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Contrasting phylogeography of two Western Palaearctic fish parasites despite similar life cycles

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Abstract

Aim: We used comparative phylogeography of two intestinal parasites of freshwater fish to test whether similarity in life cycle translates into concordant phylogeographical history. The thorny-headed worms *Pomphorhynchus laevis* and *P. tereticollis* (Acanthocephala) were formerly considered as a single species with a broad geographical and host range within the Western Palaearctic.

Location: Central and eastern parts of Northern Mediterranean area, Western and Central Europe, Ponto-Caspian Europe.

Methods: A mitochondrial marker (COI) was sequenced for 111 *P. laevis* and 50 *P. tereticollis* individuals and nuclear ITS1 and ITS2 sequences were obtained for 37 *P. laevis* and 21 *P. tereticollis*. Genetic divergence, phylogenetic relationships and divergence time were estimated for various lineages within each species, and their phylogeographical patterns were compared to known palaeogeographical events in Western Palaearctic. Biogeographical histories of each species were inferred.

Results: The two species show very different phylogeographical patterns. Five lineages were identified in *P. laevis*, partially matching several major biogeographical regions defined in the European riverine fish fauna. The early stages of *P. laevis* diversification occurred in the peri-Mediterranean area, during the Late Miocene. Subsequent expansion across Western Europe and Russia was shaped by dispersal and vicariant events, from Middle Pliocene to Middle Pleistocene. By contrast, *P. tereticollis* has differentiated more recently within the Western and Central parts of Europe, and shows weak geographical and genetic structuring.

Conclusion: Our study highlights weak to moderate similarity in the phylogeographical pattern of these acanthocephalan parasites compared to their amphipod and fish hosts. The observed differences in the timing of dispersion and migration routes taken may reflect the use of a range of final hosts with different ecologies and dispersal capabilities. By using a group underrepresented in phylogeographical studies, our study is a valuable contribution to revealing the biogeography of host–parasite interactions in continental freshwaters.

KEYWORDS
amphipod, British islands, comparative phylogeography, Cyprinidae, Danube, helminth, Mediterranean, Messinian salinity crisis, *Pomphorhynchus*, Ponto-Caspian

1 | INTRODUCTION

Comparative phylogeography is a powerful approach to unravel the effects of biogeography and evolutionary history on the genetic structure of co-distributed species. More specifically, the life history and ecology of species provide a comparative framework for testing whether their phylogeographical structure is more impacted by palaeogeographical and palaeoclimatic events, or by biotic factors (Papadopoulou & Knowles, 2016). This is particularly exemplified in parasitic taxa, where differences in life history traits such as transmission mode, life cycle and host specificity can result in discordant genetic structure in response to similar historical events (van der Mescht, Matthee, & Matthee, 2015; and references therein). Yet, comparative phylogeographical studies on parasitic species are relatively scarce when considering that metazoan parasites comprise *c.* 40% of all living metazoan species (Dobson, Lafferty, Kuris, Hechinger, & Jetz, 2008). Most of these studies investigate host–parasite co-phylogeny, and predict weak host–parasite co-evolutionary signal if host associations play little role in the dispersal and diversification of parasites (Hoberg & Brooks, 2008) as, for instance, in parasites with long-lived free-living stages, in generalist multi-host parasites, or in parasites with a complex life cycle involving intermediate host(s). By contrast, few studies have compared the phylogeographical patterns of multiple parasite species having similar life cycle.

The aim of our study is to compare the phylogeographies of two Western Palaearctic freshwater parasite species sharing a similar life cycle and ecology, and occupying overlapping ranges. Continental-scale biogeography studies have demonstrated that the palaeogeographical history of continental waters within the Western Palaearctic is complex, with numerous dispersal and diversification opportunities for freshwater and brackish-water species, determined by the alternations of isolation and interconnection among hydrographic basins (several references in Costedoat, Chappaz, Barascud, Guillard, & Gilles, 2006; Dubut et al., 2012; Levy, Doadrio, & Almada, 2009; Appendix S1). Several regions (e.g., the peri-Mediterranean, Aegean, and Ponto-Caspian regions, and the Danubian system) have been identified as important sources of diversification during a time frame spanning from the Oligocene-Miocene to the Quaternary post-glacial period (see Appendix S1). By contrast, macroparasites of fish and crustaceans have been used only exceptionally for historical biogeography reconstructions.

We address whether two species of parasites with complex life cycles, *Pomphorhynchus laevis* (Müller, 1776) and *P. tereticollis* (Rudolphi, 1809) (thorny-headed worms, Acanthocephala), responded in a similar way to the palaeogeography of continental waters within the Western Palaearctic. Because of previous taxonomic confusion (Appendix S1 in Supporting Information), *P. tereticollis* has been recorded as *P. laevis* in most parasitological surveys (Špakulová, Perrot-Minnot, & Neuhaus, 2011; Vardić-Smrzlić et al., 2015), therefore leading us to use here the general term *P. laevis s.l.* wherever taxonomic distinction between these species could not be made. *Pomphorhynchus laevis s.l.* is potentially a valuable parasite model in the phylogeography of continental waters because it exhibits a large geographical distribution, and uses a broad range of freshwater and brackish-water fish species as final hosts and gammaridean amphipods as intermediate hosts (Kennedy, 2006; Špakulová et al., 2011; Vardić-Smrzlić et al., 2015) (Appendix S1). Although the precise distribution and host range of each of the two *Pomphorhynchus* species is unclear, they appear to occur in sympatry across Europe, with verified records from France to Slovakia, where they also share the same crustacean hosts and some of the fish hosts (Špakulová et al., 2011; Perrot-Minnot, unpublished data).

We expected that the expansion and differentiation of their intermediate amphipod host and final fish host, which began as early as the Late Eocene-Middle Miocene (Buonerba et al., 2015; Dubut et al., 2012; Hou, Sket, Fiser, & Li, 2011; Kotlík & Berrebi, 2002; Levy et al., 2009; Mamos, Wattier, Burzyński, & Grabowskiand, 2016; Perea et al., 2010; Appendix S1), has provided ecological opportunities for their own dispersal and differentiation. Our study relies on a range-wide sampling both in terms of hosts and geography, to identify evolutionary lineages for *P. laevis* and *P. tereticollis*, and to compare their respective biogeographical histories. Mitochondrial cytochrome *c* oxidase I (COI) and internal transcribed spacers (ITS1 and ITS2) sequences were used for species assignment and estimation of divergence time, while only COI was used to infer intraspecific phylogenetic relationships between lineages, and plausible biogeographical scenarios accounting for the genetic diversity within each species. We compared the observed phylogeographical pattern of each species to the major biogeographical units identified in European riverine fish fauna based on a cluster analysis of fish community composition (Reyjol et al., 2007). We also related these patterns to known geological events and palaeoenvironmental conditions in Western Palaearctic, starting from the Messinian salinity crisis (MSC) in the Mediterranean around 6 Myr ago. At this time, near-desiccation of the Mediterranean Sea promoted the cutting of deep fluvial canyons, connections of drainage basins and lake or river captures (Orszag-Sperber, 2006; Rouchy & Caruso, 2006), thereby offering dispersal opportunities for freshwater and brackish-water species. The phylogeographical patterns uncovered were also related to the expansion of freshwater systems during Pliocene (Neubauer, Harzhauser, Kroh, Georgopoulou, & Mandic, 2015), and the major Quaternary glaciations during Early and Middle Pleistocene (1.7 to 0.12 Myr).

