018) and also derived from water column samples taken each core sampling day and at later time points. (*): Only one data are available. TP, total phosphorus; TN, total nitrogen; Ca, calcium; Chla, Chlorophyll-a.

TTCG-3′) and DpuND5a (ATAAAACTCCAATCAAC-CTTG-3′) primers (Colbourne *et al.*, 1998) and was performed in a total volume of 50 μL containing 5 μL NH_4, 1.5 mM $MgCl_2$, 10 nmol dNTPs, 15 pmol of each primer, 1 μL of Taq polymerase and 2 μL of DNA template, completing the volume with water. PCR amplification was carried out with a Mastercycler Pro thermocycler (Eppendorf) using 40 cycles of 30 s at 94°C, 30 s at 48°C and 1 min at 72°C, followed by one cycle of 6 min at 72°C. PCR products were directly sequenced in both directions with PCR primers from Macrogen Inc. (South Korea) using a 3730XL DNA analyzer (Applied Biosystems, USA).

ND5 sequences were compared with 257 *D. pulex–pulicaria* ND5 sequences downloaded from GenBank (Table S2 in Supplementary Information). These GenBank sequences and the ND5 sequences obtained in this study were visually examined in an individual manner and verified for protein coding frameshifts to avoid pseudogenes or stop codons. All sequences were aligned using the ClustalW algorithm in MEGA7 (Kumar *et al.*, 2016), and the alignment was trimmed to the shortest sequence in our ND5 dataset (380 bp) and then collapsed into haplotypes with FaBox v. 1.4 (Villesen, 2007). The final dataset included 45 haplotypes selected from GenBank, 1 haplotype from BG (corresponding to all 39 sequences used from BG), 1 haplotype from collapsed Sierra Nevada lake sequences, 1 haplotype from RS, 1 haplotype from CD and 1 haplotype from CB (Table S3 in Supplementary Information). We constructed a phylogenetic tree using MEGA7 (Kumar *et al.*, 2016). The best-fitting model of nucleotide substitution for the ND5 dataset was the Hasegawa–Kishino–Yano model with gamma distribution (HKY + G) (Hasegawa *et al.*, 1985), which was used to construct the tree by the maximum likelihood method. The bootstrap consensus tree inferred from 1000 replicates (Felsenstein, 1985) was taken to represent the evolutionary history of the taxa analyzed (Felsenstein, 1985). It was rooted with sequences of Eu *D. pulex* used in previous studies as an outgroup to Eu *D.* "*pulicaria*," NA *D. pulicaria* and NA *D.* "*pulex*" (Marková *et al.*, 2013; Möst *et al.*, 2015).

Microsatellite analyses

We used eight microsatellite markers developed by Colbourne *et al.* (2004) for the *D. pulex* complex, which in other studies were polymorphic, in order to analyze the genetic diversity and the difference between individuals/resting eggs. The selected microsatellites were Dp335 (Allen *et al.*, 2010), Dp522 (Dufresne *et al.*, 2011), Dp208, Dp291, Dp304, Dp339 (Allen *et al.*, 2012), Dp514alt (Dufresne *et al.*, 2011; Marková *et al.*, 2007) and Dp70

(Cristescu *et al.*, 2012). All loci were polymorphic in at least one lake under study.

Forward primers were labeled with different fluorescent dyes (6-FAM, ROX and HEX). Microsatellite primers were diluted and multiplexed in two sets of primers and were simultaneously amplified with the Qiagen© Multiplex PCR Kit (Qiagen). Each microsatellite locus was amplified in a 30 μL PCR containing 5 μL of DNA, 15 μL of 2× Qiagen Multiplex PCR Master Mix (including HotStarTaq® Polymerase, 3 mM $MgCl_2$ and dNTP) and 3 μL of the primer mix to reach a final concentration of 0.2 μM of each primer, completing the volume with water. PCR amplifications commenced with an initial step of 15 min at 95°C, followed by 30 cycles of 30 s at 94°C, 90 s at 56°C and 90 s at 72°C, with a final elongation step of 10 min at 72°C.

The size of microsatellites was assigned using Peak Scanner 1.0 (Applied Biosystems). All microsatellite alleles were manually checked and identified. The number of alleles (*A*), observed heterozygosity (H_o) and within-population gene diversity (H_s) were estimated using Arlequin version 3.5.2.2 (Excoffier and Lischer, 2010). Microsatellite genotypes of all specimens are included as Supplementary Information (Data S1).

Genetic diversity and distance

Statistical analyses were performed using R 3.5.1 (R Foundation for Statistical Computing). The number of microsatellite multilocus genotypes (MLGs) observed, the expected number of MLGs at the lowest common sample size (rarefaction) and the MLG diversity (Shannon-Wiener Index) with rarefaction were estimated using the "poppr" R package (Kamvar *et al.*, 2014, 2015). Microsatellite-based genetic distances were calculated according to Rogers' genetic distance (Rogers, 1972; Grünwald *et al.*, 2017) (Euclidean), using the "adegenet" R package (Jombart, 2008; Jombart and Ahmed, 2011). These served to construct a minimum spanning genotype network, which is highly useful to visualize relationships among individuals/resting eggs (Grünwald *et al.*, 2017).

