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High genetic variation and phylogeographic relations among Palearctic fairy shrimp populations reflect persistence in multiple southern refugia during Pleistocene ice ages and postglacial colonisation

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Abstract

1. Intense anthropogenic disturbance threatens temporary pond ecosystems and their associated fauna across the Palearctic. Since fairy shrimps (Crustacea, Branchiopoda) are endemic to temporary ponds, populations are declining due to habitat loss and it is important to define adequate units for conservation.
2. Phylogeographic reconstructions, based on genetic variation, provide valuable information for defining evolutionary and conservation units, especially for organisms with high levels of cryptic diversity like many fairy shrimps. We studied a total of 152 individuals of the fairy shrimp *Branchipus schaefferi* from 79 populations across the Palearctic and used mitochondrial (CO1) and nuclear (ITS1) DNA data to reconstruct the phylogeography of the species.
3. Our results show that *B. schaefferi* comprises four highly diverged (10.3–16.5%) evolutionary clades. The present-day haplotypes within each of the clades probably diverged from lineages that were maintained in separate refugia during the Pleistocene ice ages. While two clades represent distinct geographic regions, the two remaining clades have more wide and overlapping ranges. In addition, the limited number of shared haplotypes among populations from geographically distant regions within three of the clades suggest recent long-distance dispersal events.

4. Overall, the studied *B. schaefferi* dataset comprises high levels of genetic differentiation, without a clear morphological signal. Phylogenetic searches and pairwise genetic distances suggest that the studied lineages belong to a complex of four morphologically cryptic species. Since these four evolutionary old clades persist (±2 million years), despite overlapping geographic ranges and since they span a variety of ecological conditions, they should be considered as separate evolutionary significant units for conservation.

KEYWORDS
dispersal, freshwater, habitat destruction, molecular clock, temporary ponds

1 | INTRODUCTION

Although temporary ponds are common aquatic habitats across many regions, they are increasingly threatened by human activities including urbanisation, draining, and intensification of agriculture (Silva, Phillips, Jones, Eldridge, & O'Hara, 2007; Van den Broeck, Waterkeyn, Rhazi, & Brendonck, 2015a; Van den Broeck, Waterkeyn, Rhazi, Grillas, & Brendonck, 2015b). Due to their typically small size and the fact that their filling and drying depends on rainfall and temperature, they are also particularly vulnerable to climate change (Moss, 2012; Stoks, Geerts, & Meester, 2014; Tuytens, Vanschoenwinkel, Waterkeyn, & Brendonck, 2014). However, these systems have a high ecological importance as feeding grounds for migratory birds, stepping-stones for dispersal of aquatic organisms, and habitats of a specialised aquatic fauna and flora with high degrees of endemicity (Williams, 2006).

Large branchiopod crustaceans (Crustacea, Branchiopoda; group including the fairy shrimps) are an iconic group of temporary pond inhabitants. They typically grow and mature fast as an adaptation to the short growing seasons, determined by the time-constrained wet phase of the pond. In addition, they bridge dry periods through the production of drought-resistant dormant stages (Dumont & Negrea, 2002). Dormant stages also serve as propagules for spatial dispersal via wind, flowing water, or through animal vectors (Bilton, Freeland, & Okamura, 2001; Pinceel, Brendonck, & Vanschoenwinkel, 2016). Large branchiopods are important components of food webs, for example as a major food source for migratory birds (Horváth, Vad, Vörös, & Boros, 2013) or as competitors and predators of plankton communities (Lukić, Horváth, Vad, & Ptacnik, 2018; Sánchez & Angeler, 2007; Waterkeyn, Grillas, Anton-Pardo, Vanschoenwinkel, & Brendonck, 2011). Since temporary ponds are destroyed at a fast rate, large branchiopods are globally considered to be a vulnerable group with a constant decline in distribution (Brendonck, Rogers, Olesen, Weeks, & Hoeh, 2008).

The fairy shrimp *Branchipus schaefferi* Fischer 1934 occurs in temporary freshwaters across Europe, Northern Africa, and Asia (Al-Sayed & Zainal, 2005; Brtek & Thiéry, 1995). Given its distribution and phenology, *B. schaefferi* is considered to be a warm water species (Mura, 1999; Vanschoenwinkel, Brendonck, Pinceel, Dupriez, & Waterkeyn, 2013). In most of Europe, the species usually occurs from late spring to early autumn (Eder, Hödl, & Gottwald, 1997; Petrov & Cvetković, 1997). In warmer regions in Northern Africa, the Mediterranean, and Asia, populations have been reported throughout the year (Marrone & Mura, 2006). While the ecology, taxonomy and range of occurrence of the species have been addressed to some extent (Brtek & Thiéry, 1995; Gandolfi, Rossi, & Zarattini, 2015; Vanschoenwinkel et al., 2013), a range-wide molecular phylogeography is lacking. Given that a number of closely related species still needs to be validated (Belk & Brtek, 1995; Gandolfi et al., 2015), a full-scale genetic study would provide essential complementary information to resolve taxonomic relationships and point to meaningful taxonomic units.

