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.
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 depend 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 endemcity (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, Olsen, 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 & Cvetkovic, 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, 2013b).

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 glacial. 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

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**KEYWORDS**

dispersal, freshwater, habitat destruction, molecular clock, temporary ponds
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 hypothesised 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,  | Austria | – (2)                     | –        | –         | MK523638-MK523640 | MK643480-MK643482 |
| 4   | DR1    | Bahrain | – (1)                     | –        | –         | MK935170     | MK643510      |
| 5   | BI6,  | Belgium | Péronnes-lez-Binches (12) | 50.43    | 4.15      | MK449413–MK449424 | MK643523–MK643527 |
| 6   | KRO    | Croatia | Konjisko (1)              | 43.5865  | 16.4599   | MK564519     | MK643488      |
| 7   | FC     | France  | Arles (1)                 | 43.55    | 4.57      | MK564519     | MK643511      |
| 8   | DO(1-2)| France  | Military field            | 48.00    | 0.38      | MK564519     | MK643511      |
| 9   | ARD    | France  | Bidon (1)                 | 44.3496  | 4.534558  | MK564499     | MK643478-MK643479 |
| 10  | FP     | France  | Borce (1)                 | 42.8338  | 0.593552  | MK564502     | MK643521      |
| 11  | DK1    | France  | Le Cannet-des-Maures (1)  | 43.3480  | 6.427778  | MK564501     | MK643477      |
| 12  | FAL    | France  | Sainte-Croix-en-Plaine (1)| 48.0328  | 7.385567  | MK564501     | MK643522      |
| 13  | DI(1-3), D(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)                  | 44.5772  | 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)         | 46.8906  | 17.55569  | MK643528     | MK643531      |
| 18  | GPB,  | Italy   | Lampedusa (1)             | –        | –         | KP702853-KP702855 | – |
| 19  | GA8    | Italy   | Lecce (1)                 | 40.0805  | 18.48526  | KP702849     | – |
| 20  | GVF11  | Italy   | Monte Catabio (1)         | 42.5500  | 12.96889  | KP702864-KP702865 | – |
| 21  | GD(5-6)| Italy   | Palemo (1)                | 38.20639 | 13.2825   | KP702856-KP702857 | – |
| 22  | GD7    | Italy   | Syracuse (1)             | 37.07528 | 15.28639  | KP702858     | – |
| 23  | GB1,  | 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 |
| 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  | DB1-3, DE, DF1-2, DG1-3, DU | Morocco | Haut Atlas (5)           | 31.9      | −8.4      | MK618058, MK618060, MK618062–MK618064 | MK643495–MK643503 |
| 30  | DP1-2 | Morocco | Ifrane (1)               | 33.406699 | −5.115915 | MK618066                   | MK643507–MK643508          |
| 31  | MH  | Morocco     | Igherharen (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  | MAR1-3 | Morocco | — (3)                   | —         | —         | MK618070, MK618071         | MK643504–MK643506          |
| 35  | BIED | Poland     | Biedrusko (6)            | 52.48     | 16.85     | MK564492–MK564493, MK465095–MK465119 | MK631958–MK631967, MK643483–MK643484 |
| 36  | KON1-2 | 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  | ST1-2 | Poland | Słupsk (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  | TZ1-2 | Tunisia | Hammam Bent Djetidi (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 than 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;
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
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 Canna-des-Maure in France, and Szentbékkálla in Hungary were included in clade B. 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 Canna-des-Maure in France and Szentbékkálla in Hungary was based on the ITS1 region.

**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 Canna-des-Maure in France and Szentbékkálla in Hungary was based on the ITS1 region.

### 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.

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

| Clades      | Distances (%) | Molecular clock (mya) |
|-------------|---------------|-----------------------|
|             | Min | Max | Mean |                      |
| 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).
4.1 | 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 phylogeny as separate ESUs for directing conservation efforts. At least like to promote the recognition of the four clades within the species should be subject to a taxonomic revision, based on combined information from morphological, genetic and ecological analyses.