Genetic similarities among all individuals/resting eggs were also studied by principal component analysis (PCA) using the "ade4" R package.

Evaluation of the reproductive mode of *Daphnia*

Genetic comparison of MLGs between individuals and sedimentary resting eggs can provide evidence on the reproductive mode, because resting eggs are clonally produced by obligate parthenogenetic individuals (Decaestecker *et al.*, 2009). We compared, when possible, MLGs of individuals obtained from the water column with those obtained from the sedimentary resting eggs in each lake. We also compared MLGs among lakes. There is evidence of obligate parthenogenesis when resting eggs have identical heterozygous MLGs, because sexually produced eggs cannot be expected to have identical genotypes or identical planktonic and ephippial genotypes by chance.

In addition, we calculated the probability (P_{sex}) that repeated genotypes originate from clonal or sexual reproductive events (Arnaud-Haond *et al.*, 2007; Dufresne *et al.*, 2011). Identical MLGs reproduce clonally if this probability is lower than a given threshold (0.01) (Arnaud-Haond *et al.*, 2007; Dufresne *et al.*, 2011). Estimates of P_{sex} were obtained by using the "RClone" R package (Bailleul *et al.*, 2016), computing allelic frequencies with the round-robin method. A more conservative estimate of P_{sex} was obtained to take account of Hardy–Weinberg equilibrium departures by determining the $P_{sex}(F_{IS})$ value (Arnaud-Haond *et al.*, 2007; Dufresne *et al.*, 2011). The Hardy–Weinberg test was performed using FSTAT 2.9.4 (Goudet, 2003) to determine whether inbreeding coefficients (F_{IS}) were significantly positive or negative, measuring the heterozygote deficit (>0) or excess (<0). Highly negative F_{IS} values are also indicative of obligate parthenogenesis (Dufresne *et al.*, 2011).

Clonal distribution in relation to environmental/spatial variables

Pearson correlation analyses were performed to examine the relationship of the clonal distribution with environmental and spatial variables. Rogers' distance matrix among lakes was applied to calculate principal coordinates (PCo) of the genetic data, using the "ade4" R package (Dray and Dufour, 2007), given the recommendation to use PCo based on a genetic distance matrix in relationship analyses (Ventura *et al.*, 2014). The mean values of environmental variables in each lake were used in the correlation analyses, log-transforming (ln) some of these to obtain a better fit. Moreover, the spatial distance among the sampled lakes was used to create spatial variables by principal coordinates of neighbor matrices (PCNM), whose base functions are particular cases of Moran's eigenvector maps (Dray *et al.*, 2006). This PCNM was constructed using the *pcnm* function of the "vegan" R package (Oksanen *et al.*, 2019), including the scores of the first three PCNM axes in the Pearson correlation analyses. These first three PCNM axes explained 99.6% of the variation in the spatial model.

The Shapiro–Wilk test was applied to check the normal distribution of variables before the correlation analyses. Because the scores for the PCo axes of genetic data did

not meet the assumption of normality, permutation tests were applied to calculate the *P*-values of correlation. Permutation tests do not require a normal distribution (Berry *et al.*, 2019; Bishara and Hittner, 2012) and outperform other tests for non-normal data when samples are small (Bishara and Hittner, 2012). The "wPerm" R package (Weiss, 2015) was used to apply permutation tests for Pearson correlations. *P*-values were adjusted for multiple tests by the false discovery rate method (Benjamini and Hochberg, 1995) calculating *Q* values with the *p.adjust* R function.

RESULTS

Sedimentary *Daphnia* ephippia

We found 918 *Daphnia* ephippia in sediments from the Sierra Nevada lakes under study. The findings on the number of ephippia per gram of sediment showed no clear temporal pattern in any lake except for BG and, to a lesser extent, CD (Table S4 in Supplementary Information). In BG, ephippia were less numerous before the 1970s and not present before the 1940s, while the number of ephippia in CD had decreased over recent years (Table S4 in Supplementary Information).

Phylogeny of *Daphnia pulex* complex

Our phylogenetic results inferred from the ND5 sequences from GenBank and our study showed five lineages of the *D. pulex* complex: three located in the Palearctic and designated Eu *Daphnia pulex*, Eu *Daphnia* "*pulicaria*" and NA *Daphnia* "*pulex*," one located in the Nearctic and named NA *Daphnia pulicaria* and one arctic lineage (*D. tenebrosa*). Haplotypes of *Daphnia middendorffiana*, *Daphnia melanica* and South American *D. pulicaria* were clustered with haplotypes of NA *D. pulicaria*.

