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Distinctive genetic signatures of two fairy shrimp species with overlapping ranges in Iberian temporary ponds

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Abstract

1. Temporary lentic water bodies host biotic assemblages adapted to the transient nature of these freshwater habitats. Fairy shrimps (Crustacea, Branchiopoda, Anostraca) are one of the most important biological components of these unique environments and have a fossil record dating back to the Middle Jurassic (>150 million years).

2. Some anostracan species show a geographically restricted distribution, whereas others are widely dispersed. We aimed to investigate the relationship between different geographic extents and patterns of genetic structure in species of Anostraca. Following this objective, we selected two species with contrasting ranges but overlapping geographic distributions and similar life-history traits in the study area. We analysed additional information that, from an ecological (e.g. egg-bank, niche breadth, and pond connectivity) and evolutionary (e.g. crown-group age of each species) perspective, may explain the obtained phylogeographic patterns.

3. Between 2005 and 2018, we sampled two species of fairy shrimps (309 specimens of *Branchipus cortesi* and 264 specimens of *Tanymastix stagnalis*) from 53 temporary ponds of Portugal. We added five other locations from Spain and France to include other European locations for *T. stagnalis*. Additionally, we also sampled *Branchipus schaefferi* from two temporary water bodies (Spain and Morocco) to include in the dating analysis.

4. Reconstructed phylogenies based on mitochondrial sequence data indicate the existence of deeply divergent clades with an unequivocal phylogeographic structure in *T. stagnalis* and shallower divergences in *B. cortesi* with a less clear geographic correspondence. We found evidence of frequent local and rare long-distance dispersal events in both species and limited intermediate dispersal, which was more common in *B. cortesi*. A Bayesian dating analysis using the Branchiopoda fossil record estimated the age of the most recent common ancestors of *T. stagnalis* and *B. cortesi* at 32.4 and 12.8 million years, respectively.

5. Haplotype accumulation curves indicated that only a portion of the genetic composition of the species was sampled on each hydroperiod and showed the

existence of large, genetically diverse egg banks that remain in the soil. These egg banks represent a genetic reservoir that guarantees the survival of the species because active populations from different hydroperiods may be genetically different and adapt to a changing environment.

6. We hypothesise that the contrasting phylogeographic patterns displayed by the two fairy shrimp species may result from: (1) the earlier age of the most recent common ancestor of *T. stagnalis*, as older species have more time to accumulate mutations and, thus, are expected to exhibit higher genetic differentiation among populations; (2) slight differences in adult behaviour, life-history traits and cyst morphologies of *T. stagnalis* and *B. cortesi* favouring different animal dispersal vectors with distinct dispersal abilities. Therefore, phylogeographic patterns may be explained by both evolutionary and ecological processes, which operate in different time scales.

1 | INTRODUCTION

Temporary ponds are unique freshwater wetland habitats exposed to extreme environmental conditions where flooding occurrences are often ephemeral and alternate with periods of severe drought (Calhoun et al., 2017). Due to its transient nature, these habitats host vulnerable biotic assemblages that developed specific adaptations to cope with these conditions (Brendonck, 1996). Fairy shrimps (Crustacea: Branchiopoda: Anostraca) are characteristic inhabitants of these unique environments that produce desiccation-resistant resting eggs (cysts) that stay in diapause in the soil during the dry period. During each wet period, only a small fraction of the cysts hatch, developing the active population, while the vast majority remains dormant in the soil, remaining viable for decades (Brendonck & De Meester, 2003). This strategy of asynchronous hatching may assure the long-term survival of the populations and optimise fitness by *not putting all eggs in one basket* (diversified bet-hedging strategy; Pinceel et al., 2017; Simovich & Hathaway, 1997). In a changing and unpredictable environment, the egg bank becomes the genetic reservoir that, due to stochastic hatching or reproductive strategies (Beladjal & Mertens, 2017; Schwentner & Richter, 2015), can produce genetically distinct active populations from different reproductive events separated in time, a phenomenon called temporal dispersal (Bilton et al., 2001; Hairston & Kearns, 2002; Wang & Rogers, 2018). However, egg bank dynamics are not only affected by resting egg production, hatching, loss and senescence, but also by spatially dispersing individuals. The dispersal of fairy shrimps is mostly reliant on the passive transport of their resting propagules by a series of different vectors such as wind, water, amphibians, water birds, insects, mammals, humans, and even fish (Beladjal et al., 2007; Beladjal & Mertens, 2003, 2009; Bilton et al., 2001; Bohonak & Whiteman, 1999; Green & Figuerola, 2005; Incagnone et al., 2015;

Thiéry, 1991; Vanschoenwinkel et al., 2008; Vanschoenwinkel, Waterkeyn, et al., 2008; Waterkeyn et al., 2010). Depending on the vector, frequent dispersal among neighbouring ponds can develop the existence of true metapopulations (Rogers, 2015), or long-distance dispersal events can act as colonisation attempts of new habitats (Vanschoenwinkel et al., 2011). Nevertheless, regardless the high dispersal potential of the resting stages, there is growing evidence (based on molecular data) of the existence of significant genetic differentiation among populations even from neighbouring water bodies (De Meester, 1996; Incagnone et al., 2015; Ketmaier et al., 2012). This is because the effective colonisation of suitable habitats will not depend only on the actual dispersion of the resting stages, but also on the characteristics of the receiving population, which sometimes can hinder gene flow through the priority effects of early colonists and the adaptations of the resident populations (De Meester et al., 2002; Schwentner & Richter, 2015). However, other strategies, such as higher hatching rates of resting eggs passing the digestive tract of aquatic birds, could counteract priority effects in occupied habitats or reinforce them in unoccupied ones (Rogers, 2014; Schwentner & Richter, 2015). Moreover, only a fraction of the cyst bank, varying between 2.8% (in subtropical or desert areas) and 50%–100% (temperate areas) in fairy shrimp species, can hatch during a single hydroperiod (Brendonck, 1996; Simovich & Hathaway, 1997). As a result, an active population from a given hydroperiod may represent a particular expression of a complex genetically structured egg bank, and studies focusing on a single hydroperiod can miss a significant fraction of population genetic structure (Brendonck & De Meester, 2003). Nevertheless, given the impracticality of analysing the entire cyst bank, the best way of minimising this effect is to sample several hydroperiods and/or several years, to assess the genetic structure of the population (Schwentner & Richter, 2015).

Although some fairy shrimp species are widely distributed, more than 50% of species are only known from 10 or fewer localities (Rogers, 2013) indicating that dispersal (or colonisation capacity) is highly variable among species. Geographic ranges are the result of multiple ecological and evolutive processes and have an important role in determining the genetic structure of species (Eckert et al., 2008). It is largely expected that endemic species have lower genetic diversity and phylogeographically more structured populations than widespread species (Hamrick et al., 1992). Regarding fairy shrimps, it is difficult to make generalisations because species with small (e.g. Davies et al., 1997; Deiner et al., 2017; Ketmaier et al., 2012; Pinceel et al., 2013; Vanschoenwinkel et al., 2011) or large (e.g. Aguilar et al., 2017; Bohonak, 1998; Eimanifar et al., 2015; Lukić et al., 2019; Muñoz et al., 2008; Reniers et al., 2013) geographic ranges have generated contrasting results.

Here we sampled two species of fairy shrimps (*Branchipus cortesi* Alonso & Jaume, 1991 and *Tanymastix stagnalis* [Linnaeus, 1758]) from Mediterranean Temporary Ponds in southern Portugal, where these two co-occurring species create a characteristic branchiopod association (Cancela da Fonseca et al., 2008; Sala et al., 2017). However, these two species differ in their geographic range size: *B. cortesi* is an Iberian endemic thought to be a Tertiary relict (Alonso & Jaume, 1991) while *T. stagnalis* has a widespread distribution ranging from Scandinavia, Ireland, and south-western (SW) Russia to southern Europe and northern Africa (Algeria) (Brtek & Thiéry, 1995; Samraoui & Dumont, 2002; Young, 1976). Besides their different geographic ranges, the two species show similar strategies (Brendonck, 1996) and their life-history traits differ little (in terms of life span, time to reach maturity or hatching time) being characterised by differences among populations according to local environmental conditions (Cristo et al., 2002; Gascón et al., 2012; Mura, 1991; Mura & Zarattini, 2000; Olmo et al., 2015).