Genetic data can provide highly valuable information to study the history and diversity of a species. It can, for instance, be used to detect historical gene flow among populations and to reconstruct dispersal events more precisely than with only traditional methods based on morphological features (Freeland, Kirk, & Peterson, 2012). Conservation of the full adaptive potential of a species should have priority over simple species conservation (Moritz, 1994; Ryder, 1986; Waples, 1995). Large branchiopods are known for high levels of cryptic genetic diversity among individuals that look morphologically similar (Aguilar et al., 2017; Pinceel et al., 2013a,2013b; Schwentner et al., 2013). Such individuals could differ extensively in physiology and may represent distinct evolutionary significant units (ESUs) for conservation (Pinceel et al., 2013b). Finally, phylogeographic studies may improve our understanding of the effect of past climate events, which, in turn, may serve to forecast consequences of future environmental changes (Pinceel et al., 2013a,b).

The phylogeography of a number of large branchiopod species in Europe and North Africa has been reconstructed and many studies show limited genetic divergence among populations, especially in more northern regions of mainland Europe (Kappas et al., 2017; Reniers, Vanschoenwinkel, Rabet, & Brendonck, 2013; Vanschoenwinkel et al., 2012). This has been explained as a consequence of relatively recent range expansion from a small number of refugia after the Pleistocene glacials. Two studies have been undertaken to investigate specific aspects of the phylogeny of *B. schaefferi*, one based on allozymes and CO1 (Gandolfi et al., 2015) and another on 18S (Mioduchowska et al., 2018). These studies were, however, restricted to 11 populations in Italy, Spain, and Morocco (Gandolfi et al., 2015) and 11 populations in Poland, Italy, and Algeria (Mioduchowska et al., 2018).

Here, we conduct a large-scale phylogeographic study of the fairy shrimp species *B. schaefferi* across its range of occurrence. For this, we study the mitochondrial CO1 and nuclear ITS1 gene regions of individuals from 79 populations from wide areas in Europe and northern Africa and a single population in the Middle East. First of all, we perform phylogenetic searches and use sequence divergence based methods to verify if molecular data support the species status of the studied specimens, which were all identified as *B. schaefferi* based on morphological traits. Given the extensive geographic and ecological range of occurrence of the species, we expect high levels of genetic differentiation among certain populations. Second, we use genetic divergence data among genetically distinct groups and standard molecular clocks, to assess the likelihood of different historic scenarios as explanation for the current distribution of genetic lineages. Given the fact that *B. schaefferi* is mostly successful under relatively high temperatures, the Pleistocene ice ages would have driven *B. schaefferi* to extinction in Northern regions. Therefore, we hypothesise low levels of genetic diversity in Northern regions compared to high levels of diversity around glacial refugia. Finally, based on the level of genetic differentiation between identified haplotype groups we aim to delineate the ESUs important for conservation of the adaptive potential within *B. schaefferi*.

2 | METHODS

2.1 | Sampling procedure

Samples were collected from a total of 68 temporary ponds in Europe, northern Africa and one site in Bahrain (Asia). Most specimens were field collected between 1980 and 2016 and conserved in ethanol of variable strength. Upon reception of the samples at KU Leuven (2012–2016), all ethanol was substituted by pure grade absolute ethanol and samples were subsequently stored in a fridge at 4°C. Specimens from Morocco (Timahdite, Ifrane, Ighergharen, and unknown localities), around Alger in Algeria, El Battan in Tunisia, unknown locality in Malta, and Vars and Les Cannet-des-Maures in France (for accession numbers see Table 1) were obtained after hatching field-collected sediment with *B. schaefferi* egg banks in the laboratory.

2.2 | DNA extraction, polymerase chain reaction, DNA purification and sequencing

The molecular laboratory procedures to acquire the DNA sequences for the targeted genes were performed in two laboratories separately, at the Department of Genetics and Biosystematics, University of Gdansk in Poland (45 specimens from 10 Polish populations) and at the Laboratory for Animal Ecology, Global Change and Sustainable Development at KU Leuven in Belgium (all other specimens; see Supporting Information for both protocols).