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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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REFERENCES

Aguilar, A., Maeda-Martínez, A. M., Murugan, G., Obregón-Barboza, H., Christopher, Rogers, D., McClintock, K., & Krumm, J. L. (2017). High intraspecific genetic divergence in the versatile fairy shrimp Branchinecta lindahlia with a comment on cryptic species in the genus Branchinecta (Crustacea: Anostraca). Hydrobiologia, 801, 59–69. https://doi.org/10.1007/s10750-017-3283-3
Al-Sayed, H., & Zainal, K. (2005). The occurrence of anostracans—Fairy shrimps Branchipus schaefferi in vernal pools of Bahrain. Journal of Arid Environments, 61, 447–460. https://doi.org/10.1016/j.jaridenv.2004.06.008
Belt, D., & Brtek, J. (1995). Checklist of the Anostraca. Hydrobiologia, 298, 315–353. https://doi.org/10.1007/bf00033826
Bilton, D. T., Freeland, J. R., & Okamura, B. (2001). Dispersal in freshwater invertebrates. Annual Review of Ecology and Systematics, 32, 159–181. https://doi.org/10.1146/annurev.ecolsys.32.081501.114016
Birky, C. W. J., Adams, J., Gemmell, M., & Perry, J. (2010). Using population genetic theory and DNA sequences for species detection and identification in asexual organisms. PLoS ONE, 5, e10609. https://doi.org/10.1371/journal.pone.0010609
Birky, C. W. J., Wolf, C., Maughan, H., Herbertson, L., & Henry, E. (2005). Speciation and selection without sex. Hydrobiologia, 546, 29–45. https://doi.org/10.1007/s10750-005-4097-2
Brendonck, L., Pinceel, T., & Orteils, R. (2017). Dormancy and dispersal as mediators of zooplankton population and community dynamics along a hydrological disturbance gradient in inland temporary pools. Hydrobiologia, 796, 201–222. https://doi.org/10.1007/s10750-016-3006-1
Brendonck, L., Rogers, D. C., Olesen, J., Weeks, S., & Hoeh, W. R. (2008). Global diversity of large branchiopods (Crustacea : Branchiopoda) in freshwater. Hydrobiologia, 595, 167–176. https://doi.org/10.1007/s10750-007-9119-9
Brochet, A. L., Gauthier-Clerc, M., Guillemain, M., Fritz, H., Waterkeyn, A., Baltanás, Á., & Green, A. J. (2009). Field evidence of dispersal of branchiopods, ostracods and bryozoa by teal (Anas crecca) in the Camargue (southern France). Hydrobiologia, 637, 255. https://doi.org/10.1007/s10750-009-9976-6
Błekt, J., & Thiéry, A. (1995). The geographic distribution of the European Branchiopods (Anostraca, Notostraca, Spinicaudata, Laeviceudata). Hydrobiologia, 298, 263–280. https://doi.org/10.1007/bf00033821
Cox, A. J., & Hebert, P. D. N. (2001). Colonization, extinction, and phylogeographic patterning in a freshwater crustacean. Molecular Ecology, 10, 371–386. https://doi.org/10.1046/j.1365-294x.2001.01188.x
Dumont, H. J., & Negrea, S. V. (2002). Introduction to the Class Branchiopoda, 1st ed. Leiden: Backhuys Publishers.
Eder, E., Hödl, W., & Gottwald, R. (1997). Distribution and phylogeny of large branchiopods in Austria, Hydrobiologia, 359, 13–22. https://doi.org/10.1023/a:1003146416563
Freeland, J. R., Kirk, H., & Peterson, S. (2011). Molecular ecology. Chichester, UK: John Wiley & Sons Ltd.
Gandolfi, A., Rossi, V., & Zarattini, P. (2015). Re-evaluation of three related species of the genus Branchipus Schaeffer, 1766 (Branchiopoda: Anostraca) by morphological and genetic analyses. Journal of
A group of morphologically cryptic species with origins in the Cretaceous. Molecular Phylogenetics and Evolution, 66, 800–810. https://doi.org/10.1016/j.ympev.2012.11.005

Silva, J. P., Phillips, L., Jones, W., Eldridge, J., & O’Hara, E. (2007). Life and Europe’s wetlands. European Communities.

Song, H., Buhay, J. E., Whiting, M. F., & Crandall, K. A. (2008). Many species in one: DNA barcoding overestimates the number of species when nuclear mitochondrial pseudogenes are coamplified. Proceedings of the National Academy of Sciences of the United States of America, 105, 13486–13491. https://doi.org/10.1073/pnas.0803076105

Stoks, R., Geerts, A. N., & Meester, L. D. (2014). Evolutionary and plastic responses of freshwater invertebrates to climate change: Realized patterns and future potential. Evolutionary Applications, 7, 42–55. https://doi.org/10.1111/eva.12108

Svensson, L. (2009). Collins bird guide, 2nd ed. London: HarperCollins Publishers Ltd.