In the present study, haplotypes of seven out of the eight Sierra Nevada lakes were assigned to the Eu *D.* "*pulicaria*" lineage (Fig. 2), being closely related to several haplotypes from the Pyrenees and to haplotypes PlEU3 and PlEU11 (from High Tatra Mountains, Slovakia). Surprisingly, samples from the remaining lake, BG, which is in an easterly oriented valley, were assigned to the NA *D.* "*pulex*" lineage. This BG haplotype collapsed into the same group as an African haplotype (represented by the identical sequences PxKEN1, PxKEN2 and PxZAF1), indicating that these might be closely related haplotypes (Fig. 2). Nucleotide sequence data from the present study have been submitted to the GenBank database (see Table S3 in Supplementary Information for accession numbers).

Genetic diversity and distance of *Daphnia* inhabitants of Sierra Nevada lakes

We obtained 30 different alleles in eight microsatellites, specifically 3, 3, 5, 4, 3, 3, 5 and 4 at loci Dp208, Dp304, Dp291, Dp335, Dp339, Dp522, Dp514alt and Dp70, respectively. Within each lake, the number of alleles per microsatellite ranged between 1 and 3. The expected number of MLGs at the lowest common sample size (rarefaction) and the Shannon–Wiener Index of MLGs with rarefaction were low in all lakes (Table II).

According to Rogers' genetic distance, a high similarity was observed among most MLGs, although two broadly divergent groups were identified (Fig. 3, Table S5), assigning MLGs from BG to one group and those from the other Sierra Nevada lakes to the other. The same MLG was observed in all samples from CL, VM, RS, RSS and YE (Fig. 3). MLGs from BG exhibited heterozygosity at most microsatellite loci (Data S1).

PCA of genetic data also showed a clear separation between individuals/resting eggs from BG and those from the other Sierra Nevada lakes, according to PCA axis 1 (Fig. 4). This PCA axis explained a 91% of the genetic variation.

Genetic structure did not change over time, detecting the same MLG in all sediment layers of lakes RS, RSS and VM and observing no relevant genetic differences among sediment layers of lakes BG, CB and CD (Fig. S2 in Supplementary Information).

Reproduction mode of *Daphnia* in Sierra Nevada lakes

The same MLG(s) were found in water column and sediments in all lakes for which water and sediment samples were available (CB, CD and RS, Table S1 in Supplementary Information) with the exception of CD, where two MLGs were found in sediments but only one in the water column, although one of the former was only detected in a single resting egg (see Fig. 3 and Fig. S2 in Supplementary Information). A single MLG was found in the other lakes from which water samples were obtained (CL and YE), and it was the same as the only MLG observed in samples from RS, RSS and VM (Fig. 3). Moreover, as previously commented, virtually identical MLGs were observed in the sediment layers of each lake, and no sexually produced eggs can be expected to have the same genotypes by chance.

Microsatellite genotypes of *Daphnia* were not in Hardy–Weinberg equilibrium in any lake, and deviations from expected heterozygosities were large in all lakes, due to high negative inbreeding coefficients that were significantly lower than 0 (Table II). Moreover, highly