We tested the hypothesis that these co-occurring species with similar life cycle and ecologies shared similar biogeographical histories. On the other hand, contrasting phylogeographical patterns may reveal ecological divergence or historical contingency, and would call for a thorough assessment of their respective host specificities. By comparing the phylogenetic pattern of *P. laevis* and *P. tereticollis* to that of their gammaridean and fish hosts, we address the relative contribution of the intermediate and final hosts to their dispersal and diversification. Thus, our study makes a contribution to revealing the historical biogeography of host-parasite interactions in continental freshwaters of the Western Palaearctic.

2 | MATERIALS AND METHODS

2.1 | Data acquisition

One hundred and sixty-one *P. laevis s.l.* individuals were collected from 56 localities across Western Palaearctic (Figure 1; Table S1.1 in Appendix S1), and fixed and stored in 95% ethanol. DNA was extracted following a standard CTAB-proteinase K and phenol-chloroform protocol (Perrot-Minnot, 2004), from either a small piece of adult worm or from whole larval cystacanth. A portion of the COI and the ITS + 5.8S genes were PCR-amplified and purified according to Perrot-Minnot (2004) (Table S1.2 in Appendix S1). COI sequences and ITS + 5.8S sequences were obtained by direct sequencing of purified PCR products (MWG, Germany, or Macrogen Inc., Seoul, South Korea and Amsterdam, The Netherlands). Pseudogenes were occasionally amplified with the COI general primers in *P. laevis* samples, as confirmed by the cloning of PCR products that yielded multiple sequences. We therefore designed internal primers specific to the authentic mtDNA COI gene, and used them to analyse some *P. laevis* samples from the Balkans and Italy (Table S1.2 in Appendix S1).

The majority of amplicons of both genes were sequenced in both directions, and were manually aligned with BioEdit software (Hall, 1999). Seven of the previously collected sequences of *P. tereticollis*

used for the taxonomic revision of this species (Špakulová et al., 2011) have been included in the dataset (accession numbers: ITS, JF06705; COI; JF706706, JN695504, JN695505 to JN695508).

2.2 | Phylogenetic inference

Phylogenetic reconstructions were performed using two probabilistic methods: a maximum likelihood method (ML) implemented in PHYML 3.0 (Guindon et al., 2010) and a Bayesian approach (BA) in MRBAYES 3.1.2 (Ronquist & Huelsenbeck, 2003). Best-fitting models of sequence evolution for each marker were determined using MRMODELTEST 2.3 (Nylander, 2004). ML and BA analyses were conducted under the general time-reversible (GTR) model (Yang, 1994) with a proportion of invariable sites (I) and a gamma distribution (G) for COI, and under the HKY model (Hasegawa, Kishino, & Yano, 1985) + I + G for ITS.

Node robustness was estimated with ML bootstrap percentages (BP) after 1000 pseudo-replicates, whereas Bayesian posterior probabilities (PP) were obtained from 50% majority rule consensus trees, after discarding the first 25,000 trees as burn-in. Three independent runs of five incrementally heated Markov chains Monte Carlo (MCMC) were performed with trees sampled every 100th generation for 5,000,000 generations.

FIGURE 1 Geographical location of sampling sites for the fish acanthocephalan parasites, *Pomphorhynchus laevis* and *P. tereticollis*, within the Western Palaearctic. Outlined shapes are major biogeographical regions identified from the composition of European riverine fish fauna by Reyjol et al. (2007), and colours refer to subunits within each region. For each sampling site, the number refers to the locality, and the symbol to the affiliation of haplotypes to one of the two *Pomphorhynchus* species (circle, *P. laevis*; triangle, *P. tereticollis*) or both (square) based on sequencing data. Full details on the samples (biogeographical subunit, locality, host species, haplotype and accession numbers) are given in Table S1.1 in AppendixS1 [Colour figure can be viewed at wileyonlinelibrary.com]

2.3 | Species tree and divergence time estimation

The species tree for *P. laevis* and *P. tereticollis* and for geographically delimited populations within each species (Figure 1) was estimated using MCMC for the multispecies coalescent model implemented in STARBEAST2 (Ogilvie, Bouckaert, & Drummond, 2017). The method infers the species tree from multiple genes sampled from multiple individuals from each species (Ogilvie et al., 2017). The COI (158 sequences) and ITS (52 sequences) genes were therefore included in the analysis as independent loci. Because STARBEAST2 makes the assumption that there is no gene flow following the population divergence, COI haplotype Pl46, likely representing a recent migration from the Adriatic to Tyrrhenian population (Figure 2a), was excluded from the input datasets.

Six models corresponding to three molecular clock assumptions (strict, relaxed lognormal or relaxed exponential) and two priors for the species tree (Yule and Birth-Death), within which the gene trees follow the multispecies coalescent prior, were compared using the Akaike's information criterion through MCMC (AICM; Raftery, Newton, Satagopan, & Krivitsky, 2007) to determine which combination best fits our data. Each analysis was repeated with the same priors but with a different random seed to ensure MCMC convergence and thus robustness of the analysis. Five independent runs of 100,000,000 generations each were then performed with the best-fitting clock and speciation model combination, with a burn-in stage of 10%. Data from repeated runs were combined with LOGCOMBINER 2.4.0 (Bouckaert et al., 2014) and a maximum clade credibility consensus tree with mean node heights was generated using TREEANNO-TATOR v2.4.0 (Bouckaert et al., 2014).

The absence of acanthocephalan (or even helminth) fossils complicates construction of a time-calibrated tree for *P. laevis s.l.* Furthermore, significant rate variation among invertebrate phyla limits the reliability of an arthropod COI clock (1.4% to 2.8% sequence divergence/Myr) (Knowlton & Weigt, 1998; Solà, Sluys, Gritzalis, & Riutort, 2013 and ref. therein), for inferring divergence times within another taxa (Thomas, Welch, Woolfit, & Bromham, 2006). Alternatively, palaeogeographical events of known age can be used to calibrate a molecular clock (Solà et al., 2013). Preliminary phylogenetic analysis in *P. laevis* revealed a basal split between Italian lineages and all other lineages. One of the major palaeogeographical events in the Mediterranean basin was the MSC and its associated changes in the extent and connections of freshwater bodies. The date of 6.05 ± 0.09 Myr (6.14–5.96 Myr, Rouchy & Caruso, 2006) for the isolation of the Mediterranean Sea and the divergence of the most recently diverged Italian population of *P. laevis* (Adriatic; Pl_L4) was therefore used as the calibration point.