We analysed the genetic structure of *B. cortesi* and *T. stagnalis*, and connectivity among ponds. Phylogenetic relationships within the two fairy shrimp species and between them and other anostracans were inferred based on newly generated and available GenBank mitochondrial cytochrome *c* oxidase subunit I (COI) sequence data. We estimated the crown group age of each species based on the paleontological record of the class Branchiopoda using a Bayesian methodology that incorporates rate variation and a relaxed molecular-clock approach. We also built haplotype accumulation curves for the two species datasets to evaluate the impact of insufficient sampling on the genetic composition of active populations exhibiting dormant stages.

2 | METHODS

2.1 | Specimen collection, DNA extraction, amplification, and alignments

Between 2005 and 2018 we collected 264 specimens assigned to *T. stagnalis* and 309 specimens assigned to *B. cortesi* from 55 Iberian ponds (53 in Portugal and two in Spain) plus three in France (one in Pyrénées-Orientales and two in Corsica; sample locations in Figure 1 and Tables S1 and S3). We also included two specimens of *Branchipus schaefferi* from two temporary ponds (Catalonia, Spain and Michlifen, Morocco) in the dating analysis (Table S1). Sampling was performed by trawling with a round hand net (30.5 cm mouth diameter, 1 mm mesh size) following Machado et al. (2017). Specimens were immediately preserved in 96%–99.8% ethanol, their identity at species level being subsequently confirmed in the laboratory. Most of the sampling effort was focused on the coastal plain of SW Portugal; however, to analyse phylogenetic relationships within the species, specimens from other locations (Portugal, Spain, and France) were also included (Tables S1, S3, and S5).

Total genomic DNA was isolated from tissue using the cetyltrimethylammonium bromide protocol (Doyle & Doyle, 1987). Universal primers from Folmer et al. (1994) were used to amplify a portion of the mitochondrial COI gene. Polymerase chain reaction amplicons were purified using ethanol/sodium acetate precipitation and directly sequenced with the corresponding primers. Sequencing was performed in an automated sequencer (ABI PRISM 3700, Applied Biosystems) using the BigDye Deoxy Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems), and following the manufacturer's instructions. Sequences of *B. cortesi* and *T. stagnalis* were deposited in GenBank under the accession numbers MW069714–MW070022 and MW066477–MWO66749, respectively. The two sequences of *B. schaefferi* obtained from this study have the accession numbers MW659110–MW659111. Sequences of *T. stagnalis* from different localities available in GenBank (there are no records of *B. cortesi*) were collapsed using FaBox (https://users-birc.au.dk/~palle/php/fabox/index.php) and included in the analyses (accession numbers in Table S5). For the phylogenetic analyses we also retrieved from the GenBank COI sequences of other Branchiopoda (Anostraca: *Artemia parthenogenetica_* KF691338 and KF691343, *Artemia urmiana_*JX512748, *Branchipodopsis wolfi_*GU171358, *Branchipodopsis drakensbergensis_*GU139737, *Branchinecta paludosa_*AF209064, *Branchinecta lynchi_*FJ439751, *Streptocephalus proboscideus_*AY519829, *Streptocephalus dorothae_*AF209065, *Thamnocephalus platyurus_*AF209066; Laevicaudata: *Lynceus macleayanus_*DQ467697, *Lynceus biformis_*EF189672; Notostraca: *Triops granarius_*HF911379 and *Triops cancriformis_*DQ148291; Cladocera: *Daphnia angulata_*AF217115). *Armadillidium vulgare* (AF255779) from the order Malacostraca was selected as the outgroup in all analyses.

Although the use of a single gene might not be ideal for phylogenetic reconstruction as each gene tree reflects a unique story that might not be the only one for the species (Szöllősi et al., 2014), we used a portion of the COI gene because it would allow the inclusion of sequences from previous studies. Also, nuclear introns (ITS2) yielded similar phylogenetic relationships within Iberian populations of *T. stagnalis* (Rodríguez-Flores et al., 2020). Nonetheless, we may still get the wrong species tree when using the whole genome and a gene tree represents one, among several phylogenetic hypotheses, not the definitive tree for the species.
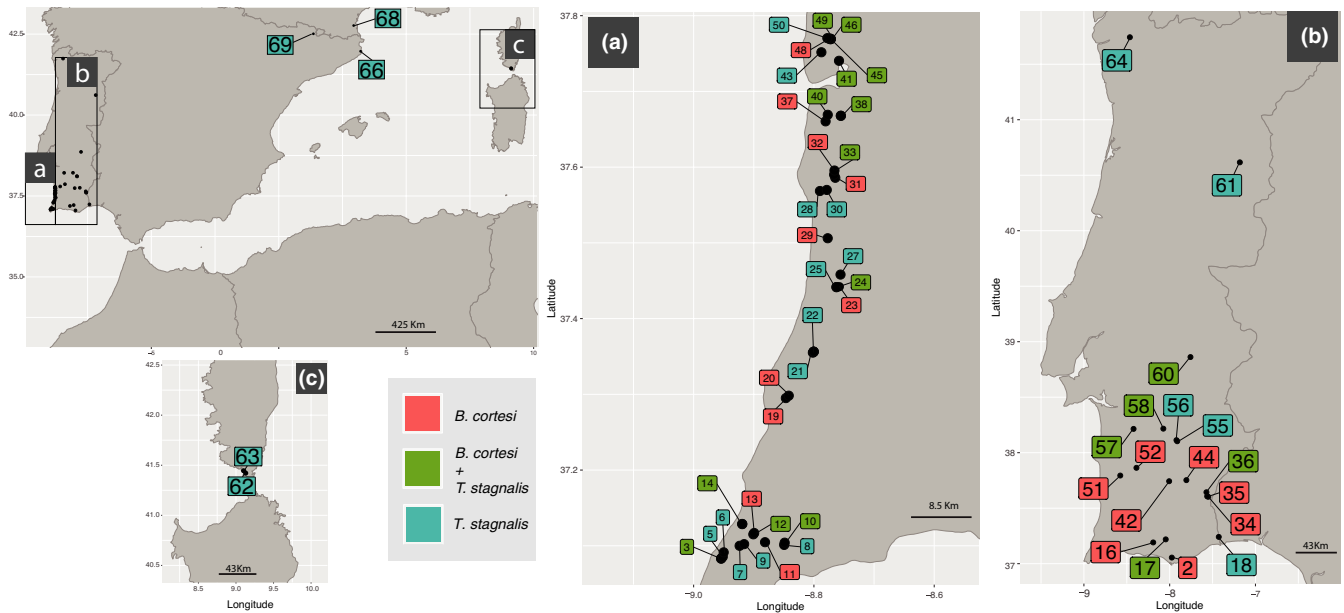
FIGURE 1 Map of the sampling area for the two fairy shrimp species (*Tanymastix stagnalis* and *Branchipus cortesi*). Ponds are represented by coloured numbers in squares: red squares correspond to samples of *B. cortesi* only, green to both *B. cortesi* and *T. stagnalis*, and light blue to *T. stagnalis* only. (a) South-western Portuguese coastal plain; (b) Portugal, excluding south-western Portuguese coastal plain, and (c) Corsica, France

All COI sequences were aligned with Mafft v7.245 (Multiple alignment using Fast Fourier Transform; Katoh & Toh, 2010) using the --auto option that automatically selects the appropriate strategy according to data size. Three different COI alignments were produced: dataset (1) that included 478 base pairs (bp) from 278 *T. stagnalis* specimens plus one haplotype from the following taxa: *S. dorothae*, *D. angulata*, and *L. biformis*; dataset (2) that included 556 nucleotide positions from 309 *B. cortesi* specimens and one haplotype from the following taxa: *S. dorothae*, *D. angulata*, and *L. biformis*; and dataset (3), which included 624 bp from all unique haplotypes of *B. cortesi* (121), *T. stagnalis* (84), *B. schaefferi* (2), and one haplotype from the following Branchiopoda (Anostraca: *Artemia parthenogenetica*, *A. urmiana*, *B. wolfi*, *B. drakensbergensis*, *B. paludosa*, *B. lynchi*, *S. proboscideus*, *T. platyurus*; Laevicaudata: *L. macleayanus*; Notostraca: *T. granarius*, and *T. cancriformis*). Amino acid translations were checked using Mesquite v.3.04 (Maddison & Maddison, 2005). The Akaike information criterion (Akaike, 1973) as implemented in jmodeltest-2.1.10 (Darriba et al., 2012; Guindon & Gascuel, 2003) selected the GTR+G as the evolutionary model that best fitted datasets (1) and (2), and GTR+I + G for dataset (3).