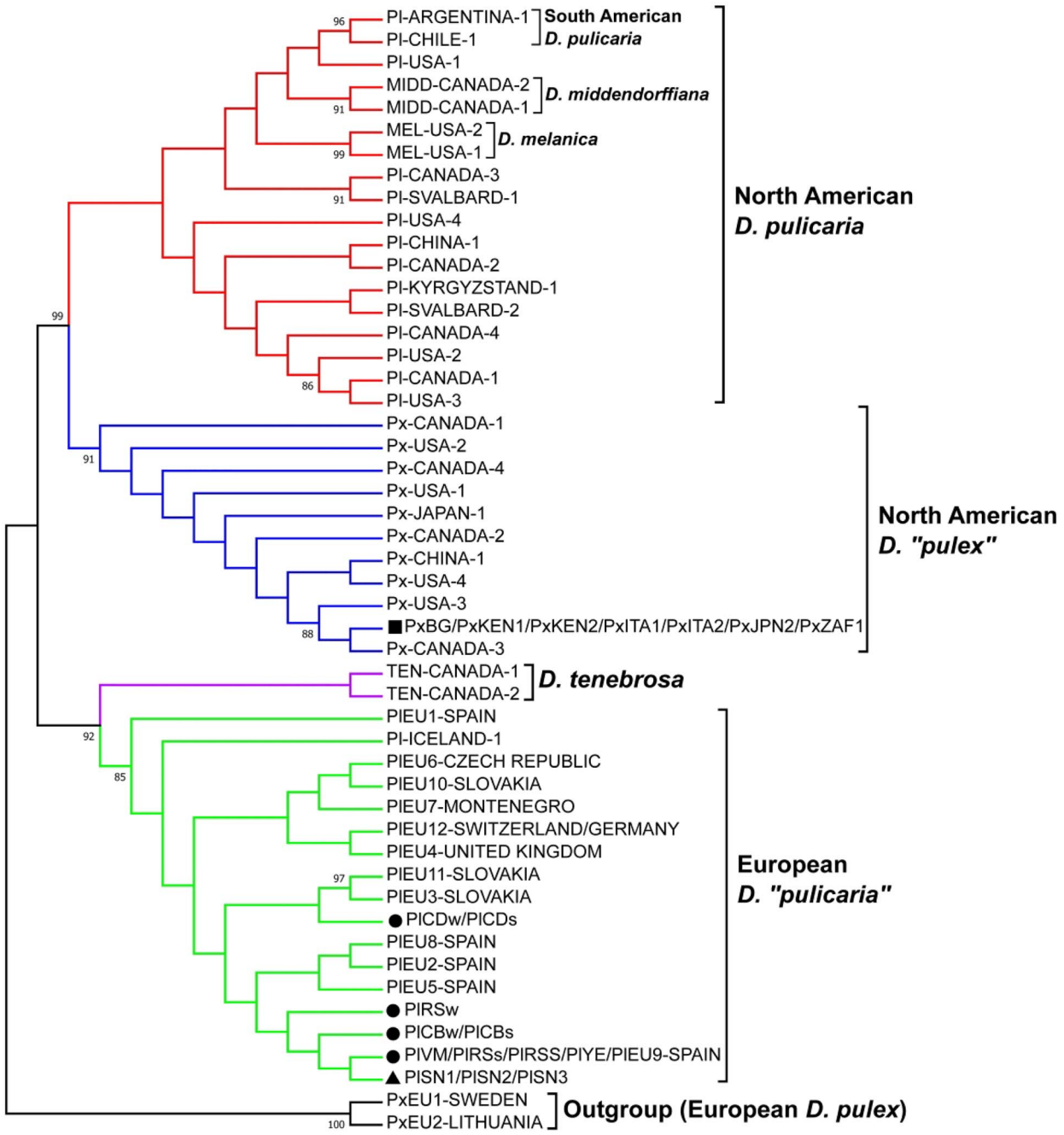

Fig. 2. Phylogenetic tree of *D. pulex* complex based on ND5 sequence. The evolutionary history was inferred by using the maximum likelihood method based on the Hasegawa–Kishino–Yano model (Hasegawa *et al.*, 1985). The bootstrap consensus tree inferred from 1000 replicates (Felsenstein, 1985) is taken to represent the evolutionary history of the taxa analyzed (Felsenstein, 1985). Branches corresponding to partitions reproduced in less than 50% of bootstrap replicates are collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the maximum composite likelihood (MCL) approach and then selecting the topology with superior log likelihood value. A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories (+G, parameter = 0.3046)). The analysis involved 50 haplotypes (Table S3 in Supplemental Information). All positions containing gaps and missing data were eliminated. There were a total of 380 positions in the final dataset. Evolutionary analyses were conducted in MEGA 7 (Kumar *et al.*, 2016). Sequences obtained in this study are indicated with black circles except that from Borreguil lake (PxBG), which is indicated with a black square (within a set of collapsed sequences). Sierra Nevada sequences obtained by Marková *et al.* (2013) are indicated with a black triangle.

Table II: Mean number of alleles per locus (A), within-population gene diversity (H_s) for all loci, mean inbreeding coefficient (F_{IS}) for all polymorphic loci in each population, number of MLGs observed, expected number of MLGs (eMLG) at the lowest common sample size (rarefaction), Shannon–Wiener Index of MLG with rarefaction (Hest) and sample sizes for each lake (n)

	A	H_s	F_{IS}	MLG	eMLG	Hest	*n*
BG	1.750 (0.46)	0.379 (0.234)	-0.876*	5	1.65	0.25	43
CB	1.750 (0.46)	0.357 (0.231)	-0.913*	2	1.98	0.59	29
CL	1.625 (0.52)	0.323 (0.267)	-1.000*	1	1.00	0.00	16
CD	1.750 (0.71)	0.324 (0.268)	-0.980*	2	1.35	0.13	20
VM	1.625 (0.52)	0.331 (0.274)	-1.000*	1	1.00	0.00	9
RS	1.625 (0.52)	0.316 (0.261)	-1.000*	1	1.00	0.00	50
RSS	1.625 (0.52)	0.337 (0.279)	-1.000*	1	1.00	0.00	7
YE	1.625 (0.52)	0.329 (0.272)	-1.000*	1	1.00	0.00	10

Standard deviations are given in brackets.
*Significant values after adjusting the nominal level (5%) for multiple comparisons ($P < 0.0018$), standard Bonferroni correction, indicating that F_{IS} values are significantly lower than zero (heterozygote excess).