2.4 | Genetic characterization of *Pomphorhynchus laevis* and *P. tereticollis* populations

Due to the limited intraspecific variation in the ITS sequences these analyses were performed with the COI dataset only. A Kimura-2-parameter distance was chosen for the estimation of the genetic divergence within and between *P. laevis* and *P. tereticollis* lineages using MEGA v5.2.2 (Tamura et al., 2011). The haplotype number (nh), nucleotide (π) and haplotype (h) diversities and the average number of nucleotide differences between two sequences (k) were obtained for each lineage using DNASP 5.10.01b (Librado & Rozas, 2009). The demographic history of lineages (stability or expansion) was investigated using three neutrality tests and mismatch indices (see Appendix S2 for details). Finally, population structures were evaluated with a median-joining network with NETWORK v4.5.1.6 (http://www.fluxus-engineering.com/sharenet.htm; Bandelt, Forster, & Röhl, 1999) for the haplotypes of *P. laevis* and *P. tereticollis*.

2.5 | Biogeographical analysis

To infer the geographical origin of *P. laevis* and *P. tereticollis*, the distribution of both species were subdivided into, respectively, 13 and five geographical areas, corresponding to the main drainages and/or recipient seas (Table S1.1 in Appendix S1). Considering the complex history of the Danubian system and its importance in most phylogeographical studies, four subareas were distinguished: Carpathian, Pannonian, and Balkan areas, and the Danube itself. Biogeographical inferences were obtained with the programs Statistical Dispersal–Vicariance Analysis (S-DIVA; Yu, Harris, & He, 2010, 2014) and Bayesian Binary MCMC (BBM) as implemented in the RECONSTRUCT ANCESTRAL STATE IN PHYLOGENIES software (RASP 3.0; Yu et al., 2014; Yu, Harris, Blair, & He, 2015). S-DIVA analyses were performed with Bayesian trees obtained for phylogenetic reconstructions. The maximum number of ancestral areas was set to two. In BBM analyses, MCMC was carried out using 10 chains (temperature 0.1) for 100,000 cycles with state sampled every 100th cycle, and a burn-in stage of 100. The Jukes-Cantor model + G was used, while the maximum number of ancestral areas and the root distribution were set, respectively, to 2 and as null. In both cases, the possible ancestral ranges were thus obtained at each node of S- DIVA and BBM trees.

3 | RESULTS
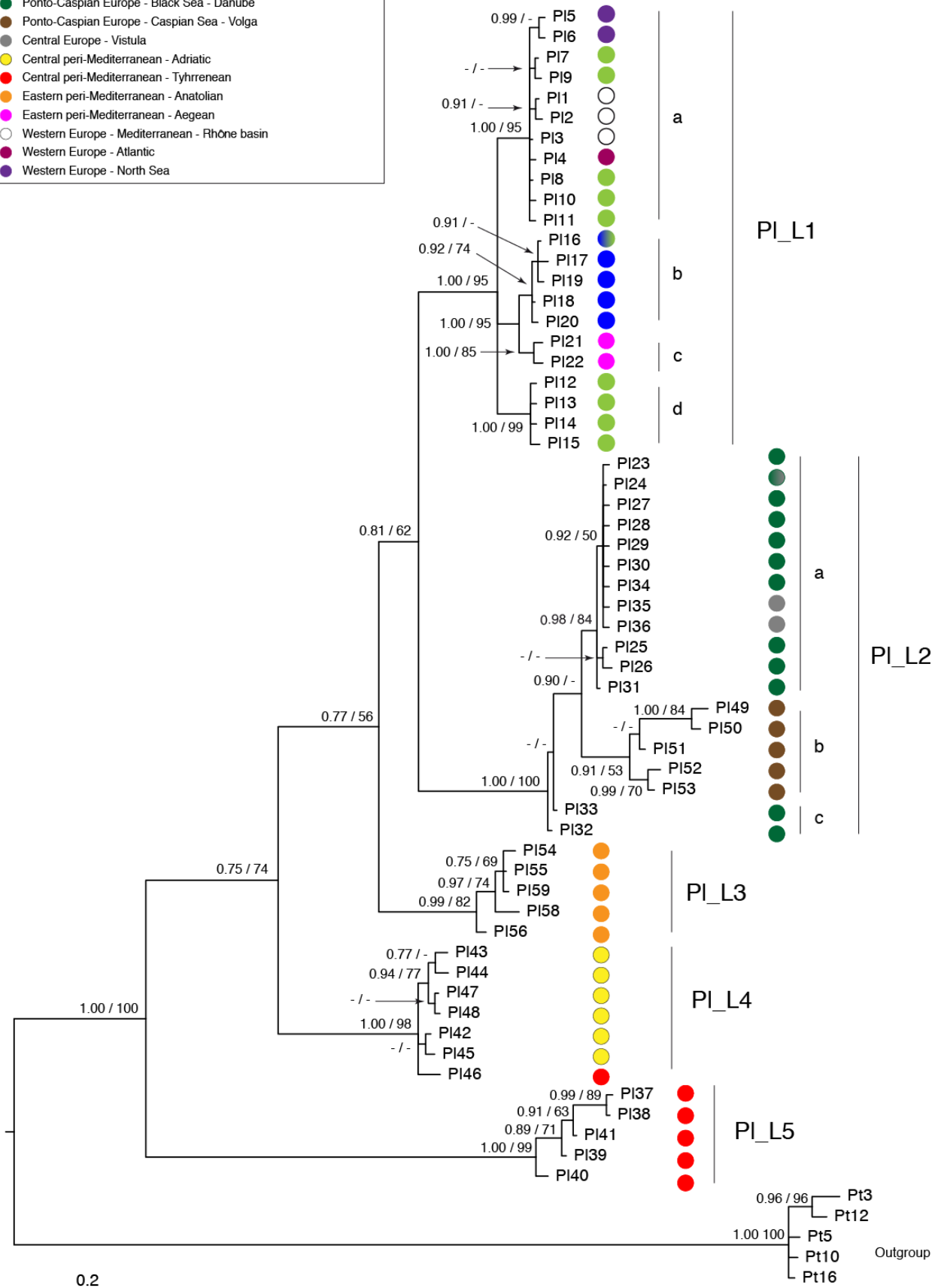
3.1 | Phylogenetic inference

Among the 161 sequences of *P. laevis s.l.*, 111 belong to *P. laevis* and 50 to *P. tereticollis*, based on the interspecific differences at ITS