2.3 | Phylogenetic and phylogeographical reconstructions

All generated *B. schaefferi* CO1 sequences were assembled and visually checked for quality using SeqScape v2.5. Consensus sequences were edited in BioEdit Sequence Alignment Editor (Hall, 1999). All sequences that contained insertions and/or deletions (15 in total) were removed from the CO1 alignments to avoid the risk of co-amplified nuclear mitochondrial pseudogenes interfering with the analyses (Song *et al.*, 2008). The newly generated sequences of *B. schaefferi*, together with existing *B. schaefferi* sequences from GenBank (Gandolfi *et al.*, 2015), one sequence of *Branchipus blanchardi* Daday 1908 (KP702861.1) and one outgroup taxon (CO1: *Branchipodopsis drakensbergensis* GU139737.1 and ITS1: *Branchipodopsis wolfi* MN325155), were aligned with the CLUSTALW multiple alignment tool in BioEdit. All sequences were uploaded to GenBank (for accession codes see Table 1). The most probable evolutionary model for both markers was determined in PhyML (Lefort, Longueville, & Gascuel, 2017) based on both the Bayesian information criterion and Akaike information criterion (AIC). For CO1, the AIC selected for a general time reversible model (GTR) with discrete γ model (+G; γ = 1.83) with invariable sites (I = 0.57) which was used to assemble the Bayesian inference (BI) and maximum likelihood (ML) tree. To assemble neighbour joining (NJ) trees, we used a Tamura Nei evolutionary model (TN93; Tamura & Nei, 1993) with a discrete γ distribution, which was the best scored available model for the NJ method. For ITS1, the Bayesian information criterion selected a Kimura 2-parametric model (K2P; Kimura, 1980), which was used for constructing the ML and NJ tree. The GTR with invariable sites was selected as most suitable evolutionary model by the AIC and used for assembling the BI tree since K2P models are not embedded within MrBayes. Substitution saturation was tested in DAMBE v. 7.0.28 (Xia & Kumar, 2018). The index of substitution saturation was significantly smaller than the critical index of substitution saturation, indicating little saturation (Xia & Lemey, 2009; Xia, Xie, Salemi, Chen, & Wang, 2003) for both markers. The haplotype number was determined based on calculated pairwise distances in MEGA X (Kumar, Stecher, Li, Knyaz, & Tamura, 2018).

The consensus phylogeny was constructed based on CO1 sequences by comparing phylogenetic trees obtained with four different methods of inference: NJ, ML, maximum parsimony (MP), and BI. ML analyses were performed in MEGA X and PhyML (Guindon et al., 2010) according to the GTR + G + I evolutionary model for the CO1 and K2P model for ITS1 with 1,000 bootstrap replicates. The MP analyses for CO1 were performed in PAUP* v4.0 (Swofford, 2001) and for ITS1 in MEGA. The settings included Heuristic search, Tree-Bisection-Reconnection, 1,000 saved trees, and 100 bootstrap replicates. The number of polymorphic and parsimony informative sites was also determined in PAUP*. NJ analyses were performed in MEGA X including 1,000 bootstrap replicates and partial deletion

TABLE 1 Overview of newly generated and GenBank sequences of *Branchipus schaefferi* with details on the localities

No.	ID	Country	Localities (pond number)	Latitude	Longitude	CO1 Acc. Nr.	ITS1 Acc. Nr.
1	A11	Algeria	Around Alger (1)	36.71	3.06	MK564523	–
2	A22	Algeria	Tassili N'Ajjer (1)	25.82	9.13	MK564489	MK643493
3	OOS1, OOS2	Austria	– (2)	–	–	MK523638–MK523640	MK643480–MK643482
4	DR1	Bahrain	– (1)	–	–	MK935170	MK643510
5	BI6, BI7, BI12	Belgium	Péronnes-lez-Binches (12)	50.43	4.15	MK449413–MK449424	MK643523–MK643527
6	KRO	Croatia	Konjsko (1)	43.586530	16.459980	–	MK643488
7	FC	France	Arles (1)	43.55	4.57	–	MK643476
8	DO(1-2)	France	Military field Auvours (1)	48.00	0.38	MK564519	MK643511
9	ARD	France	Bidon (1)	44.349675	4.534558	MK564499	MK643478–MK643479
10	FP	France	Borce (1)	42.833875	0.593552	MK564502	MK643521
11	DK1	France	Le Cannet-des-Maures (1)	43.348000	6.427778	–	MK643477
12	FAL	France	Sainte-Croix-en-Plaine (1)	48.032819	7.388567	MK564501	MK643522
13	DI(1-3), DJ(1,3), DL(1-3), DM(1-2), DN(1-2), DS(1-3), FS(1-2)	France	Military field Sissonne (7)	49.59	3.94	MK564504–MK564509, MK564511–MK564518, MK564520–MK564522	MK643512–MK643520
14	ALP	France	Vars (1)	44.577265	6.742022	MK564496–MK564497	MK643474–MK643475
15	KO3	Germany	Cologne (6)	50.88	7.14	MK523626–MK523637	MK643528–MK643531
16	HA	Hungary	Apaj (1)	47.12	19.08	MK564490	MK643533
17	DX	Hungary	Szentbékkálla (1)	46.89064	17.55569	–	MK643485
18	GPB, GPC	Italy	Lampedusa (1)	–	–	KP702853–KP702855	–
19	GA8	Italy	Lecce (1)	40.08056	18.48528	KP702849	–
20	GVF11	Italy	Monte Catabio (1)	42.5500	12.96889	KP702864–KP702865	–
21	GD(5-6)	Italy	Palermo (1)	38.20639	13.2825	KP702856–KP702857	–
22	GD7	Italy	Syracuse (1)	37.07528	15.28639	KP702858	–
23	GB1, GA10	Italy	Teramo (1)	42.6575	13.44889	KP702851–KP702852	–
24	GD11	Italy	Trapani (1)	38.18222	12.72389	KP702860	–
25	GA4	Italy	Trieste (1)	45.7775	13.59139	KP702848	–
26	GVF(4-5)	Malta	Il Qaliet (1)	–	–	KP702862–KP702863	–
27	MAL	Malta	– (1)	–	–	MK564491	MK643491–MK643492

(Continues)