2.2 | Phylogenetic and dating analyses

Maximum likelihood (ML) analyses based on datasets (1), (2), and (3) were performed with RAxML v8.2.10 (Stamatakis, 2014). Following the manual recommendations, the analyses were run under the GTRCAT evolutionary model for the three datasets. The best-scoring ML tree was determined from 100 randomised maximum-parsimony starting trees using the rapid hill-climbing algorithm, and

500 bootstrap replicates were drawn on the best-scored ML tree using the exhaustive bootstrap algorithm.

Bayesian inference (BI) analyses were performed with MrBayes v.3.1.2 (Ronquist & Huelsenbeck, 2003) under the GTR + G model (lset nst = 6 rates = γ) for datasets (1) and (2). The Metropolis coupled Markov chain Monte Carlo was sampled for 15×10^6 generations (two simultaneous MC chains) with a sampling frequency of 1,500. The robustness of the inferred trees was tested using Bayesian posterior probabilities.

The Multidistribute package that includes the software Estbranches and Multidivtime (Thorne et al., 1998) was used to estimate the crown-group age of *T. stagnalis* and *B. cortesi* based on dataset (3). We used the inferred ML topology based on dataset (3) as the starting phylogeny. This Bayesian method "estimates the changes in evolutionary rates over time using the Markov chain Monte Carlo (MCMC) to derive the posterior distribution of rates and times" (Rutschmann, 2005). First, the software PaML v.4.8 (Yang, 2007) estimated the ML parameters using a discrete γ distribution with five rate categories and the F84 model of nucleotide substitution because of computational tractability (Wiegmann et al., 2003). Those parameters were used by Estbranches (Kishino et al., 2001) to estimate branch lengths and subsequently, Multidivtime estimated divergence times. The Multidistribute package requires a prior assumption for the calibration of the root of the tree (the parameter rttm), which was set to 48.8-time units, where in this analysis, one-time unit represented 10 million years (myr). This value was based on the fossil record of small carbonaceous branchiopod appendages from the Cambrian of Canada corresponding to the crown Branchiopoda, originated at least 488 myr ago (Harvey et al., 2012; Sun et al., 2016). The earliest appearance on the paleontological

record of the Laevicaudata during the Middle Jurassic (174.1–163.5 myr) (Shen & Chen, 1984) was used to calibrate the stem node corresponding to *L. macleayanus*. The 200,000-year record of brine shrimp *Artemia* remains (faecal carbonate pellets and organic cysts) from Lake Urmia, NW Iran (Djamali et al., 2010) was used to calibrate the crown group node including *Artemia urmiana* and *A. parthenogenetica*. These are the only *Artemia* species that occur in this lake and nearby lagoons (Agh et al., 2007). We used the fossil of *Archaebranchinecta barstowensis* (Branchinectidae) from the Middle Miocene (15.97–11.60 myr) Barstow Formation, California (Belk & Schram, 2001; Sun et al., 2016) to calibrate the stem node of the two Branchinectidae *B. paludosa* and *B. lynchi*. The MCMC method was employed to approximate both prior and posterior distributions (Kishino et al., 2001). Initial parameter values were randomly selected to initialise the Markov chain and then, a burn-in period of 100,000 cycles was completed before parameters were sampled from the MCMC chain. Afterwards, the state of the Markov chain was sampled every 100 cycles until a total of 10,000 generations. The analysis was run twice to assure that MCMC chains have converged.

2.3 | Genetic variability, genetic structure among ponds, Bayesian clustering, and haplotype networks

Indices of genetic diversity were estimated for each pond (Tables S2 and S4). The number of polymorphic sites, number of haplotypes (NH), and haplotype diversity (h, the probability that two haplotypes chosen at random from the sample are different, Nei, 1987) and nucleotide diversity (π, the mean number of nucleotide differences among all haplotypes in a sample, Nei, 1987) were calculated for mtDNA COI sequences using a combination of R-packages v.3.6.1 (R Core Team, 2019): pegas (Paradis, 2010); mmod (Winter, 2012); and haplotypes (Aktas, 2015).

To identify groups of populations within southern Portuguese populations that are phylogeographically homogeneous and maximally differentiated from each other, we used for both species the Spatial Analysis of MOlecular VAriance (SAMOVA) with K (groups) = 2 to K = 20, 10,000 iterations and ten repetitions (Dupanloup et al., 2002), as implemented in SPADS (Dellicour & Mardulyn, 2014). The SAMOVA algorithm assigns sampling sites to groups based on geographic proximity and sequence similarity. The number of clusters reflecting the most-likely genetic structure was chosen based on the maximised among-group variation (Φ_{CT} = the proportion of total genetic variance resulting from differences among groups of populations) while excluding configurations with single-population groups because this indicates that the group structure is disappearing (Magri et al., 2006).

Population genetic connectivity of southern Portuguese populations of *T. stagnalis* and *B. cortesi* was investigated by reconstructing haplotype networks with a statistical parsimony algorithm (Templeton et al., 1992), as implemented in TCS v.1.21 (Clement et al., 2000). Resulting networks were overlaid with groups resulting

from SAMOVA and with sampling years. The raw output was visualised in the web implementation of tcsBU (Múrias dos Santos et al., 2015). A visualisation of all connections was sought, the connection limit was set as the known maximum distance between any two variants in that network (a forced connection).

2.4 | Haplotype accumulation curves

Haplotype accumulation curves were obtained by 10,000 random permutation subsampling using the functions haploAccum() and plotted with the plot.haploAccum() as implemented in the spider R-package (Brown et al., 2012). Based on the total NH detected in the species and the number of singletons and doubletons in the dataset, we used the Chao 1 estimator (Chao, 1984), with chaoHaplo() function to determine the putative total NH. To evaluate the coverage of haplotype sampling, haplotype accumulation curves are extensively used (Phillips et al., 2015). These curves, similar to rarefaction curves, use the extent of saturation as a function of the number of individuals sampled and the NH accumulated (Gotelli & Colwell, 2001). Curves displaying weak asymptotic behaviour suggest that more sampling is necessary to capture the genetic variation of the species. In contrast, curves with rapid saturation indicate that much of the intraspecific haplotype diversity is represented (Phillips et al., 2015). The Chao1 estimates the appropriate minimum sample size to account for all haplotype diversity based on the sample size and number of detected haplotypes as well as the number of singleton and doubleton sequences (those occurring once and those appearing twice) in the dataset.

3 | RESULTS

3.1 | Phylogenetic reconstructions and dating analysis

Maximum likelihood and BI analyses based on *T. stagnalis* dataset (1) retrieved similar topologies (likelihood values, ML: −ln L = 3,889.73; BI: −ln L = 4,495.78). The three deeply divergent *T. stagnalis* clades depicted in the ML analysis (Figure 2a) showed a clear geographic correspondence. Clade 1 included Portuguese specimens from central Alentejo and central Algarve that grouped with samples from Spain and France. Clade 2 grouped specimens from ponds of the SW Portuguese coastal plain with a more northern distribution (SW–north) with a single exception (pond 8, SW–south). Clade 3 included specimens from ponds exhibiting the south-westernmost Portuguese distribution (SW–south). Specimen from the south-easternmost pond (pond 18) had a sister position in relation to all SW specimens and those from northernmost pond (pond 64) were retrieved as the sister group to all remaining *T. stagnalis* specimens (Figure 2a).