FIGURE 2 Bayesian tree reconstructed from COI sequences of (a) *Pomphorhynchus laevis*, (b) *P. tereticollis*, and the species used as outgroup (*P. tereticollis* and *P. laevis*, respectively). Branch support values are maximum-likelihood bootstrap (≥50%) percentages and Bayesian posterior probabilities (≥0.70). Haplotypes are indicated by numbers as given in Table S1.1 in Appendix S1. Colour chart is corresponding to biogeographical subunits as defined in Figure 1 and Table S1.1 in AppendixS1 [Colour figure can be viewed at wileyonlinelibrary.com]
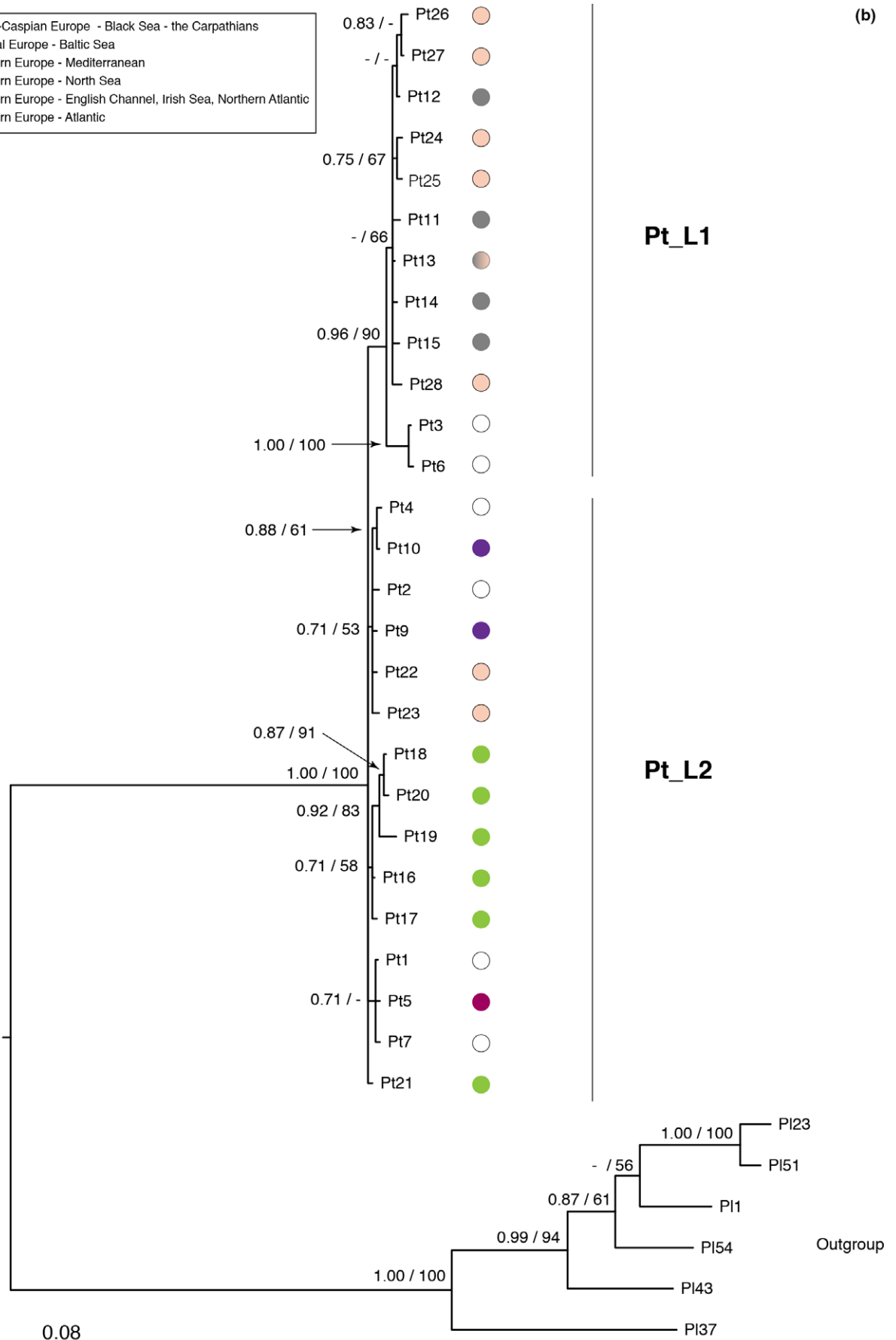
(a)

and COI reported in Špakulová et al. (2011). The gene tree constructed for 58 concatenated sequences of ITS1 and ITS2 confirmed the reciprocal monophyly of *P. tereticollis* and *P. laevis* (Figure S2.1. in Appendix S2).

The COI alignment represents 614 positions for both *P. laevis* and *P. tereticollis* with, respectively, 172 and 25 phylogenetically informative sites. For both species, BA analysis (Figure 2a and b) and ML (Figure S2.2 in Appendix S2) provided congruent tree topologies. When included in the same analysis (results not shown), COI sequences of *P. laevis* and *P. tereticollis* present a reciprocal monophyly with high support (BP = 100%; PP = 1.00). In *P. laevis*, five highly supported clades (Pl_L1 to Pl_L5) are identified (Figure 2a and Figure S2.2a in Appendix S2), suggesting strong geographical structuring of the populations. Clades Pl_L4 and Pl_L5 are two basal lineages found in Italian populations, one with Tyrrhenian (Pl_L5), and one with Tyrrheno-Adriatic (Pl_L4) distribution. Clade Pl_L3 is found in the Aegean and south-western Anatolian parts of Turkey. Clade Pl_L2 is distributed in Ponto-Caspian Europe, and includes haplotypes from the Volga (Pl_L2b) and Danube and Vistula rivers (Pl_L2a and c). Finally, clade Pl_L1 has a wide geographical distribution covering three major biogeographical areas: Eastern peri-Mediterranean, Ponto-Caspian, and Western Europe. It comprises haplotypes from the Balkan tributaries of the Danube (Pl_L1b), from an Aegean river (Pl_L1c), from Pannonian–Western Carpathians to Western Europe (Pl_L1a), and from Eastern Carpathians (Pl_L1b, Pl_L1d) (Figure 2a). By contrast, the COI gene tree of *P. tereticollis* shows much shallower structure with only one well-supported clade (Pt_L1) including haplotypes from Western to Central Europe, while the remaining haplotypes (collectively labelled as Pt_L2) are distributed in Western and Ponto-Caspian Europe (Figure 2b and Figure S2.2b in Appendix S2).

3.2 | Molecular dating

AICM values suggested that the relaxed exponential clock and the Yule model were significantly more likely given our COI and ITS datasets than the other priors. Consequently, the time-calibrated species tree for *P. laevis* and *P. tereticollis* was estimated with these models (Figure 3). With Tracer v.1.6 (Rambaut, Suchard, Xie, & Drummond, 2014), Markov chain convergence was ascertained by visual inspection and comparison of the traces and posterior distributions, and the effective sample size was >200 for all parameters.

The species tree (Figure 3) is largely concordant with the COI gene tree estimated by ML and BA (Figures 2 and S2.2). The calibration point gave an estimate of 0.0201 (0.0199–0.0202) substitution/ bp/Myr, similar to the substitution rate calibrated for invertebrate mitochondrial genes (1.4% to 2.8%: Knowlton & Weigt, 1998; Solà et al., 2013). The analyses suggested that the split between *P. laevis* and *P. tereticollis* took place during the Late Miocene (8.40 Myr). The major geographical populations of *P. laevis* began to diverge during Late Miocene (from 6.82 Myr). However, the divergence within groups of populations characterized by the two major COI clades (Pl_L1 and Pl_L2) seems to be related to Middle (0.50 Myr) and Early (1.27 Myr) Pleistocene, respectively (Figure 3). By contrast, the populations of *P. tereticollis* appear to have diverged much later, in Middle Pleistocene (0.42 Myr) (Figure 3).