Swofford, D. L. (2001). PAUP*: Phylogenetic Analysis Using Parsimony (and other methods) 4.0a165. Sinauer Associates, Sunderland: Massachusetts.

Tamura, K., & Nei, M. (1993). Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees. Molecular Biology and Evolution, 10, 512–526. https://doi.org/10.1093/oxfordjournals.molbev.a004023

Tuytens, K., Vanschoenwinkel, B., Waterkeyn, A., & Brendonck, L. (2014). Predictions of climate change infer increased environmental harshness and altered connectivity in a cluster of temporary pools. Freshwater Biology, 59, 955–968. https://doi.org/10.1111/twb.12319

Van den Broeck, M., Waterkeyn, A., Rhazi, L., & Brendonck, L. (2015a). Distribution, coexistence, and decline of Moroccan large branchiopods. Journal of Crustacean Biology, 35, 355–365. https://doi.org/10.1163/1937240x-00002316

Van den Broeck, M., Waterkeyn, A., Rhazi, L., Grillas, P., & Brendonck, L. (2015b). Assessing the ecological integrity of endorheic wetlands, with focus on Mediterranean temporary ponds. Ecological Indicators, 54, 1–11. https://doi.org/10.1016/j.ecolind.2015.02.016

Vanschoenwinkel, B., Brendonck, L., Pinceel, T., Dupriez, P., & Waterkeyn, A. (2013). Rediscovery of Branchipus schaefferi (Branchiopoda: Anostroca) in Belgium - notes on habitat requirements and conservation management. Belgian Journal of Zoology, 143, 3–14.

Vanschoenwinkel, B., Pinceel, T., Vanhove, M. P. M., Denis, C., Jocque, M., Timms, B. V., & Brendonck, L. (2012). Toward a global phylogeny of the “living fossil” crustacean order of the Notostraca”. PLoS ONE, 7, e34998. https://doi.org/10.1371/journal.pone.0034998

Waples, R. S. (1995). Evolutionarily significant units and the conservation of biological diversity under the Endangered Species Act. In J. L. Nielsen (Ed.), Evolution and the aquatic ecosystem: Defining unique units in population conservation (pp. 8–27). Bethesda: American Fisheries Society.

Waterkeyn, A., Grillas, P., Anton-Pardo, M., Vanschoenwinkel, B., & Brendonck, L. (2011). Can large branchiopods shape microcrustacea communities in Mediterranean temporary wetlands? Marine and Freshwater Research, 62, 46–53. https://doi.org/10.1071/mf10147

Waterkeyn, A., Vanschoenwinkel, B., Elson, S., Anton-Pardo, M., Grillas, P., & Brendonck, L. (2010). Unintentional dispersal of aquatic invertebrates via footwear and motor vehicles in a Mediterranean wetland area. Aquatic Conservation: Marine and Freshwater Ecosystems, 20, 580–587. https://doi.org/10.1002/aqc.1122

Williams, D. D. (2006). The biology of temporary waters. Oxford, UK: Oxford University Press.

Xia, X., & Kumar, S. (2018). DAMBE7: New and improved tools for data analysis in molecular biology and evolution. Molecular Biology and Evolution, 35, 1550–1552. https://doi.org/10.1093/molbev/msy073

Xia, X., & Lemey, P. (2009). Assessing substitution saturation with DAMBE. In P. Lemey, M. Salemi, & A.-M. Vandamme (Eds.), The phylogenetic handbook (pp. 615–630), Cambridge, UK: Cambridge University Press. https://doi.org/10.1107/cbic09780511819049

Xia, X., Xie, Z., Salemi, M., Chen, L., & Wang, Y. (2003). An index of substitution saturation and its application. Molecular Phylogenetics and Evolution, 26, 1–7. https://doi.org/10.1016/s1055-7903(02)00326-3

Zacharias, I., & Zamparas, M. (2010). Mediterranean temporary ponds. A disappearing ecosystem. Biodiversity and Conservation, 19, 3827–3834. https://doi.org/10.1007/s10531-010-9933-7

SUPPORTING INFORMATION

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