Maximum likelihood and BI analyses (likelihood values: ML −ln L = 4,192.61; BI −ln L = 4,805.69) based on *B. cortesi* dataset (2)
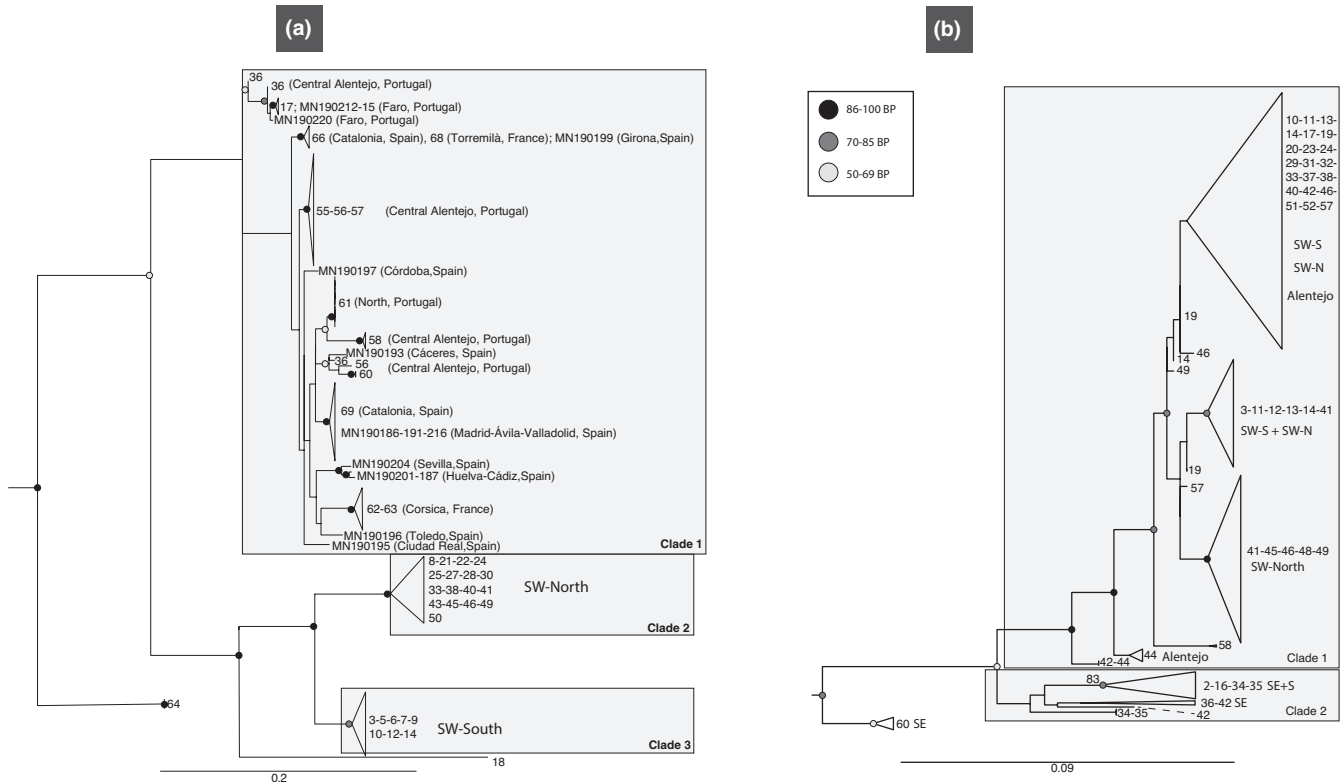
FIGURE 2 Phylogenetic relationships of the two fairy shrimp species *Tanymastix stagnalis* (a) and *Branchipus cortesi* (b) based on the maximum likelihood analysis of a fragment of the mitochondrial cytochrome *c* oxidase subunit I gene. Circles at the nodes represent bootstrap proportions (BP) with values >50%. Black circles represent BP values between 86 and 100; dark grey: BP values between 70 and 85; and light grey: BP values between 50 and 69. Numbers at the tips of the trees indicate pond locations (see Figure 1 for details) assigned to each analysed individual, and accession numbers of sequences from other Iberian specimens retrieved from GenBank

(Figure 2b) yielded similar topologies. The ML topology depicted in Figure 2b indicated shallow divergences within *B. cortesi*. The phylogeographic structure was not as clear as in *T. stagnalis* because clades from the same geographic area were interspersed throughout the tree. Nonetheless, two main clades may be considered: Clade 1, which includes all samples from the SW Portugal (including SW–north samples intermingled with SW–south samples) plus samples from central Alentejo (ponds 42, 44, 51, 52, 57 and 58) and one from central Algarve (pond 17); Clade 2, which includes samples from central Algarve (ponds 2 and 16),

central Alentejo (pond 42), and south-east Alentejo (ponds 34, 35 and 36). Pond 42 from central Alentejo has specimens belonging to both clades. Specimens from the northernmost pond (pond 60) were retrieved as the sister group to all remaining *B. cortesi* specimens (Figure 2b).

Dating analysis based on dataset (3) estimated the crown-group age (most recent common ancestor, MRCA) of *B. cortesi* at 12.8 (14.7–9.7) myr during the Miocene while the MRCA of *T. stagnalis* dated back to the Lower Oligocene (32.4 [85.7–14.9] myr; Figure 3).

FIGURE 3 Bayesian divergence dating analysis based on a portion of the mitochondrial cytochrome *c* oxidase subunit I (COI) gene obtained with Multidivtime using the topology inferred by a maximum likelihood (ML) analysis. The dataset comprised 219 COI Branchiopoda sequences plus the outgroup *Armadillidium vulgare*. It includes all *Tanymastix stagnalis*, *Branchipus schaefferi*, and *Branchipus cortesi* haplotypes obtained in this study. It also includes *T. stagnalis* sequences retrieved from GenBank (Table S5). Other Branchiopoda required for fossil calibration (Anostraca: *Artemia parthenogenetica*, *Artemia urmiana*, *Branchipodopsis wolfi*, *Branchipodopsis drakensbergensis*, *Branchinecta paludosa*, *Branchinecta lynchi*, *Streptocephalus proboscideus*, *Thamnocephalus platyurus*; Laevicaudata: *Lynceus macleayanus*; Notostraca: *Triops granarius* and *Triops cancriformis*) were also retrieved from GenBank (accession numbers in Methods). Numbers on top of the nodes represent age estimates (in million years) and corresponding 95% confidence intervals in square brackets. Numbers below the nodes represent ML bootstrap proportions (values <50% are represented by a dash). Only the most relevant age estimates are shown. Numbers at the terminal tips of the tree represent pond locations (see Figure 1 for details), and asterisks denote calibration points. Lineages A, B, C, D, E, F, and G refer to meaningful groups shown in figure 1 of Rodríguez-Flores et al. (2020). Branches corresponding to basal nodes are broken with double dashes to accommodate the scale
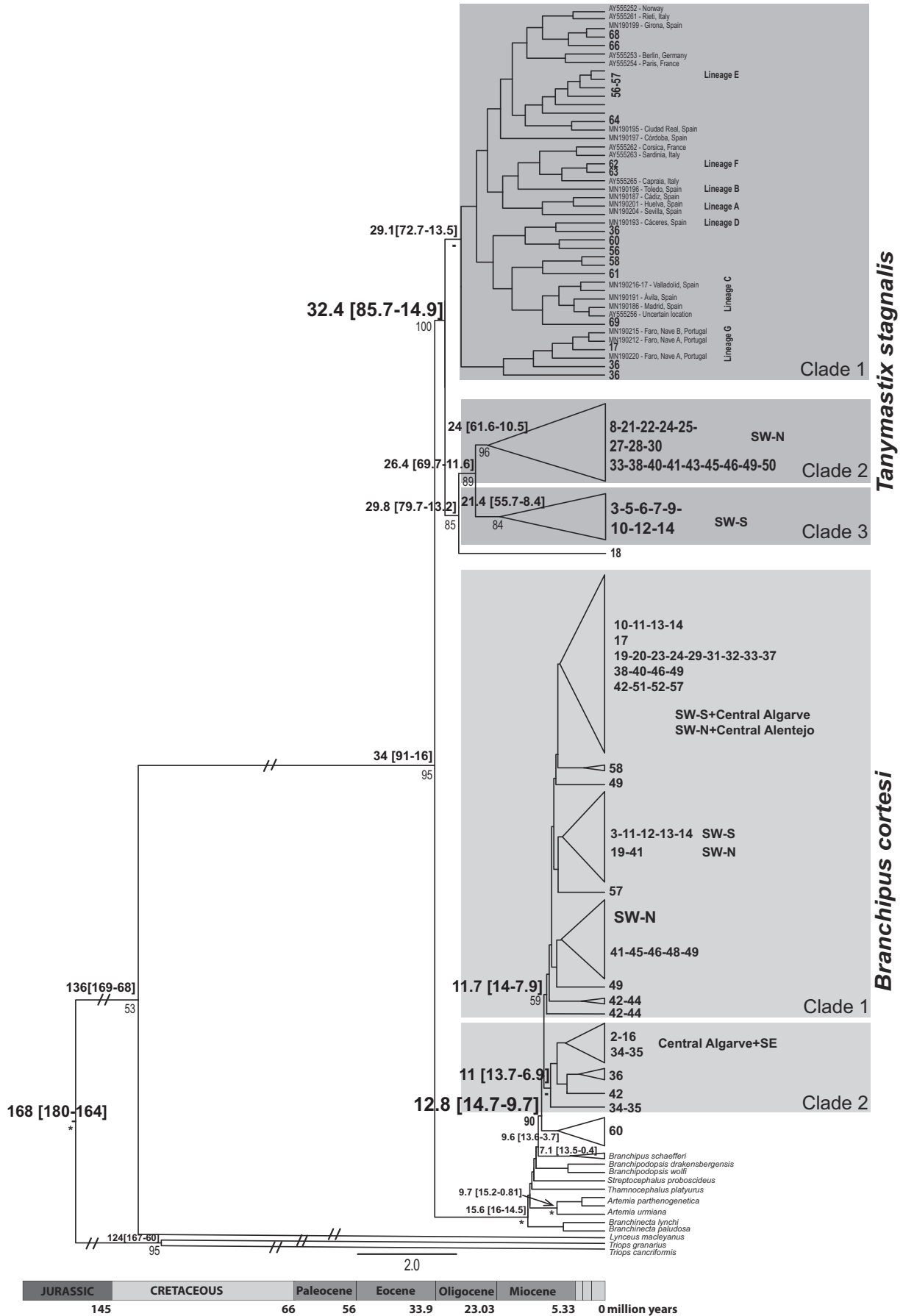
3.2 | Genetic variability, population connectivity and haplotype networks

The 264 *T. stagnalis* individuals were collected from 40 ponds (Iberia: 37 ponds; southeastern continental France: one pond; Corsica: two ponds). The sequences were collapsed into 65 haplotypes, of which 54 (84%) are private haplotypes restricted to single ponds, and 33 (52%) are singletons (represented by a single individual). The analysis of 309 *B. cortesi* individuals sampled in 35 southern Portuguese ponds revealed the existence of 122 haplotypes, which include 108 private haplotypes restricted to single ponds (89%), and 90 (74%) singletons.