3.3 | Genetic diversity, demographic history and population structure

In *P. laevis*, percentage divergence is from 1.2 ± 0.3% to 2.4 ± 0.4% within clades, and from 10.5 ± 1.2% to 20.3 ± 2.1% between clades (Table 1a). The genetic divergence is 1.6 ± 0.3% within *P. tereticollis*, and reaches 24.4 ± 1.8% between the two species (Table 1b). For both species, the nucleotide (π) and haplotype (*h*) diversities are rather homogeneous, whereas the average number of nucleotide differences between two sequences is relatively high (Table 2). The three neutrality tests and mismatch distribution reject population expansion for the five *P. laevis* lineages and for *P. tereticollis* (Table S2.3 in Appendix S2).

For both species, the median-joining network showed the same general pattern as the gene tree, with five *P. laevis* and two *P. tereticollis* haplogroups separated by 125 mutational steps (Figure 4). *Pomphorhynchus laevis* groups are separated by 47 to 83 mutational steps, while the two *P. tereticollis* groups are separated by only seven mutational steps (Figure 4).

Although the demographic analysis performed for each lineage of *P. laevis* rejects population expansion, the star-like network and the polytomic tree topology of the Danubian sublineage Pl_L2a suggest population expansion (Figure 4). By contrast, the three basal peri-Mediterranean lineages in Italy and Turkey, and the derived Volga sublineage could have experienced a loss of diversity, as suggested by the number of mutational steps and missing haplotypes within each lineage (Figure 4).

3.4 | Biogeographical analyses

For *P. laevis*, the geographical origin of ancestral populations is poorly resolved both in S-Diva and BBM analyses, as evidenced in widespread distribution range and occurrence frequencies less than 10% (nodes 120, 115, 108 in Figure S2.3 in Appendix S2). Both analyses generally suggest, however, that the ancestral range encompassed the peri-Mediterranean area (BBM) or the peri-Mediterranean to Ponto-Caspian area (S-Diva). The ancestral population of the Turkish and all non-Mediterranean populations was distributed in several alternative biogeographical areas, all anchored in the Eastern peri-Mediterranean (node 108 in Figure S2.3 in Appendix S2).

The ancestral population of all non-Mediterranean lineages could have been in the Danubian-Carpathian area within Ponto-Caspian Europe (EH in Table 3; node 103 in Figure S2.3 in Appendix S2). This population gave origin to a Danubian population, ancestral to the Ponto-Caspian lineage (Pl_L2), and a Carpathian–Western Europe population, ancestral to the widely distributed Ponto-Caspian and Western Europe lineage (Pl_L1) (H & EC in Table 3; nodes 81-80 and 102 in Figure S2.3 in Appendix S2). Genetic differentiation within the former occurred in the Danubian–Caspian Sea area,
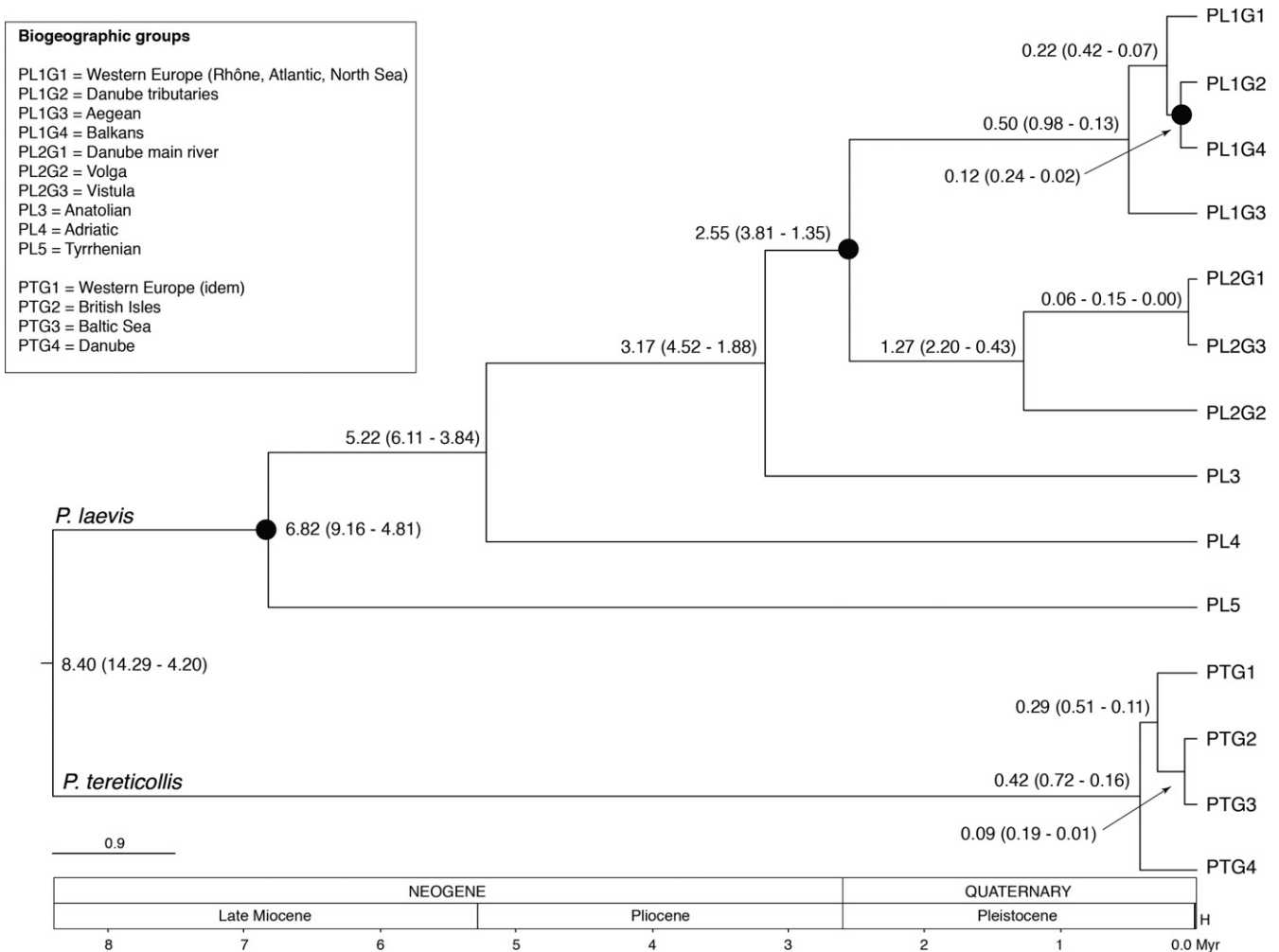
FIGURE 3 Species tree chronogram for *Pomphorhynchus laevis* and *P. tereticollis* estimated by STARBEAST2 based on COI and ITS data. Numbers at nodes are for divergence times (Myr) and their 95% confidence intervals estimated from a geologic calibration point, 6.05 ± 0.09 Myr (Messinian salinity crisis, Rouchy & Caruso, 2006). Black circles are for nodes supported with PP < 0.70 meaning that molecular dating at these nodes should be taken with caution