Table III: Permutation correlation tests between PCo scores of the Daphnia genetic data and environmental/spatial variables (n = 8)

	PCo axis 1			PCo axis 2			PCo axis 3		
	r	perm *P*	*Q* value	*r*	perm *P*	*Q* value	*r*	perm *P*	*Q* value
Alkalinity (log)	-0.384	0.237	0.711	-0.165	0.666	0.984	0.554	0.104	0.624
Altitude	0.275	0.980	0.986	-0.279	0.545	0.984	0.602	0.155	0.636
Lake area	-0.234	0.597	0.984	0.022	0.693	0.984	0.313	0.621	0.984
Conductivity (log)	-0.220	0.523	0.984	0.014	0.978	0.986	0.835	**0.004**	0.056[m]
Calcium (log)	-0.280	0.518	0.984	-0.062	0.908	0.986	0.787	**0.002**	**0.046**
Catchment area	0.614	0.197	0.636	0.044	0.773	0.984	0.365	0.380	0.887
Chlorophyll-a (log)	0.047	0.905	0.986	-0.361	0.313	0.876	0.706	0.087	0.609
Maximum depth	-0.257	0.352	0.887	-0.078	0.847	0.986	0.128	0.953	0.986
pH	-0.211	0.423	0.935	-0.578	0.161	0.636	-0.619	0.195	0.636
Total nitrogen (log)	0.036	0.531	0.984	0.077	0.926	0.986	0.957	**0.002**	**0.046**
Total phosphorus (log)	0.310	0.379	0.887	0.107	0.841	0.986	0.129	0.723	0.983
Axis 1PCNM	-0.255	0.986	0.986	0.102	0.665	0.984	-0.921	**0.028**	0.298
Axis 2PCNM	0.607	**0.037**	0.309	0.197	0.764	0.984	0.197	0.764	0.983
Axis 3PCNM	-0.237	0.569	0.984	-0.492	0.194	0.636	-0.493	0.194	0.636

Axis 1PCNM. 2PCNM and 3PCNM: PCNM axis scores obtained from geographic distances among Sierra Nevada lakes; *r*: Pearson correlation coefficient; perm *P*, permutation *P* value; *Q* value, permutation *P* value adjusted by the false discovery rate method (Benjamini and Hochberg, 1995). Significant *P* or *Q* values highlighted in bold (<0.05); m, marginal significant *Q* value (<0.1).

significant $P_{sex}(F_{IS})$ values ($P_{sex}(F_{IS}) \leq 0.001$, $P < 0.001$) were found in all of the repeated MLGs from Sierra Nevada lakes; therefore, it appears unlikely that individuals or resting eggs originated from distinct sexual events.

Clonal distribution in relation to environmental/spatial variables

Pearson correlations of PCo axes scores (based on Rogers' genetic distance matrix) with geographic distance (PCNM axis 1 and 2) obtained significant *P* values, although *Q* values were not significant, (Table III). PCo axis 3 scores were correlated with conductivity, TN and Ca, and all *Q* values were significant, except for conductivity, which was marginally significant (Table III).

DISCUSSION

This is the first time that a NA *D.* "*pulex*" haplotype is found in the Sierra Nevada and in a European high mountain system. Our study of its historical colonization reveals genetic homogeneity over time, which was also observed for the native Eu *D.* "*pulicaria*" in Sierra Nevada lake populations. Various environmental factors may be related to the global genetic structure of the *D. pulex* complex in Sierra Nevada, including water mineralization. There has been scant published research on these issues.

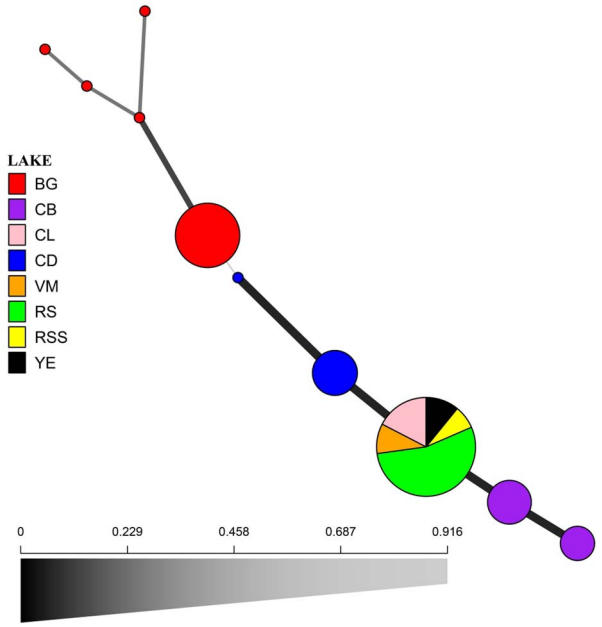

Fig. 3. Minimum spanning network using Rogers' genetic distance for *Daphnia* microsatellite markers. Each node represents a unique MLG. The node sizes are related to the number of samples. Colors represent lake membership, while edges (lines) represent the minimum genetic distance between individuals/resting eggs. Lake name abbreviations: BG, Borreguil; CB, Caballo, CL, La Caldera; CD, Cuadrada; VM, Virgen Media; RS, Río Seco; RSS, Río Seco Superior; YE, Las Yeguas.