TABLE 1 (Continued)

No.	ID	Country	Localities (pond number)	Latitude	Longitude	CO1 Acc. Nr.	ITS1 Acc. Nr.
28	DW	Morocco	Essaouira (1)	31.38503	−9.71633	MK618053	MK643494
29	DB(1-3), DE, DF(1-2), DG(1-3), DU	Morocco	Haut Atlas (5)	31.9	−8.4	MK618058, MK618060, MK618062–MK618064	MK643495–MK643503
30	DP(1-2)	Morocco	Ifrane (1)	33.406699	−5.115915	MK618066	MK643507–MK643508
31	MH	Morocco	Ighergharen (1)	30.661842	−9.414123	MK618055	—
32	GA9, GD10	Morocco	Marrakech (1)	32.08	−8.66	KP702850, KP702859	—
33	DH2	Morocco	Timahdite (1)	33.24	−5.06	MK618069	MK643509
34	MAR(1–3)	Morocco	— (3)	—	—	MK618070, MK618071	MK643504–MK643506
35	BIED	Poland	Biedrusko (6)	52.48	16.85	MK564492–MK564493, MK465095–MK465119	MK631958–MK631967, MK643483–MK643484
36	KON(1–2)	Poland	Drawsko (2)	53.53	15.8	MK465085–MK465094	MK631970–MK631973
37	PA	Poland	Piła (1)	53.13	16.8	MK465075–MK465077	MK631968–MK631969
38	ST(1–2)	Poland	Slupsk (2)	54.43	17.05	MK465078–MK465084	MK631974–MK631976
39	SRB2	Serbia	Bačko Gradište (1)	45.53669	20.06996	MK564495	MK643486–MK643487
40	SRB1	Serbia	Northern Banat (1)	46.04736	20.19385	MK564494	MK643532
41	GG1	Spain	Vinaròs (1)	40.46806	0.474722	KP702866	—
42	ELB1	Tunisia	El Battan (1)	35.748417	9.940022	MK523642	MK643489
43	TZ(1–2)	Tunisia	Hammam Bent Djedidi (1)	36.369444	10.440000	MK523643	MK643490

of 90% (<10% alignment gaps, missing data, and ambiguous bases were allowed at any position). Bayesian inference was performed in MrBayes (Huelsenbeck & Ronquist, 2001; Ronquist & Huelsenbeck, 2003; Ronquist et al., 2012). Markov Chain Monte Carlo analysis ran for 2×10^6 generations with a sampling frequency of 1,000 generations and a 25% burn-in. Pairwise genetic distances (based on K2P model) between all generated sequences and the mean genetic distances within and among the main groups in the phylogeny of *B. schaefferi* were calculated in MEGA X (Kumar, Stecher, & Tamura, 2016) with partial deletion of 90% (373 positions in the final data set).

To estimate the approximate timing of divergence among phylogenetic groups we applied various molecular clocks to the minimum and maximum of the calculated pairwise distances. For CO1, molecular clocks of 1.4 and 2.6% divergence per million years were applied as in Reniers et al. (2013). Eventually, the time range of the group split was estimated between the highest pairwise distance (D/2) divided by the lowest rate of evolution, for the most distant time scenario, and the lowest pairwise distance divided by the highest rate of evolution for the most recent likelihood of the events.

We used the automatic barcoding gap discovery method (ABGD) (Puillandre, Lambert, Brouillet, & Achaz, 2012) and the *4×-rule* (Birky, Adams, Gemmel, & Perry, 2010; Birky, Wolf, Maughan, Herbertson, & Henry, 2005) to assess whether the most distinct *B. schaefferi* groups within the phylogeny warrant a separate species status based on the studied CO1 fragment. The 4×-rule states that the lineages can be considered as separate species when the mean distance between them is at least four times larger than the mean divergence within the lineages (Birky et al., 2005). The ABGD method separates species based on the *barcode gap* that occurs when the divergence between individuals of the same species is lower that the divergence between the individuals of the different species (Puillandre et al., 2012). To run the ABGD method, we used the online version (http://wwwabi.snv.jussieu.fr/public/abgd/abgdweb.html) following the default settings.

3 | RESULTS

We generated 117 CO1 and 79 ITS1 sequences (accession codes Table 1) of *B. schaefferi* from 13 countries and 35 regions. All ponds in a region are counted as one locality and NAs are counted as separate localities. Generated ITS1 consensus sequences ranged from 494 to 695 bp. The length of the produced consensus sequences for CO1 ranged from 242 to 658 bp. The partial CO1 sequence from the Bahrain specimen was excluded. Combined with sequences drawn from GenBank (*Branchipus* populations primarily identified as *Branchipus visnyai* and *Branchipus pasai* were here referred as *B. schaefferi* since their synonymy was recently confirmed; Gandolfi et al., 2015), we compiled an alignment with 134 CO1 sequences from 71 populations distributed over 13 countries (40 regions).