TABLE 1 Genetic distance based on COI sequences within and between (a) *Pomphorhynchus laevis* and (b) *P. tereticollis* lineages. The species used as outgroup for *P. laevis* and *P. tereticollis* were *P. tereticollis* and *P. laevis*, respectively. Genetic distance between lineages is given below the diagonal, and standard error above the diagonal

	Lineage	Distance within lineage (SE)	Distance between lineages					
			Pl_L1	Pl_L2	Pl_L3	Pl_L4	Pl_L5	Outgroup
(a)								
Pomphorhynchus laevis	Pl_L1	0.024 (0.004)		0.011	0.012	0.015	0.019	0.022
	Pl_L2	0.023 (0.004)	0.107		0.014	0.015	0.021	0.023
	Pl_L3	0.017 (0.004)	0.105	0.117		0.014	0.019	0.020
	Pl_L4	0.012 (0.003)	0.118	0.148	0.125		0.016	0.020
	Pl_L5	0.015 (0.003)	0.193	0.203	0.186	0.181		0.022
Outgroup		0.017 (0.003)	0.236	0.266	0.217	0.231	0.230	
		Distance within lineage (SE)			Distance between Pt and outgroup			
(b)								
Pomphorhynchus tereticollis		0.016 (0.003)						0.018
Outgroup		0.142 (0.011)			0.244			

TABLE 2 Genetic diversity indices from COI sequences for each lineage of *Pomphorhynchus laevis* and for *P. tereticollis*

Species	Lineage	n[a]	nh[b]	π (SD)[c]	h[d]	k[e]
Pomphorhynchus laevis	Pl_L1	50	22	0.021 (0.001)	0.922	13.049
	Pl_L2	39	19	0.014 (0.003)	0.830	8.669
	Pl_L3	8	5	0.011 (0.002)	0.857	6.964
	Pl_L4	9	7	0.013 (0.001)	0.917	7.778
	Pl_L5	5	5	0.017 (0.004)	1.000	10.500
Pomphorhynchus tereticollis		50	28	0.015 (0.001)	0.968	9.220

[a]Number of sequences.
[b]Number of haplotypes.
[c]Nucleotide diversity (*SD*, standard deviation).
[d]Haplotype diversity.
[e]Mean number of pairwise differences.

resulting in one sublineage in the Danube and Vistula rivers (Pl_L2a), and the other strictly Caspian (Volga, Pl_L2b), with no evidence of subsequent mixing (Figure 2a and Figure S2.3 in Appendix S2). Genetic differentiation within the Carpathian–Western Europe ancestral population occurred in a widespread area, and resulted in an Eastern Carpathian sublineage (Pl_L1d), a Western Carpathian-Pannonian-Western European sublineage (Pl_L1a) and an Eastern Carpathian-Northern Aegean sublineage (Pl_L1b, c) (Figure 2a; Table 3; nodes 102 and 91 in Figure S2.3 in Appendix S2). The latter sublineage experienced at least two biogeographical events: the splitting of a Northern Aegean lineage (Pl_L1c), and the dispersal towards the Balkans (Pl_L1b) (Table 3; nodes 87 and 85 in Figure S2.3 in Appendix S2, respectively). The Pannonian-Western European sublineage further dispersed towards its western margins, to the Atlantic river drainage (Pl_L1a; Figure S2.3 in Appendix S2).

For *P. tereticollis*, S-DIVA and BBM analyses suggest one possible ancestral range in Western Europe, more specifically in the Rhone drainage basin, possibly reaching as far as the Ponto-Caspian area (Table 3; node 59 in Figure S2.4 in Appendix S2). The geographical expansion of this ancestral population resulted in the formation of a Central and Western European lineage (Pt_L1), and a Western and Ponto-Caspian European lineage (Pt_L2) (node 56 in Figure S2.4 in Appendix S2). The Central and Western European lineage subsequently underwent repeated dispersal, vicariance and admixture events between the Baltic Sea and the British Isles (Figure 2b and Figure S2.4 in Appendix S2). The Western and Ponto-Caspian European lineage also experienced a complex history of dispersal, vicariance, admixture and extinction events within an area comprising the Rhone and Rhine systems, the Carpathians, and the British Isles (Table 3; nodes 55 and 50 in Figure S2.4 in Appendix S2).

4 | DISCUSSION

The present study aimed at comparing the range-wide phylogeographical structure of two Western Palaearctic acanthocephalan parasites with a similar complex life cycle. Our results supports the

evolutionary relationships between *P. laevis* and *P. tereticollis* inferred from species-specific features (Špakulová et al., 2011). Phylogenetic and biogeographical analysis, and molecular dating using the Messinian salinity crisis (6 Myr) to age-calibrate the phylogeny, revealed contrasted biogeographical histories between these species. Their distinct differentiation pattern at both geographical and time scales highlights their potential contribution to our understanding of the Western Palaearctic historical biogeography, as detailed below.

4.1 | The origin and comparative biogeographical structuring of *P. laevis* and *P. tereticollis*

Pomphorhynchus laevis and *P. tereticollis* diverged during the Late Miocene from a common ancestral lineage, possibly within the peri-Mediterranean area. Despite the low resolution of biogeographical analysis at deep nodes, three lines of evidence support this geographical origin: (1) in the haplotypic network, the closest relative of *P. tereticollis* to *P. laevis* is from the Rhone drainage, and is related to Turkish *P. laevis* haplotypes, (2) in *P. tereticollis*, the highest number of haplotypes is found in the Rhone drainage, and (3) the basal lineages of *P. laevis* are found in the Central and Eastern Mediterranean area.

In *P. laevis*, five lineages of Pleistocene age with 10.5% to 20.3% divergence were identified. They are distributed in the Central and Eastern peri-Mediterranean and throughout most of the Ponto-Caspian and Western Europe, but with almost completely non-overlapping geographical distribution except in the Danubian system. Such high divergence and phylogeographical structure provide evidence for multiple populations with a history of long-term persistence and separation. By contrast, *P. tereticollis* has no clear biogeographical structuring, as evidenced in a basal polytomy restricted mainly to Western, Central and Ponto-Caspian Europe. The overall genetic diversity of *P. tereticollis* is comparable to the within-lineage genetic diversity of *P. laevis*.