Haplotype diversity was estimated in ponds with more than four individuals. In *T. stagnalis*, those values ranged from 0.000 to 0.833 (overall $h_{(N=264)} = 0.930$), while in *B. cortesi* varied between 0.000 and 1.000 (overall $h_{(N=309)} = 0.958$). Nucleotide diversity within ponds ranged from 0.000 to 0.037 (overall $\pi = 0.086$) for *T. stagnalis* and from 0.000 to 0.043 (overall $\pi_{(N=309)} = 0.029$) for *B. cortesi* (Tables S4 and S2, respectively).

For the SAMOVA, although there was a gradual increase in Φ_{CT} values with *K*, there was a local maximum for both species at $K = 7$ (*T. stagnalis* $\Phi_{CT} = 0.935$ and *B. cortesi* $\Phi_{CT} = 0.685$) and this was the highest Φ_{CT} when no groupings contained single ponds. The only exceptions were ponds 18 (*T. stagnalis*) and 60 (*B. cortesi*) that were extremely different from all other ponds (Figure 4).

The haplotype networks based on the groups defined by SAMOVA were particularly diversified for both species with no discernible central, more abundant haplotype (Figure 5). The haplotypes within each species' network could not all be connected within 95% confidence limits of parsimony, suggesting that many haplotypes were not sampled (black circles represent missing haplotypes). Both networks had a reticulate pattern, and *B. cortesi* displayed more shared haplotypes among groups (Figure 5b). *Branchipus cortesi* also revealed a small star-like shaped network for group 2 located in the SW tip of Portugal (Figure 5b). Networks based on the samples collected in the same year/hydroperiod indicated that most of the rarer haplotypes occurred in a single year (e.g. 2009 in Figure 5c and 2010 in Figure 5d), while the more abundant haplotypes appeared for several years (e.g. Figure 5c—circle including haplotypes from 2005, 2008, 2014, 2015, and 2018; Figure 5d—circle including haplotypes from 2008, 2015, and 2016).

3.3 | Haplotype accumulation curves

Haplotype accumulation curves (Figure S1) from *B. cortesi* and *T. stagnalis* showed no asymptotic behaviour and a steep ascent. However, estimated total haplotype richness was higher in *B. cortesi* (470 haplotypes) compared to *T. stagnalis* (190 haplotypes: Figure
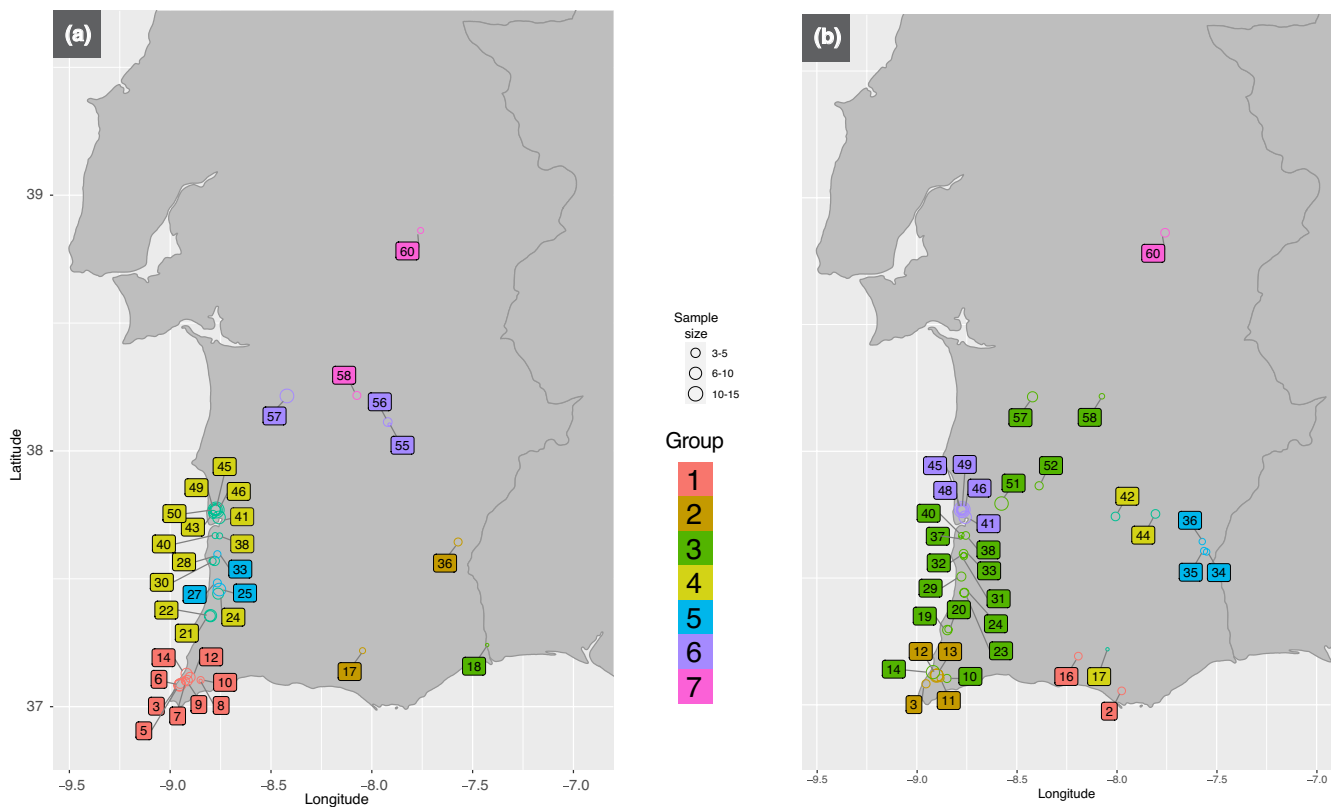