3.1 | Genetic diversity

We identified a total of 31 unique CO1 haplotypes among the studied *B. schaefferi* individuals, based on calculated pairwise distances with K2P model and partial deletion of 90%. The overall average intraspecific genetic divergence was 10.9% considering all lineages of *B. schaefferi*. A total of 57 (+1 *B. blanchardi* sequence and one outgroup) lineages were included in phylogenetic reconstructions since multiple identical sequences of one locality were considered as a single lineage. In each case, the longest assembled sequence was chosen as representative lineage. Of 237 polymorphic sites, 178 were parsimony informative with a proportion of constant characters of 63.98%. Among individuals, genetic differentiation ranged from 0 to 19.0%. The lowest genetic differentiation was generally found among individuals from geographically clustered localities in France, Belgium, Morocco, Poland, and Austria. The highest difference was found between one French individual from Bidon and an individual from Morocco.

Based on the ITS1 marker, we identified 13 unique haplotypes. The overall average genetic divergence was 1.0% considering all lineages. A total of 59 (and one outgroup) lineages was included in the phylogenetic reconstructions, selected in the same manner as for CO1. There were 91 polymorphic sites, of which 20 were parsimony informative. Pairwise distances ranged from 0 to 2.6%, with the highest divergence between one specimen from the Camargue area (Arles) in France and one from the High Atlas mountain range in Morocco.

3.2 | Phylogenetic analyses based on CO1

The four different methods of phylogenetic inference (ML, MP, NJ, and BI) produced trees with a highly similar topology for the studied *B. schaefferi* populations (Figure 1). The phylogenetic search methods group the studied haplotypes in four clades (A–D; Figure 2), except when the population from Vars in France was placed as a separate group (i.e. clade) in MP tree. The most basal clade A within the evolutionary tree groups a total of seven haplotypes from the Mediterranean islands (Sicily, Lampedusa, Malta) and Tunisia. Subsequently, a clade B grouping the studied *B. schaefferi* populations from central (Austria, northern Italy, and Poland) and southern Europe (France and Spain) and a single haplotype from northern Serbia appears to have diverged. This clade represents seven distinct CO1 haplotypes. Next in line comes a clade C with 10 Moroccan haplotypes from six localities and a single population from Algeria and the extreme South of mainland Italy. Finally, the remaining seven haplotypes are grouped in a fourth monophyletic clade D. Although the majority of haplotypes in this clade originate from all across Europe, also two specific Moroccan (+1 shared with European populations) haplotypes and a single Algerian haplotype are included. Mean within-group K2P distances were 1.59% for clade A, 1.69% for clade B, 2.14% for clade C, and 1.64% for clade D. Mean between group (K2P) distances ranged from 10.3% between clades C and D to 16.5% between clades B and D (Table 2;