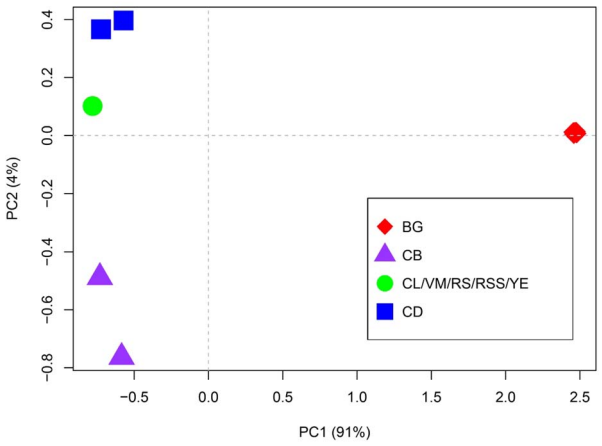

Fig. 4. PCA of genetic variations in individuals or resting eggs of *Daphnia*. Lake name abbreviations: BG, Borreguil; CB, Caballo, CL, La Caldera; CD, Cuadrada; VM, Virgen Media; RS, Río Seco; RSS, Río Seco Superior; YE, Las Yeguas.

The NA *D.* "*pulex*" lineage was only present in one lake (BG) and included five microsatellite genotypes, with one being much more abundant than the others. Eu *D.* "*pulicaria*" inhabited the other seven Sierra Nevada lakes under study. In a previous study, Marková *et al.* (2013) genetically identified individuals of Eu *D.* "*pulicaria*" in two Sierra Nevada lakes (according to their geographical coordinates, CL and RS), in agreement with the present results.

According to the phylogenetic tree created, these NA *D.* "*pulex*" individuals are closely related to individuals described as a hybrid of NA *D.* "*pulex*" and NA *D. pulicaria* and considered an invasive species in Africa (Mergeay *et al.*, 2006); therefore, NA *D.* "*pulex*" in BG may be also a hybrid. In fact, MLGs from BG exhibited heterozygosity at most microsatellite loci, suggesting their hybrid origin (Ma *et al.*, 2019). This lineage has an almost worldwide distribution, being present in every continent except Antarctica (Crease *et al.*, 2012). In the Mediterranean region, NA *D.* "*pulex*" has already been reported in the east of Spain (Schwenk *et al.*, 2000), north of Italy (Vergilino *et al.*, 2011) and Sardinia (Fadda *et al.*, 2011). However, it was only found in medium- to lowland waterbodies (altitude <714 m.a.s.l.), whereas we now show the presence of NA *D.* "*pulex*" in a European high-altitude lake (3020 m.a.s.l). According to an analysis of cladoceran remains in BG sediments over the past ∼180 years, daphniid remains apparently disappeared in sediments dated to ∼1957 (Jiménez *et al.*, 2018), and no *Daphnia* ephippia were found in earlier BG sediments in the present study.

In Sierra Nevada, sedimentary resting eggs have identical heterozygous MLGs to those of individuals retrieved, when possible, from the water column. Moreover, sedimentary resting eggs of Eu *D.* "*pulicaria*" were identical among most of the lakes. We also observed a low genotype richness and high deviations from Hardy–Weinberg equilibrium (excess of heterozygotes) in all *Daphnia* haplotypes of Sierra Nevada studied in addition to highly significant values of $P_{sex}(F_{IS})$. All of these features characterize populations that reproduce by obligate parthenogenesis (Dufresne *et al.*, 2011).

In Europe, Dufresne *et al.* (2011) showed that this reproduction mode was common in populations of Eu *D.* "*pulicaria*" in the High Tatra Mountains but not in those in the Pyrenees. We now show that obligate parthenogenesis is the reproductive mechanism of Eu *D.* "*pulicaria*" in Sierra Nevada, the southernmost alpine mountain range in Europe, complying with geographical parthenogenesis, i.e. the distribution of asexual populations at high altitudes and latitudes and in extreme areas (Vandel, 1928; cited by Dufresne *et al.*, 2011). Dufresne *et al.* (2011) suggested that obligate parthenogens are confined to High Tatra Mountains due to their superior colonization abilities or fitness in comparison with cyclic parthenogens in the region. Obligate parthenogenesis may be advantageous to colonize remote areas with harsh conditions because resting eggs are directly produced by mitosis, saving the time spent on parthenogenesis to produce males that would then mate with females. However, sexual individuals may show higher fecundity and/or survivorship

with respect to obligate parthenogens (Lehtonen *et al.*, 2012), and high abortion rates were observed for obligate parthenogenetic Eu *D.* "*pulicaria*" in a Sierra Nevada lake (Conde-Porcuna *et al.*, 2011). There is no evident explanation for the presence of obligate parthenogenetic Eu *D.* "*pulicaria*" in the High Tatra Mountains and Sierra Nevada but not in the Pyrenees. Dufresne *et al.* (2011) suggest that obligate parthenogenesis takes place in High Tatra Mountains because they have been recolonized from multiple sources after glaciation. Further information is needed on other high mountain systems in Europe.