FIGURE 4 Map of the southern part of Portugal showing the groups inferred by SAMOVA ($K = 7$) for *Tanymastix stagnalis* (a) and *Branchipus cortesi* (b). On each map, the circles represent ponds, and coloured squares define group ponds by genetic affinity. The size of the circles is proportional to the number of samples
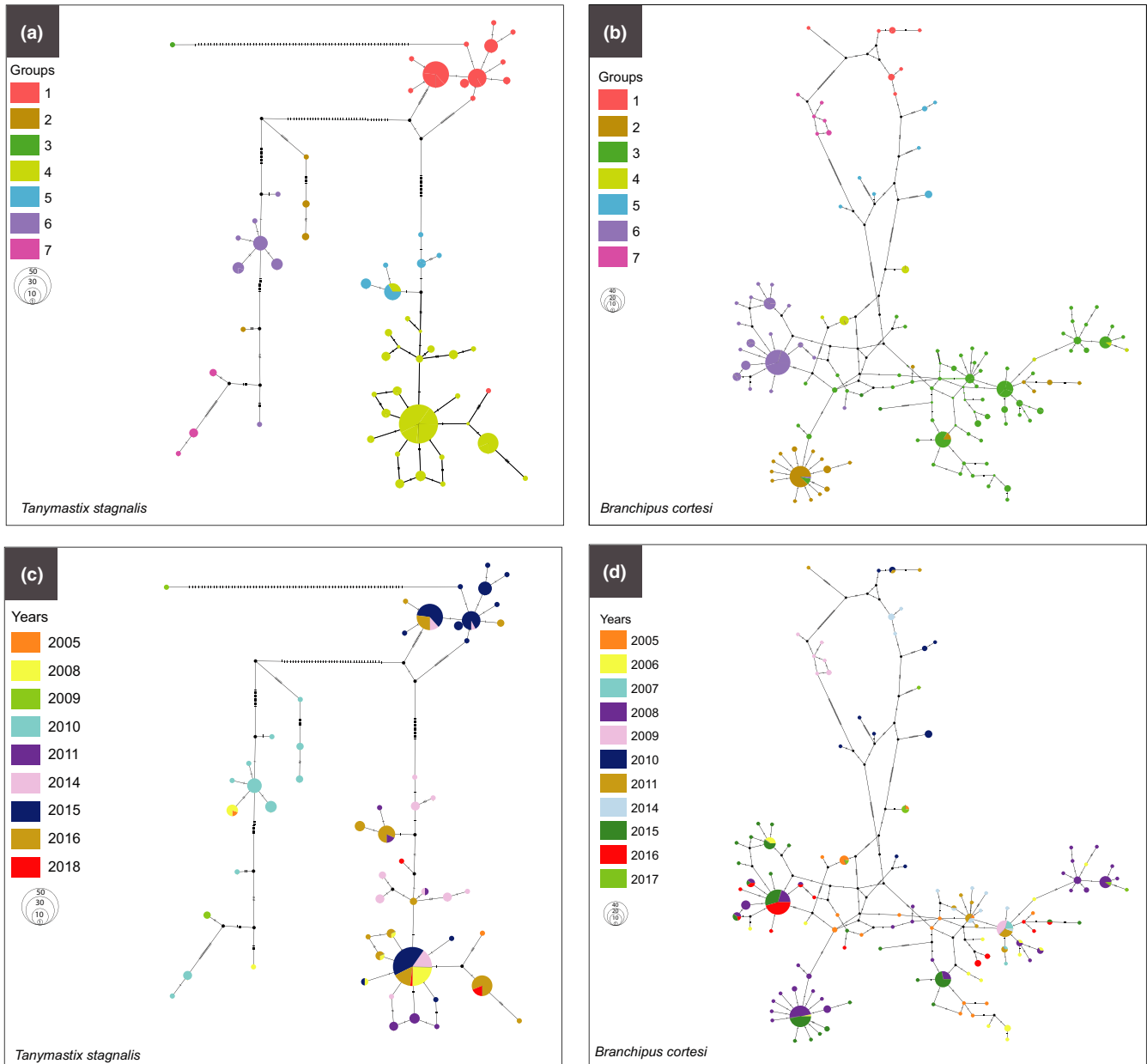
FIGURE 5 Cytochrome *c* oxidase subunit I median-joining networks of *Tanymastix stagnalis* (a and c) and *Branchipus cortesi* (b and d). Active populations per year (between 2005 and 2018) are depicted in networks (c and d). Networks (a) and (b) correspond to the groups inferred by the SAMOVA analysis. The area and slices of each circle are proportional to the number of individuals sharing a particular haplotype. The dashes represent the number of mutational steps between haplotypes, and black dots represent hypothetical connecting haplotypes that were not detected in our study

S1). Regardless of the large sample size (*B. cortesi* = 309 specimens; *T. stagnalis* = 264 specimens), the NH detected was only a fraction of the estimated haplotype diversity (*B. cortesi*: observed haplotypes = 122, estimated = 482; *T. stagnalis*: observed = 65, estimated = 117).

4 | DISCUSSION

In the light of the *everything is everywhere* hypothesis originally sustained by Darwin (1859), no genetic structure would be expected

in organisms from temporary freshwater habitats, because the large-scale dispersal potential of these organisms determined by the passive transport of their resting eggs would be expected to maintain constant gene flow between populations (Louette & De Meester, 2005). However, despite the high potential dispersal capacity of these organisms, low gene flow has usually been observed among populations in close proximity (Incagnone et al., 2015), leading to the description of the *dispersal-gene flow* paradox (De Meester et al., 2002). Our results are in accordance with reduced gene flow and limited long and intermediate dispersal events, especially for *T. stagnalis*.

Our COI-based phylogenies revealed contrasting genetic patterns in the two studied fairy shrimp species. The three deeply divergent clades retrieved in *T. stagnalis* molecular phylogenies showed geographic correspondence. In contrast, we found shallower divergences in *B. cortesi* phylogenies with individuals from different geographic areas grouping together. Phylogenies with strong geographic correspondences have been regularly observed in freshwater anostracans (e.g. *B. lynchi*, *B. sandiegonensis*, *B.* cf. *wolfi*, *B. schaefferi*, *Chirocephalus diaphanus*) (Andrews, 2013; Deiner et al., 2017; Lukić et al., 2019; Reniers et al., 2013; Vanschoenwinkel et al., 2011). Nonetheless, shallower divergences have also been detected, particularly in species with widespread distributions (e.g. *B. paludosa*, *Eubranchipus vernalis*, *Streptocephalus torvicornis*) (Kappas et al., 2017; Lindholm et al., 2016; McCafferty et al., 2010).

Both fairy shrimp species exhibited high haplotype and nucleotide diversities regardless of their different geographic ranges. Due to population-level processes that include genetic drift and selection, species with narrower geographic ranges are expected to exhibit lower genetic diversity (Doorenweerd et al., 2020; Nekola & White, 1999). Nonetheless, the Iberian endemic *B. cortesi* did not conform to this general expectation showing high haplotype and nucleotide diversities. This pattern has also been shown for other endemic species found in the Mediterranean, where a high number of glacial refugia have been described (Médail & Diadema, 2009), preventing significant population contractions during Quaternary glacial periods (Fady-Welterlen, 2005) and therefore no loss of intraspecific genetic diversity. A similar pattern of high genetic diversity within populations has also been observed in other large branchiopod taxa inhabiting Mediterranean and arid climates such as *B. lynchi*, *B. sandiegonensis*, *Branchinella longirostris*, or *B.* cf. *wolfi* (Andrews, 2013; Deiner et al., 2017; Pinceel et al., 2013; Vanschoenwinkel et al., 2011), although different results were found for some other taxa (e.g. *B. drakensbergensis* or *Chirocephalus kerkyrensis*; Ketmaier et al., 2012; Vanschoenwinkel et al., 2011).

Networks from both fairy shrimp species show a large number of singleton haplotypes (occur in a single individual). Particularly in *B. cortesi*, singletons are connected to more abundant haplotypes by more than ten mutational steps, suggesting that haplotype diversity was only partially sampled. The observed steep slope of the haplotype accumulation curves for the two species revealed that irrespective of the large sample size, the NH was only a fraction of the estimated haplotype diversity. Moreover, the two species showed complex haplotype networks suggesting deep coalescences and long stable demographic histories. Nonetheless, the *B. cortesi* network contained one star-shaped subnetwork corresponding to group 2 from the SW tip of Portugal, suggesting that those populations underwent a bottleneck followed by a recent expansion. Often, the imprint of more recent population expansion is overridden in a genealogy with deeper partitions (Grant, 2015).

Despite the high passive dispersal potential of fairy shrimps, the existence of a large number of unique haplotypes has been reported in several species (Aguilar, 2011; Arukwe & Langeland, 2013; Ketmaier et al., 2005; Lukić et al., 2019; Rodríguez-Flores et al., 2020;

Vanschoenwinkel et al., 2011). In our case, the absence of a central most common haplotype and the presence of unique haplotypes in the networks of *T. stagnalis* and *B. cortesi* points to reduced gene flow among ponds. The *monopolisation hypothesis* proposed by De Meester et al. (2002) could provide one plausible explanation for this apparent paradox of *high dispersal potential–low gene flow*. According to this hypothesis, genetic differentiation is facilitated among neighbouring ponds, specially at the initial stages of colonisation. This is due to the potential stochastic differences of founding migrants which adapt to local conditions, grow rapidly and monopolise existing resources, hindering subsequent colonisation and gene flow from nearby ponds (De Meester et al., 2002; Montero-Pau et al., 2018; Ortells et al., 2014; Rossi et al., 2006). However, this process may have more significance in clonal-reproducing organisms, such as cladocerans or rotifers, than in sexually reproducing organisms such as fairy shrimps (Brendonck et al., 2017). Moreover, the effect of the monopolisation of the habitat by founding migrants may decay throughout time due to subsequent gene flow from successful new immigrants, effectively providing large propagule pools with higher genetic diversity, especially in temporally variable environmental conditions (Brendonck et al., 2017). In this sense, the small fraction sampled from the estimated haplotype diversity inferred from the haplotype accumulation curves may also explain the high genetic differentiation (suggested by the SAMOVA analysis) between some neighbouring ponds inhabited by either *T. stagnalis* or *B. cortesi*. Active populations are the result of stochastic hatching from a putative highly genetically diversified cyst bank that may be similar to those in neighbouring temporary ponds (Schwentner & Richter, 2015). High genetic diversity and phylogeographic breaks within taxa occupying reduced geographic areas often indicate long-term historical persistence or restricted gene flow with periods of isolation (Rovito et al., 2012). We have detected stronger phylogeographic structure in *T. stagnalis* that exhibits a broader distribution than the Iberian endemic *B. cortesi*. Older species, having more time to accumulate mutations, are expected to exhibit higher genetic differentiation among populations (Cádiz et al., 2018); therefore, we expected an earlier origin for *T. stagnalis* considering the three deep-divergent clades that showed clear geographic correspondence.