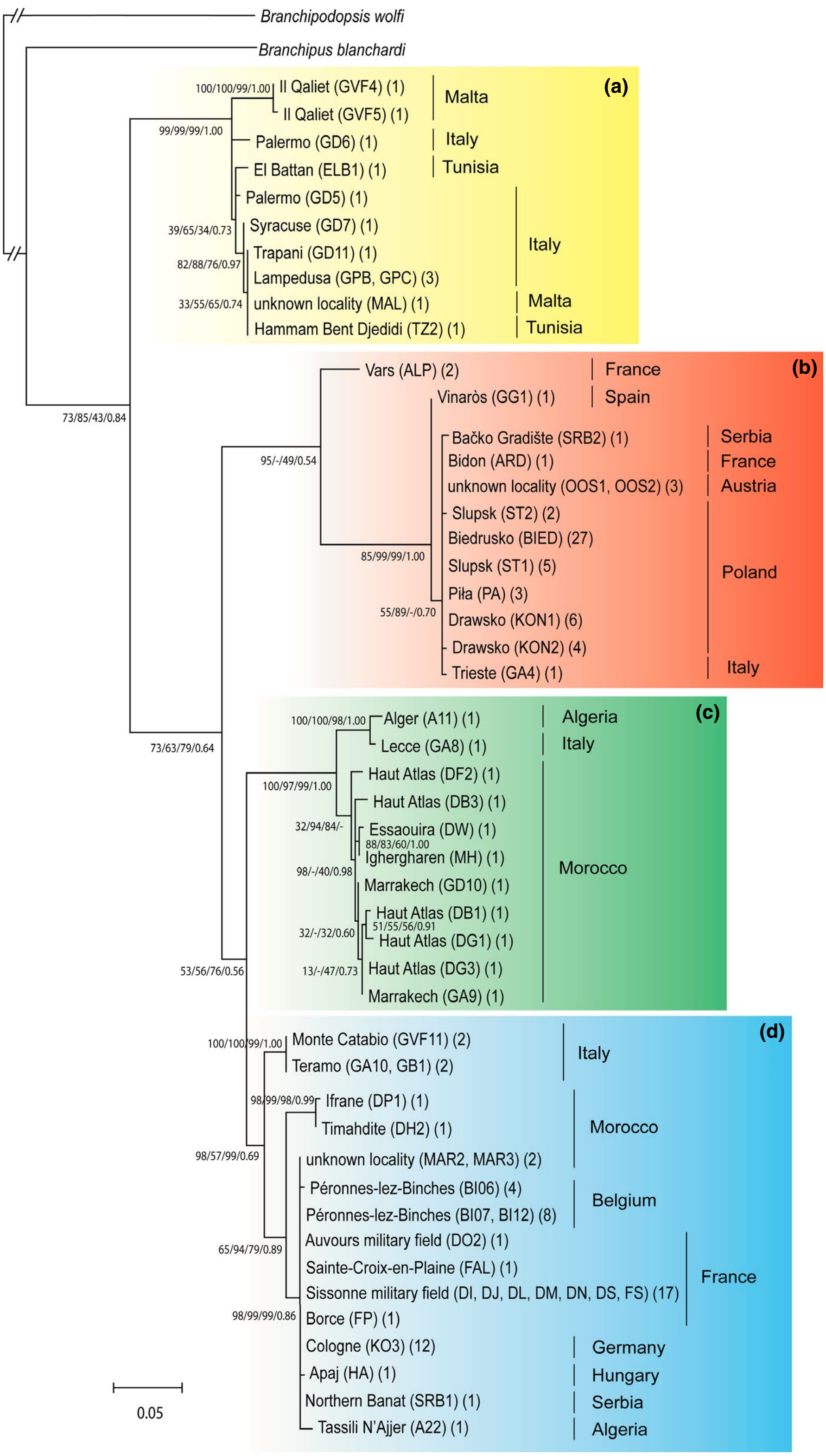

FIGURE 1 Consensus phylogenetic tree for *Branchipus schaefferi*, based on the mitochondrial CO1 gene fragment (maximum likelihood—ML, maximum parsimony—MP, neighbour joining—NJ and Bayesian inference—BI). The ML tree was used as a template. The supporting values of four evolution reconstruction methods are included close to the nodes (ML/MP/NJ/BI). The unsupported groupings are indicated with '–'. Codes within the first pair of brackets indicate the codes of sequenced specimens and numbers in the second pair of brackets specify the number of specimens from the same region that belong to the same haplotype. The groups (clades) identified by the phylogenetic search methods are indicated with the same colour-coding as in Figure 2: yellow—Clade A, red—Clade B, green—Clade C and blue—Clade D [Colour figure can be viewed at wileyonlinelibrary.com]

for TN93 + G between group distances see Table S2). Mean between group distances of *B. blanchardi* and four *B. schaefferi* clades were overall higher than distances between the *B. schaefferi* clades (Table 2). Both ABGD (prior maximal distance $p = 0.035$; Table S1) and the 4x-rule suggest that the clades should be considered as different species.

3.3 | Phylogenetic analyses based on ITS1

All phylogenetic search methods divided the studied haplotypes in two groups. The first group corresponded to the clade B recognised for CO1 (three haplotypes), while the second group included all other groups (A, C, and D, overall 10 haplotypes; Figure S1). Mean

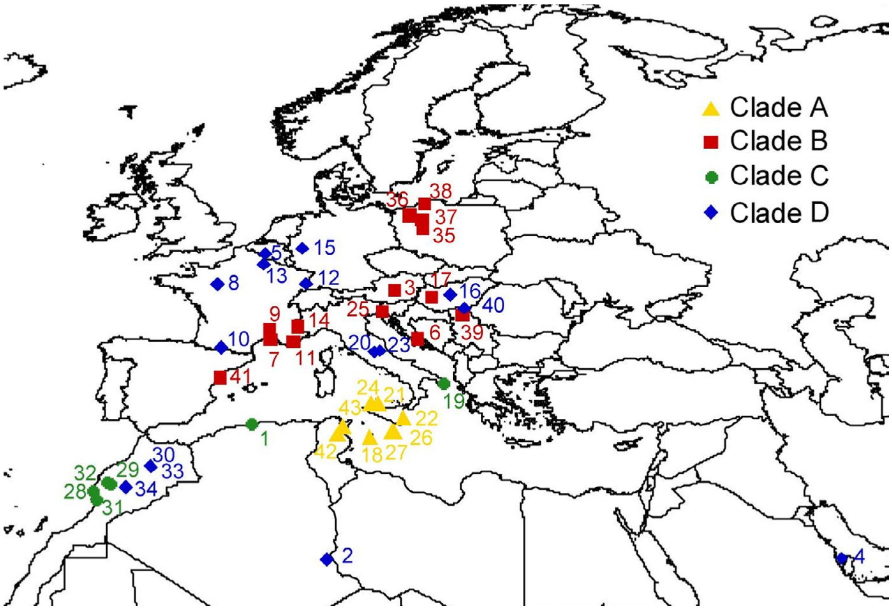

FIGURE 2 Distribution of sequenced *Branchipus schaefferi* populations. Locality numbers match to the population numbers in Table 1. Specimens were grouped based on the divergence of the CO1 gene (showed in different colours and shapes). Colours of the clades: (A) yellow, (B) red, (C) green, (D) blue. Shapes of the clades: (A) triangle, (B) square, (C) circle, (D) diamond. The coordinates for which exact localities were unknown were chosen based on country codes (https://developers.google.com/public-data/docs/canonical/countries_csv). The phylogenetic position of the specimen from Bahrain was determined based on the ITS1 and the partial CO1 sequence and the position of the specimens from Konjsko in Croatia, Arles and Le Cannet-des-Maures in France and Szentbékkálla in Hungary was based on the ITS1 region [Colour figure can be viewed at wileyonlinelibrary.com]