The finding that all haplotypes of the *D. pulex* complex in Sierra Nevada lakes use this reproduction mode was expected, given previous reports on the virtual absence of males of *D.* "*pulicaria*" in Sierra Nevada lakes (Pérez–Martínez *et al.*, 2007). However, we detected males of NA *D.* "*pulex*" in BG, despite this lineage being obligate asexual, because it can produce males that transmit asexuality to sexual lineages (Innes and Hebert, 1988; So *et al.*, 2015; Xu *et al.*, 2015). Mergeay *et al.* (2006) described a rapid spread throughout Africa of hNA *D.* "*pulex*" and the apparent disappearance of the native cyclical parthenogenetic *D. pulex* within a similar time scale. Further research is needed to know if there is a competitive advantage of hNA *D.* "*pulex*," because some European alpine lakes are inhabited by cyclical parthenogenetic populations of Eu *D.* "*pulicaria*", such as those in the Pyrenees (Dufresne *et al.*, 2011), which could be vulnerable to colonization by NA *D.* "*pulex*".

The colonization route of NA and/or hNA *D.* "*pulex*" from North America to other continents is variable or unknown. Human actions are involved in some cases (Mergeay *et al.*, 2006; So *et al.*, 2015), while colonization appears to be mediated by natural dispersal in others, mainly via migratory birds (Ma *et al.*, 2019; Mergeay *et al.*, 2006; So *et al.*, 2015). At any rate, there has been no research on the mechanisms by which NA *D.* "*pulex*" entered the Mediterranean region.

Despite suggesting that the colonization of NA *D.* "*pulex*" is related to natural dispersal, So *et al.* (2015) employ the term *invader*, while many authors consider that this term should be reserved for species introduced through human action (Blackburn *et al.*, 2011; Lockwood *et al.*, 2013; Occhipinti-Ambrogi and Galil, 2004). We use the term *colonizer* hereafter to refer to NA *D.* "*pulex*" in Sierra Nevada, which appears to be restricted to a single lake, following Hoffmann and Courchamp (2016),

Diapausing propagules of *Daphnia* species can be naturally dispersed by wind, fish and waterbirds, among other vectors (Mellors, 1975; Moreno *et al.*, 2019; Vanschoenwinkel *et al.*, 2008). However, there are no fish or waterbirds in Sierra Nevada lakes, and other less likely biotic vectors include non-aquatic birds present at high altitude (>2500 m.a.s.l.), mainly the dotterel (*Charadrius morinellus*), a migrant species that arrives at the end of summer before flying to northern Africa (Garzón Gutiérrez and Henares Civantos, 2012; Whitfield *et al.*, 1996). Other bird species seen at altitude in Sierra Nevada make numerous stops during their migration (e.g. *Oenanthe oenanthe*), but this migration pattern is thought to have low potential for propagule long-distance dispersal (Viana *et al.*, 2016). In consequence, long-distance dispersal by birds from sub-Saharan Africa to Sierra Nevada lakes appears very unlikely, and abiotic vectors, such as winds, are more likely to be responsible for the transport of NA *D.* "*pulex*" from Africa.

Although the dispersal of zooplankton diapausing propagules by wind appears to be infrequent (Jenkins and Underwood, 1998; Moreno *et al.*, 2016), a water system can be colonized by a single individual (Ortells *et al.*, 2014). The geographical location of the lake and chance may likely determine "propagule" colonization via winds from different directions. The passive deposition of propagules appears to be more influenced by wind direction than by wind speed (Moreno *et al.*, 2016), and the winds in Sierra Nevada are predominantly northeast or southwest (Conde-Porcuna *et al.*, 2014). NA *D.* "*pulex*" is present in a lake (BG) that is to the east of the line of peaks and can therefore be more easily reached by northeast winds in comparison with the other study lakes (Fig. 1), and the wind transportation of a few zooplankton individuals could potentially result in the random colonization of a lake by a species over a long time period (Moreno *et al.*, 2016). Awareness of this colonization in Sierra Nevada is important because of the risk that NA *D.* "*pulex*" could reach other Sierra Nevada lakes in the future, placing the native Eu *D.* "*pulicaria*" in danger. It is surprising that the most abundant clone of Eu *D.* "*pulicaria*" is present in the central region of Sierra Nevada alone, suggesting a possible spatial pattern of *Daphnia* clones that is not influenced by the wind direction. Dispersal limitation, founder effects and/or environmental lake conditions may explain why NA *D.* "*pulex*" has not spread from BG to other Sierra Nevada lakes.