Our dating analysis estimated the crown-group age of *T. stagnalis* at 32.4 [85.7–14.9] myr, during the Lower Oligocene. The shallower divergences obtained in *B. cortesi* phylogenies were consistent with a more recent origin, estimated at 12.8 [14.7–9.7] myr, during the Miocene. A previous study (Rodríguez-Flores et al., 2020) based on fixed clock rates (ranging from 0.007 to 0.013 mutations/site/myr) indicated a much more recent estimate for the origin of *T. stagnalis* during the Pliocene (4.38 myr). In that study, the chosen mutation rates were calibrated for snapping shrimps (order Decapoda, genus *Alpheus*) using the closure of the isthmus of Panama (Knowlton & Weigt, 1998). It is widely recognised that many pairs of geminate species diverged prior to the severance of this seaway (Collins et al., 1996); therefore, mutation rates based on this biogeographic event may be misleading, and the use of the fossil record is considered a more reliable source of information for tree calibration

(Forest, 2009). Our estimates were based on a relaxed molecular-clock model (Brown & Yang, 2011) in a Bayesian framework that incorporates paleontological information (Branchiopoda fossil record) and associated uncertainties through priors, producing more reliable age estimates with a better fit to real data (Drummond et al., 2006; Drummond & Suchard, 2010). Furthermore, our estimates for the age of the MRCA of *Branchipus schaefferi* (7.1 [13.5–0.4] myr) agrees with estimates by Lukić et al. (2019). Also, our estimate of the age of the MRCA of the genus *Triops* is consistent with estimates by Korn et al. (2013). Despite the differences in the dating analyses, the *T. stagnalis* clades retrieved in our phylogenetic analyses are largely consistent with lineages from Rodríguez-Flores et al. (2020). Noticeably, the subclade grouping some individuals from ponds 36 and 17 fits Rodríguez-Flores et al. (2020) lineage G. As in their paper, this clade is the sister group of all the other *T. stagnalis* samples included in clade 1.

Our estimates place the divergence of the *T. stagnalis* clades 2 (SW–north) and 3 (SW–south) at the end of the Oligocene (26.4 [69.7–11.6] myr), which corresponds to a period of acute cooling and aridification that caused the extinction of many taxa and substantial floristic changes in the Iberian Peninsula (Postigo Mijarra et al., 2009). The climatic deterioration may have caused a vicariant effect in *T. stagnalis* populations inhabiting the leading edges of southwest Iberia due to habitat discontinuities, originating the two clades. Those detrimental effects did not affect the Portuguese populations of *B. cortesi*, given their more recent origin (12.8 myr). The persistence of the two *T. stagnalis* clades throughout the Ice Ages (Pleistocene) until the present in the SW tip of the Iberian Peninsula may be explained by the *refugia-within-refugia* scenario proposed by Gómez and Lunt (2007). We hypothesise that one of these refugia could have been located at the SW tip of the Iberian Peninsula. This is an area known as a hotspot of biodiversity (Schmitt, 2007) for several groups of organisms such as plants and hexapods (da Gama et al., 2000), reptiles (Sillero et al., 2009), and large branchiopods (Korn et al., 2010; Sala et al., 2017). *Tanymastix stagnalis* populations corresponding to the SW–south clade (Clade 3) may have survived in temporary ponds formed by the deposition of Plio-Pleistocene sediments upon poorly permeable Palaeozoic strata (carboniferous shale and greywacke; Salvador et al., 2011). We propose another refugium in temporary ponds formed in a similar way in the coastal plain located north to the SW Portuguese domain and the Cenozoic Aljezur *Graben* (González-Clavijo & Dias, 2003). There, tectonic events brought the impermeable paleozoic strata to the surface (P. Fernandes, personal communication; Pimentel & Amaro, 2000), shaping morphological characteristics (orography) unsuitable for the occurrence of temporary ponds. That second putative refugium is an area rich in plant endemism (Santo et al., 2012) and probably allowed the persistence of the *T. stagnalis* SW–north clade (Clade 2).

Similarly, our results pointed to the existence of a differentiated clade in SW Portugal for *B. cortesi* (although not as narrowly distributed as those for *T. stagnalis*) highlighting the importance of the region for biodiversity conservation. *Branchipus cortesi* is an endemic species with a considerable number of populations located in the central and southern areas of the Iberian Peninsula (Sala et al., 2017). Bayesian dating analyses indicate an earlier origin for specimens from ponds 60 (SE Portugal), 34, 35, and 36 (easternmost ponds), which are the closest locations to Spanish populations, which we were not able to include in our analyses. Considering our results, a central or southern Iberian origin for *B. cortesi* is very likely, although this requires further confirmation with the inclusion of samples from the rest of Iberia.

The contrasting phylogeographic patterns between the two anostracan species are puzzling because they co-occur in the same habitat and share similar ecological requirements (Cancela da Fonseca et al., 2008; Gascón et al., 2012), which suggests similar dispersal vectors. Nonetheless, Alonso (1985) stated that co-existing species with similar environmental requirements had to present some type of segregation in feeding or behaviour. In co-existing congeneric species, differences in feeding strategies or in size can mostly avoid competition (Rogers et al., 2006; Timms & Sanders, 2002; White et al., 1969), but other species with similar biological traits have been observed living together (Maeda-Martínez et al., 1997; Nhiwatiwa et al., 2014; Timms & Sanders, 2002). This situation has been explained by differences in life cycles, ecological tolerances or in mesohabitat use, among others (Maeda-Martínez et al., 1997). These aspects seem to be also valid for co-existing non-congeneric species (Mura, 1991), although it is not clear if the different phylogenetic history is important in facilitating the co-existence of species with similar biological characteristics. In our case, slight differences in behaviour and life history traits, in addition to the earlier origin of *T. stagnalis* (which explains the deeper divergences of the three recovered clades separated by long branches, i.e., higher number of substitutions/site), may account for these contrasting patterns. In the geographic area shared by both species in Portugal, *T. stagnalis* and *B. cortesi* are found in the same or very similar habitats, mainly oligotrophic temporary ponds (rarely on flooded fields), where they co-occur forming a characteristic large branchiopod association (Cancela da Fonseca et al., 2008; Sala et al., 2017). In its remaining distributional range, *T. stagnalis* can also be found in other temporary habitat types (e.g. rockpools, dolines, dune or sand slacks, and road ditches; Boix, 2002; Brtek & Thiéry, 1995; Ketmaier et al., 2005; Mura & Zarattini, 2000), which would explain its broader geographic distribution. Although the two species frequently co-occur in the study area, *B. cortesi* prefers deeper (>50 cm) and more densely vegetated ponds (Gascón et al., 2012). *Tanymastix stagnalis* is mainly found in shallower marginal areas whereas *B. cortesi* mainly occurs in the deeper zones (personal observation). Nevertheless, cysts of *T. stagnalis* are less abundant in the marginal areas of the ponds than in deeper areas, whereas cysts of *B. cortesi* are evenly distributed across the pond bed (Pérez-Bote et al., 2008; J. Sala, M. Machado and M. Cristo, personal observation). The two fairy shrimp species show some differences in life span (1–2.75 months for *T. stagnalis* and 2.5–4 months for *B. cortesi*), and size and age to reach sexual maturity (6–7 mm length, 14–24 days for *T. stagnalis* and 8–9 mm length,