within-group (K2P) distances were 0.09% for clade B and 0.5% for the second group containing all remaining sequences. The clade B (for the CO1 gene) was also supported by all phylogenetic reconstructions based on the ITS1 region. This group contains three different ITS1 haplotypes, two from localities in France and a third, which represents all other specimens. Specimens from Konjsko in Croatia, Arles and Le Cannet-des-Maures in France, and Szentbékkálla in Hungary, for which no CO1 sequences could be generated, were included in clade B (Figure 2). The second (A, C, and D) group contains 10 haplotypes. All phylogenetic searches grouped lineages from Tunisia together as a sub-group with two haplotypes. This is consistent with the reconstructions based on CO1, with the exception that the haplotype from Malta is not separated from the other lineages. It should be noted that, based on the ITS1 region and the partial CO1 fragment, the studied specimen from Bahrain (R1) belongs to clade D.

TABLE 2 Divergence between the groups (clades) within *Branchipus schaefferi* and between *Branchipus blanchardi* (*B.b.*) and *B. schaefferi* clades

	Distances (%)			
Clades	Min	Max	Mean	Molecular clock (mya)
A–B	11.2	18.6	14.0	6.6–2.2
A–C	12.5	16.9	15.1	6.0–2.4
A–D	12.6	14.9	13.8	5.3–2.4
B–C	13.0	16.4	14.7	5.9–2.5
B–D	10.4	19.0	16.5	6.8–2.0
C–D	9.0	13.2	10.3	4.7–1.7
B.b.–A	16.5	18.9	17.8	6.7–3.2
B.b.–B	19.0	22.6	20.0	8.1–3.6
B.b.–C	21.3	25.2	23.0	9.0–4.1
B.b.–D	20.2	22.0	20.7	7.9–3.9

The table contains minimum, maximum, and mean genetic distances between groups (in %) and an assessment of the timing of divergence among groups (millions of years ago, mya). The number of base substitutions per site, averaged over all sequence pairs between groups, represents the mean distances. Analyses were conducted using the Kimura 2-parameter model (Kimura, 1980). Fewer than 10% alignment gaps, missing data, and ambiguous bases were allowed at any position. Sequences were 373 base pairs in length in the final alignment. Molecular clocks ranged between 1.4% and 2.6% of substitution per my (cf. Reniers et al., 2013).

4 | DISCUSSION

Our results demonstrate high levels of genetic differentiation among populations of the widely distributed Palearctic fairy shrimp species *B. schaefferi*. Pleistocene ice ages and long-distance dispersal events appear to have shaped the present-day diversity and distribution of genetic lineages. Phylogenetic searches and analyses of molecular divergence identified four major evolutionary groups within *B. schaefferi* which, according to sequence divergence-based species concept methods, could represent separate species. These clades should at least be considered separate ESUs for conservation purposes, especially since they have persisted for millions of years in separation under highly diverse ecological conditions.

4.1 | Pleistocene glaciations shaped the evolutionary history of *B. schaefferi*

Temperate Europe was characterised by extreme climatic fluctuations throughout the Pleistocene (2.6 million years ago [mya]–11,000 years ago). Periods of glaciation and extending ice cover were alternated with milder interglacial periods (Paillard, 1998). The Arctic ice cover was formed 2.4 mya and until 0.9 mya the ice coverage advanced and retreated in cycles of approximately 41,000 years (Hewitt, 2000). Later on, 0.9 mya—present, the glacial/interglacial cycles became more severe and typically lasted around 100,000 years (Paillard, 1998). Most species from temperate regions were affected by prolonged ice cover, which resulted in local extinctions, genetic bottlenecks, and range shifts. In contrast, warmer interludes were typically associated with demographic and range expansions (Hewitt, 2000). Even based on the least conservative molecular clocks available for CO1, the four major *B. schaefferi* clades identified in our study diverged before or during the first Pleistocene ice ages, around 6.8–1.7 mya. This suggests that at least four separate *B. schaefferi* refugia may have existed during the last glacial periods. An alternative—and non-mutually exclusive—explanation could be that the clades were reproductively isolated prior to the ice ages.

It seems reasonable to assume that the clade A and C lineages would have been least affected by the consequences of glaciation. Representatives from these clades all originate from localities in the Mediterranean and in northern Africa, areas far less affected by climate change throughout the Pleistocene than the more northern regions where *B. schaefferi* occurs today (Hewitt, 2000). The high genetic similarity among most *B. schaefferi* from the northern parts of Europe (Poland in clade B and Belgium and Germany in clade D) suggests that these areas were colonised relatively recently. In contrast, several of the southern populations (e.g. Morocco in clade C and Italy in clade A) are characterised by relatively high pairwise divergences. Furthermore, both within clades B and D, haplotypes from the most southern locations (Clade B: France and Spain; Clade D: Morocco and Italy) appear to be most basal in position. This is consistent with the notion that the Iberian and Apennine peninsula and the Balkans served as Pleistocene glacial refugia for many taxa (Hewitt, 2000), including fairy shrimps (Muñoz et al., 2008; Reniers et al., 2013).