4.2 | In and out of the Mediterranean Sea: The pre-Quaternary evolutionary history of *P. laevis*

The two oldest genetic lineages of *P. laevis* find their origin in Late Miocene in the Italian Peninsula, one strictly Tyrrhenian and one Tyrrheno-Adriatic. According to a first scenario of colonization of Southern Europe (Gante, 2011; Levy et al., 2009; Tsigenopoulos, Durand, Ünlü, & Berrebi, 2003), dispersal from North Africa during the Messinian lacustrine phase of the Mediterranean Sea could account for the colonization of the Western Mediterranean. Under this 'southern Sea dispersal scenario', the Italian peninsula would have been colonized during the Lago Mare stage (6 Myr). However, the oldest lineage to all *P. laevis* has here a pre-Messinian origin, and the two basal Italian lineages are too divergent to both originate from this short-time event. Alternatively, the Italian peninsula could have been colonized prior to the intensification of Alpine orogenesis during Miocene, through river capture across north-eastern Europe (Perdices, Doadrio, Economidis, Bohlen, & Banarescu, 2003; Perea
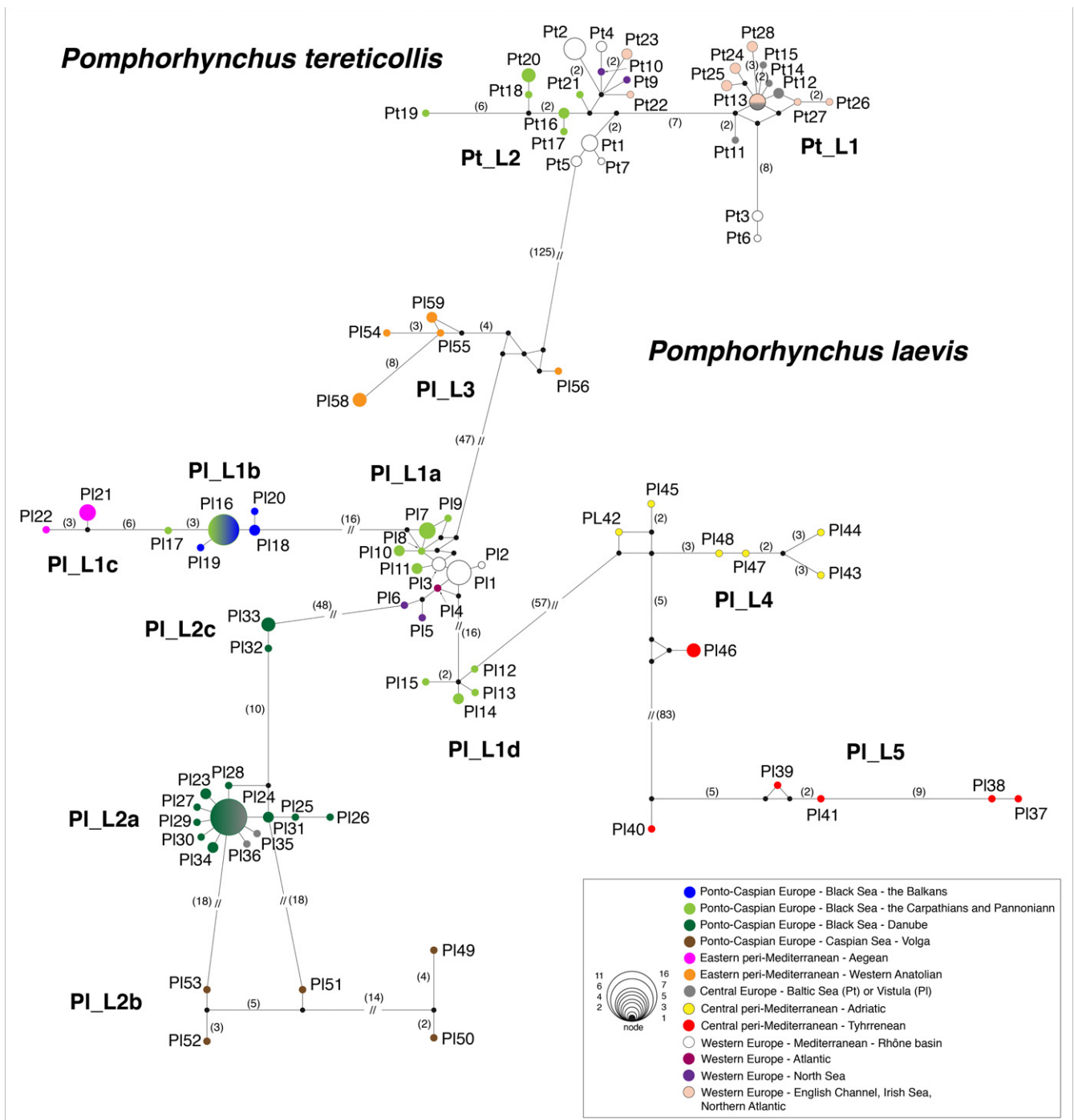
FIGURE 4 Median-joining network of the COI haplotypes from *Pomphorhynchus laevis* and *P. tereticollis*. Line lengths in the network reflect the number of mutational changes (in brackets when >1) between haplotypes, and the size of the circles is proportional to the frequencies of the represented haplotype. Black dots represent hypothetical missing or unsampled ancestral haplotypes. Haplotypes are indicated by numbers as given in Table S1.1 in Appendix S1. Colour coding corresponds to geographical areas as defined in Figure 1 and Table S1.1 in AppendixS1 [Colour figure can be viewed at wileyonlinelibrary.com]

et al., 2010). The Adriatic and Tyrrhenian populations could have subsequently diverged from a widespread ancestral population, at a time coincident with the emergence of the Northern Apennine Chain in Late Miocene; the flowing of Tyrrhenian and Adriatic rivers through opposite side of the Apennines would have precluded later exchange, as suggested for the speciation of endemic fluvio-

lacustrine *Barbus* species (Buonerba et al., 2015). Our data, however, do not allow us to favour one scenario over the other, and both a 'southern route' and a 'northern route' have been proposed for the colonization of the Mediterranean by leuciscine freshwater fish (Durand, Bianco, Laroche, & Gilles, 2003; Gante, 2011; Levy et al., 2009; Perdices et al., 2003; Perea et al., 2010).

TABLE 3 Biogeographical history inferred from S-DIVA analysis based on COI sequences for (a) *Pomphorhynchus laevis* lineages (except for peri-Mediterranean ones), (b) *P. tereticollis* lineages. The reconstructed history includes the divergence time estimates (from the analysis with STARBEAST2) and geographical distribution of the most recent common ancestor (MRCA), and the biogeographical scenario leading to derived populations (disp. dispersal, vic. vicariance, ext. extinction) and its associated probability (>17%). Abbreviations for biogeographical units are detailed in Table S1.1 in Appendix S1 (a): B, Pannonian area of Ponto-Caspian Europe; C, Western Europe; E, Carpathian area of Ponto-Caspian Europe; F, Balkan area of Ponto-Caspian Europe (Danubian southern tributaries); G, Eastern peri-Mediterranean (North Aegean-Anatolian); H, river Danube in Ponto-Caspian Europe; J, Caspian part of Ponto-Caspian Europe; K, Eastern peri-Mediterranean (North Aegean and West Anatolian). (b): A, British Isles; B, Baltic Sea; C, Western Europe; D, North Sea; E, Carpathian area. The symbol 'x' combines two or more biogeographical units covered by a derived population; na: not available