The physical barrier of the line of peaks may limit dispersal in lakes to the west of the Sierra Nevada but would not account for the absence of NA *D.* "*pulex*" in eastern lakes that are close to BG in the same valley, although these were recently reported to lack the environmental conditions (deep water with no permanent outlet) required for the presence of *Daphnia* in Sierra Nevada lakes (Morales-Baquero *et al*., 2019; Pérez-Martínez *et al.*, 2020a).

The absence of new colonization in Sierra Nevada may be attributable to founder effects (De Meester *et al.*,

2002; Haileselasie *et al.*, 2018; Ventura *et al.*, 2014). However, besides founder effects, genetic differences in *Daphnia* among Sierra Nevada lakes may be related to colonization by pre-adapted genotypes of obligate parthenogens of *Daphnia* across an environmental gradient, because no temporal genetic changes were observed in any lake. Correlations between environmental factors and genetic variations are expected to be stronger in asexual than sexual organisms (De Meester *et al.*, 2002; Haileselasie *et al.*, 2018), and we observed a possible influence of water mineralization and TN concentration on the genetic structure of *Daphnia* in the present study. The same MLG was observed for *Daphnia* in lakes with higher water mineralization (Ca concentration and conductivity) and higher TN concentrations, whereas different MLGs were observed in the other lakes. As expected, a major genetic differentiation was observed between the MLGs in BG (NA *D.* "*pulex*") and those in other Sierra Nevada lakes studied (Eu *D.* "*pulicaria*"). BG, CB and CD were all characterized by low mineralization, with low concentrations of Ca and nitrogen (Table I, Fig. 3). It is therefore possible that these three lakes have not been colonized by the most abundant clone of Eu *D.* "*pulicaria*" in Sierra Nevada because of their water conditions.

Our observation of the relationship between total nitrogen concentrations and the genetic differentiation of *Daphnia* in Sierra Nevada lakes is in line with the previous observation by Haileselasie *et al.* (2016) of an association between total nitrogen and the clonal composition of NA *D. pulicaria* in Greenland. At any rate, nitrogen-limited conditions may even increase the survivorship and resistance to food limitation of *D. pulex* (Groeger *et al.*, 1991) and other zooplankton populations (Conde–Porcuna, 2000; Ramos-Rodríguez and Conde-Porcuna, 2003). Consequently, the lower nitrogen concentrations in BG, CB and CD cannot, in our view, be considered harsh conditions for *Daphnia* populations.

Previous studies have also demonstrated that water Ca concentrations (Ashforth and Yan, 2008; Prater *et al.*, 2016) and conductivity (Dionne *et al.*, 2017; Jose and Dufresne, 2010) affect populations of the *Daphnia pulex* complex. Ashforth and Yan (2008) established a 0.0025–0.0125 mM Ca threshold for their survival and reproduction, although discrepant findings have been published on the survival threshold of this complex (Azan *et al.*, 2015; Pérez-Fuentetaja and Goodberry, 2016). It has also been observed that clonal groups of *D. pulex* complex may be restricted to specific conductivity values (Weider and Hebert, 1987). Further laboratory experiments are needed on the tolerance of these Sierra Nevada clones to low ambient Ca and conductivity to elucidate the response of the *D. pulex* complex to mineralization levels.

Changes in plankton species in Sierra Nevada lakes over the past century indicate alkalinization and increased Ca concentrations, which have been attributed to warming and to Saharan Ca inputs (Jiménez *et al.*, 2018; Pérez-Martínez *et al.*, 2020b). In this regard, Jiménez *et al.* (2018) found that the number of *Daphnia* in some Sierra Nevada lakes increased with higher atmospheric Ca deposition. Future increases in Ca and conductivity could lead to changes in microcrustacean populations in Sierra Nevada, as expected by Dionne *et al.* (2017) for subarctic and arctic lakes.

CONCLUSIONS

We describe for the first time the presence of NA *D.* "*pulex*" in a high-mountain lake of Europe, which most likely resulted from natural colonization. The restriction of this colonizer to a single Sierra Nevada lake since its arrival ~65 years ago may have been due to dispersal limitation, environmental lake conditions (colonization by pre-adapted genotypes) and/or founder effects. In Sierra Nevada, *Daphnia* exhibit a low genetic diversity and an asexual reproduction mode (clonal growth). Genetic differences between *Daphnia* inhabitants of Sierra Nevada lakes may be mediated by the colonization of pre-adapted genotypes to water mineralization, while the lack of temporal differences in the genetic structure of NA *D.* "*pulex*" and Eu *D.* "*pulicaria*" in Sierra Nevada lakes indicates either a high plasticity or a low capacity for variation or a combination of both. Further research is warranted not only to compare demographic traits of these lineages but also to test the tolerance of the species and genotypes to different degrees of water mineralization. This is important to evaluate the risk of colonization by NA *D.* "*pulex*" of other high-mountain lakes in Sierra Nevada and in the rest of Europe.

SUPPLEMENTARY DATA

Supplementary data is available at *Journal of Plankton Research* online.

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