Branchipus schaefferi and *Chirocephalus diaphanus* are both widespread species in Europe. However, they differ in some ecological traits. While *C. diaphanus* is typically a cold-tolerant species and appears in fall and early spring, *B. schaefferi* is a thermophilic species, generally present during late spring and summer in Europe (e.g. Petrov & Cvetković, 1997). Phylogenetic reconstructions revealed genetic differentiation between *C. diaphanus* populations from eastern and western Europe (Reniers et al., 2013). Our reconstructions for *B. schaefferi* are largely consistent with this observation. However, a number of shared haplotypes do occur among eastern and western regions (e.g. specimens from Serbia and Hungary present in clade D and the specimen from Spain in clade B). This is suggestive of recent long-distance dispersal events via vectors such as migrating water birds (Brochet et al., 2009; Green et al., 2005) and motorised vehicles (Waterkeyn et al., 2010).

4.2 | Indications for long distance dispersal

Within clades B and D, genetic variation is very limited among haplotypes from different localities, especially when excluding the basal haplotypes from France, Italy, and Spain. Relatively rapid postglacial recolonisation of northern regions may be a likely historic explanation underpinning this pattern. The fact that haplotypes are highly similar or even shared among distant regions such as among France and Morocco, underlines the potential for long-distance dispersal events of *B. schaefferi*. Recent gene flow across large geographic distances was also observed in other European fairy shrimp species including *Streptocephalus torvicornis* (Kappas et al., 2017) and *Branchinecta orientalis* (Rodríguez-Flores, Jiménez-Ruiz, Forró, Vörös, & García-París, 2017). In both studies, it is argued that effective long-distance dispersal through migratory birds is the most likely explanation. Fairy shrimp species produce small (typically ±200 μm for *B. schaefferi*), drought-resistant dormant eggs that are highly resistant to drying and adverse environmental conditions for periods of up to several years (Brendonck, Pinceel, & Ortells, 2017; Vanschoenwinkel et al., 2013). These eggs act as propagules for passive dispersal and can easily be ingested by water birds or sporadically stick to their feathers (Sánchez, Hortas, Figuerola, & Green, 2012). Results from field studies demonstrate that eggs of the fairy shrimps can be dispersed across long distances in such a way (Brochet et al., 2009; Green et al., 2005; Lovas-Kiss et al., 2018; Rogers, 2014). It is also likely that long-distance dispersal of *B. schaefferi* is mediated by migratory birds. For instance, haplotype links between populations from Algeria and those in Belgium and Hungary in clade D match the yearly migration routes of some wader birds (Svensson, 2009).

4.3 | The *B. schaefferi* species status and delineating ESUs

Although delineating new species surpasses the aim of this study, we would like to phrase a number of critical remarks with regard to the current grouping of all studied *B. schaefferi* individuals as a single species. First of all, the mean genetic differentiation, on the standard barcoding marker CO1, among the four different *B. schaefferi* phylogenetic clades ranged between 10.3 and 16.5%. This is in line with, or exceeds, commonly accepted CO1 species divergence thresholds of 7–10% for freshwater fairy shrimps (Cox & Hebert, 2001; Pinceel et al., 2013a; Reniers et al., 2013). Second, the results from the ABGD searches and the 4×-rule support a separate species status for the four separate clades. Third, while the degree to which the clades are reproductively isolated remains to be assessed experimentally, the reconstructed phylogeography suggests a degree of isolation, at least among clade B and clade D lineages. Despite the fact that clade B and clade D have a largely overlapping geographic range, individuals from both clades are genetically highly distinct, which implies that there is no interbreeding among representatives from the clades.

Combined, our results suggest that the studied *B. schaefferi* lineages probably represent four morphologically cryptic species, which correspond to the four clades in the reconstructed phylogeny. Therefore, the species should be subject to a taxonomic revision, based on combined information from morphological, genetic and ecological analyses. Clades A and C are especially vulnerable due to their restricted distribution in the Mediterranean region where their habitats are disappearing at an alarming rate (2015a; Van den Broeck, Waterkeyn, Rhazi, Grillas, et al., 2015b; Zacharias & Zamparas, 2010). Considering the current threats to *B. schaefferi* across its range of occurrence, we would for now at least like to promote the recognition of the four clades within the phylogeny as separate ESUs for directing conservation efforts.

5 | CONCLUSIONS

Overall, our results illustrate the importance of assessing the phylogeography of a species for the development of conservation strategies, especially for morphologically cryptic taxa with high genetic diversity. Temporary ponds contain many rare and specialist species, such as the studied freshwater crustacean. However, across the studied regions temporary ponds are also essential as sources of food, water, and breeding grounds to many other organisms including threatened birds and amphibians. Therefore, their protection should be considered a priority in nature management plans.

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CONFLICT OF INTEREST

Authors declare no conflict of interest.

DATA AVAILABILITY

The DNA sequence data that support the findings of this study are openly available in GenBank at https://www.ncbi.nlm.nih.gov/genbank/, accession numbers are listed in Table 1.

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SUPPORTING INFORMATION

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