20–45 days for *B. cortesi*; Cancela da Fonseca et al., 2008; Cristo et al., 2002; M. Machado, personal observation). Cyst morphology and sizes are different for both species: larger and smooth-lenticular in *T. stagnalis* (Alonso, 1996; Mura, 1986; Thiéry & Gasc, 1991); and smaller and spherical-polyhedral in *B. cortesi* (Alonso & Jaume, 1991). The dispersal capacity of the cysts may be modulated by these morphological characteristics, as those cysts with polygonal depressions are more prone to adhere to the substrate (Brendonck et al., 1992; Pinceel et al., 2016). Sculptured patterns and/or projections on the surface of resting stages of several organisms have been considered to favour dispersal, although they also offer protection against predation or sinking (e.g. Belmonte et al., 1997; Dumont et al., 2002; Incagnone et al., 2015). Recently, Meyer-Milne et al. (2021) found a relation between cyst morphologies and geographic size range in South African large branchiopods. Cysts with larger sizes and/or without surface ornamentations showed larger distributional ranges, possibly due to wind-mediated dispersal. Although these results match ours (i.e. species with larger and smooth cysts more widespread than species with smaller ornamented ones), the characteristics of the ponds in our study area (i.e. frequent and longer hydroperiods, vegetated basins) would decrease the importance of wind as a dispersal agent. Therefore, other factors may be promoting cyst dispersal. For example, all these behavioural, morphological and life-history characteristics, along with a peak in abundance earlier in the hydroperiod (Cancela da Fonseca et al., 2008; Cristo et al., 2002; Gascón et al., 2012), may enable *T. stagnalis* to colonise more ephemeral ponds that would not allow the survival of *B. cortesi* due to its longer life span and later sexual maturity. As such, *T. stagnalis* seems to exhibit a wider niche breadth (Gascón et al., 2012).

The SAMOVA analysis identified groups of haplotypes that may represent frequent local and rare long-distance dispersal events for both species of fairy shrimp. However, intermediate-distance dispersal events seem to be much more common for *B. cortesi* rather than for *T. stagnalis*. Several vectors may contribute to local dispersal (<10 km) of both anostracan species. Local dispersal may involve endozoochory (dispersal via ingestion) or ectozoocory (external transport) processes. The most probable endozoochory events may be performed by predatory amphibians, especially the newt *Pleurodeles waltl* Michahelles, 1830, which is a common species in the study area (Beja & Alcazar, 2003; Cancela da Fonseca et al., 2008; Cristo et al., 2002), and/or by adult insects, especially Dytiscidae, that are common top predators in temporary ponds (Beladjal & Mertens, 2009; Boix et al., 2006; Cristo et al., 2002; Culler et al., 2014). Cysts of fairy shrimps are able to survive passage through the digestive tract of amphibians and insects, which promotes their local dispersal (Beladjal & Mertens, 2009; Bohonak & Whiteman, 1999; Thiéry, 1991). Wild boars frequently use aquatic habitats for wallowing (mud bathing; Vanschoenwinkel, Waterkeyn, et al., 2008) and the mud attached to hair and feet may carry a considerable number of cysts from both species that may be washed away in the nearby temporary ponds. Wild boars can easily travel daily distances between 2 and 15 km (Vanschoenwinkel, Waterkeyn, et al., 2008). Similarly, cattle frequently visit temporary ponds for

feeding and drinking at them, carrying away mud with large branchiopods cysts attached to their hooves (Thiéry, 1991).

Rare long-distance dispersal events (considered here as distances >40 km) were identified for both species of fairy shrimp involving connections between ponds separated by 60 to 80 km. These findings are in agreement with results obtained by Rodríguez-Flores et al. (2020) for *T. stagnalis*, particularly the ones referring to the distribution of specimens outside the Iberian Peninsula. Combining data from Rodríguez-Flores et al. (2020) and our own, we were also able to detect a relationship between eastern Alentejo and Spanish Extremaduran populations, and between populations inhabiting the mountain ranges of the Central System and the Pyrenees (Clade 1). As for plant and other invertebrate species (Brochet et al., 2010; Figuerola et al., 2003), there are some examples of birds dispersing large branchiopod species over long distances (Green et al., 2005; Proctor et al., 1967; Rogers, 2014; Viana et al., 2013). Ducks (e.g. mallards) and wading birds (e.g. *Pluvialis apricaria* L., 1758, *Charadrius dubius* Scopoli, 1786, *Tringa ochropus* L., 1758) have been frequently observed in the southern Portuguese temporary ponds (J. Ministro, personal communication; Cristo et al., 2002) and they may be responsible for the long-distance dispersal of both fairy shrimp species (J. Ministro, personal communication).

Differences in the use of the various mesohabitats in temporary ponds by aquatic birds could be responsible for the contrasting results in regard to intermediate dispersal events, considered here as distances ranging from 10 to 40 km. Mallard (*Anas platyrhynchos* L., 1758) is the most common duck species in Portugal (Rodrigues & Ferreira, 1993). They are opportunistic omnivore (Rodrigues & Ferreira, 1993) feeding on seeds and vegetative parts of aquatic plants, as well as on aquatic invertebrates such as insects, molluscs, and crustaceans, and occasionally also on amphibians (Eldridge, 1990; Rogers, 2014). Due to their capacity to dive, they can easily prey on *B. cortesi* which prefers the deeper zones of the ponds. Although some individuals are migratory, wintering in Portugal and flying north through the East Atlantic flyway at the end of the winter (thus, contributing putatively to long-distance dispersal), most mallards found on Portuguese territory are residents and 90% of their dispersing movements are up to 60 km between aquatic habitats (Rodrigues et al., 2000). However, their movements are adjusted to the wetland distribution in the landscape, thus increasing flight distances with decreasing wetland availability (Kleyheeg, van Dijk, et al., 2017). This characteristic implies a landscape-dependent dispersal of propagules in a landscape of temporary ponds (Kleyheeg et al., 2017), compatible with the phylogeographical structure of *B. cortesi*. By contrast, wading birds feed mainly on insects but also on other invertebrates, such as crustaceans, and on plant material (Proctor et al., 1967) at the shallower zones of the habitat (J. Ministro, personal communication), where *T. stagnalis* is usually found. We hypothesise that waders could be less efficient intermediate distance dispersing vectors than mallards, because, although capable of performing intermediate distance flights, they seem to remain more faithful to a particular site during non-migrating periods (Pearce-Higgins & Yalden, 2003; Smith et al., 1992), increasing their

likely contribution to local dispersal events. A portion of the cysts ingested by ducks and wading birds are viable after passing through the digestive tract (Beladjal & Mertens, 2009; Proctor et al., 1967; Rogers, 2014). As retention times in the digestive tract of birds can span from several minutes to several hours (Charalambidou et al., 2005; Green & Figuerola, 2005; Proctor et al., 1967), cysts can reach new sites because these birds move selectively among aquatic habitats (Rogers, 2014). Nonetheless, knowledge of the role of aquatic birds in the dispersal of large branchiopods in this protected habitat (according to Habitat European Directive—92/43/CEE), Mediterranean Temporary Ponds, is still almost non-existent.

5 | CONCLUSIONS

Our analyses revealed the existence of contrasting genetic patterns in two species of fairy shrimp with overlapping ranges in Iberian temporary ponds. The reconstructed phylogenies indicate shallower divergences in *B. cortesi*, whereas the three deeply divergent clades inferred for *T. stagnalis* show a clear geographic correspondence. The estimated earlier origin of the MRCA of *T. stagnalis* during the Oligocene provides a plausible explanation for its clear phylogeographic structure, given that older species are expected to exhibit higher genetic differentiation among populations. The severe cooling and aridification observed at the end of Oligocene in the Iberian Peninsula may have triggered a vicariant effect on *T. stagnalis* originating two divergent clades in populations inhabiting the leading edges of the Portuguese southern distribution. This genetic imprint is not observed in the Portuguese *B. cortesi* populations given their more recent origin during the Miocene. We hypothesise that temporary ponds from the SW Portuguese coastal plain might have played an essential role for the persistence of the two species in the area during Pleistocene glacial periods, acting as *refugia within refugia* when declining temperatures led to widespread population extinction outside those areas due to severe droughts. Spatial analysis of molecular variance identified frequent local and rare long-distance dispersal events for both fairy shrimp species. Slight differences in adult behaviour, life-history traits, and cyst morphologies of *T. stagnalis* and *B. cortesi* may further account for the observed differences in phylogeographic patterns, favouring different dispersing vectors with distinct dispersal abilities. Nevertheless, a multidisciplinary case study involving terrestrial ecologists, limnologists and geneticists is needed to further investigate the feeding behaviour and/or movements of potential vectors, as well as the viability of anostracan cysts retrieved from them, together with a deeper knowledge on the biology, ecology, behaviour and genetic structure of the anostracan species inhabiting those ponds. The need for this kind of joint research performed by researchers with different specialisations has also been emphasised by Green and Figuerola (2005).

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.