Pomphorhynchus laevis ancestral population (MRCA to derived populations)	Time to MRCA	Ancestral biogeographical distribution (% of alternative biogeographical area)	Biogeographical events		Derived populations or lineages	Ref. node[a]
(a)						
Ponto-Caspian × Western Europe	2.55 Myr	EH − BH − CH (33:33:33)	EH -> ECH -> EC | H (p = .17)	Disp. (1) vic. (1)	Carpathian x Western Europe//Danubian (Pl_L1, Pl_L2)	103
Danubian × Caspian	1.27 Myr	HJ (100)	HJ -> H | J (p = 1)	Vic. (1)	Danube, Vistula/Volga(Pl_L2a, Pl_L2b)	79–81
Carpathian × Western Europe	0.22 Myr	EC − EB (50:50)	EC - > BEC -> E | BC (p = .25)	Disp. (1) vic. (1)	Carpathian//Pannonian x Western Europe	102
Carpathian	0.50 Myr	E (100)	E -> E ^E -> E ^EG -> E | EG (p = 1)	Disp. (1)	Eastern Carpathian//Eastern Carpathian x North Aegean(Pl_L1d, Pl_L1b + c)	91
Eastern Carpathian × North Aegean	0.50 Myr	EG (100)	EG -> EGF -> EF | G (p = 1)	Disp. (1) vic. (1)	Eastern Carpathian x Balkans//North Aegean (Pl_L1b, Pl_L1c)	87
Pannonian × Western Europe	na	BC − B (50:50)	BC -> B ^BC -> B | BC (p = .25)	Disp. (1)	Pannonian//Pannonian x Western Europe (within Pl_L1a)	101
Eastern Carpathian × Balkans	na	EF (100)	EF - > E | F (p = 1)	Vic. (1)	Eastern Carpathian//Balkans (within Pl_L1b)	85

Pomphorhynchus tereticollis ancestral population (MRCA to derived populations)	Time to MRCA	Ancestral biogeographical distribution (% of alternative biogeographical area)	Biogeographical events		Derived populations or lineages	Ref. node[b]
(b)						
Ancestral to all lineages	0.42 Myr	C (100)	C->C^C->CE^C->C|CE (p = .6)	Disp. (1)	Western Europe (Rhone drainage)//Western Europe × Carpathian	59
Western Europe × Carpathian	0.42 Myr	CE or C (61:39)	CE->CE^C->CEB^C-> CB|CE (p = .34)	Disp. (2)	North-western Europe (Pt_L1)//Western and Central Europe (Pt_L2)	56
North-western Europe	0.29 Myr	CB (100)	CB->C|B (p = 1) and B->B^B-> AB^B->AB|B (p = 1)	Disp. (1) vic. (1)	Western Europe (Rhone drainage)//North-western Europe (Baltic sea, British Isles)	44, 41
Western and Central Europe	na	CE or E (52:48)	CE->E->E^E->EA^E->E|AE (p = 0.23)	Disp. (2) vic. (1) ext. (1)	Eastern Carpathian//Southern Carpathian – British Isles	55
Central Carpathian – British Isles	na	AE or CE or DE (40:35:25)	AE->EAC->E|AC (p = 0.17)	Disp. (1) vic. (1)	Eastern Carpathian//Western and North Europe, British Islands (within Pt_L2)	50

[a]Ref. node in Figure S2.3 in Appendix S2.
[b]Ref. node in Figure S2.4 in Appendix S2.

The third distinct Mediterranean lineage is specific to the Turkish populations of the North Aegean–West Anatolian area, and is basal in the phylogenetic tree relative to the non-Mediterranean lineages. Its geographical origin is unclear given insufficient resolution of the biogeographical analysis. However, the Lago Mare stage of the MSC has possibly allowed the dispersal of freshwater hosts of *P. laevis* within the eastern part of the Mediterranean basin. The presence of contiguous freshwater bodies at a regional scale, suspected from biogeographical studies and faunal assemblages (Neubauer et al., 2015; Solà et al., 2013), could have promoted the dispersal and subsequent isolation of freshwater populations between adjacent areas (Levy et al., 2009; Perea et al., 2010; Tsigenopoulos et al., 2003), or at a circum-Mediterranean scale (Buonerba et al., 2015; Durand et al., 2003; Gante, 2011). Additionally, conditions for dispersal for freshwater organisms were less restricted within the Eastern Mediterranean than in the Western Mediterranean (Levy et al., 2009; Orszag-Sperber, 2006). For instance, several cyprinid fish species have dispersed in the Eastern Mediterranean during the Lago Mare between 5.5 and 5.3 Myr ago, and subsequently differentiated after the refilling of the Mediterranean Sea (Dubut et al., 2012; Durand et al., 2003). This is consistent with the molecular dating obtained for the split between the Turkish population and the non-Mediterranean populations around 3.17 Myr, that postdates the Lago Mare event.

The Late Miocene marine flooding of the Mediterranean Basin and the final settling of geomorphological barriers during Miocene (Alps in Northern Italy) and Pliocene (Turkish straits and Marmara Sea) have precluded any subsequent peri-Mediterranean dispersal, and promoted the independent evolution of these three populations. Their long-term persistence in isolation is reflected in their low contribution to the recent genetic pool of *P. laevis* in Western and Ponto-Caspian Europe. This result is consistent with the high level of endemism of the Mediterranean freshwater ichthyofauna (Gante, 2011; Perea et al., 2010; Reyjol et al., 2007). To confirm this pattern, more samples need to be analysed from other peri-Mediterranean localities. During Mid-Pliocene, the expansion and differentiation of one or several populations ancestral to the two non-Mediterranean lineages matches the expansion of landmasses and associated networks of new freshwater lacustrine-riverine habitats (Neubauer et al., 2015).

4.3 | Pleistocene expansion and differentiation of *P laevis* and *P. tereticollis* throughout Europe

As for many Western Palaearctic taxa during the Pleistocene, *P. laevis* and *P. tereticollis* have experienced post-glacial range expansion alternating with more or less severe range contraction and fragmentation. In *P. laevis*, the Danubian-Caspian populations (Pl_L2) began to diversify during the Pleistocene around 1.27 Myr ago, possibly within the northern Black Sea-Caspian Sea region. This scenario agrees with the existence of Ponto-Caspian refugium in several freshwater fishes (e.g. Durand, Persat, & Bouvet, 1999; Kotlík, Bogutskaya, & Ekmekci, 2004; Perdices et al., 2003; Seifertová,

Bryja, Vyskočilová, Martínková, & Šimková, 2012). The pattern of Pleistocene differentiation between the Western and Ponto-Caspian European populations (Pl_L1) appears to be geographically more widespread. Our molecular dating and ancestral biogeographical analysis suggest several expansion and differentiation events around 0.50 Myr ago from a Carpathian-Western Europe ancestral population, south-eastward into several sublineages covering the Eastern Carpathian, Balkan and North Aegean areas, and westward into a Pannonian-Western Europe area. Given the history of these Pleistocene lineages, the occurrence within the Danubian system of two differentiated populations (Pl_L2 and Pl_L1), in the Danube itself and in its tributaries, respectively, is puzzling. One scenario that remains to be tested is the replacement of Pl_L1 in the Danube by Pl_L2 from the Vistula or Volga Rivers along with introduced Ponto-Caspian amphipods and/or fish hosts. Replacement followed by rapid population expansion could account for the star-like network and polytomic gene tree of this Danubian population.

For *P. tereticollis*, the haplotype network, molecular dating and biogeographical analysis suggest an ancestral population in the Rhone basin, which began to diversify around 0.42 Myr ago. The maintenance of ancestral polymorphism in the Rhone drainage is evidenced in the highest haplotypic diversity found in its tributaries at a very local scale. One population descending from the Rhone ancestral population appears to have dispersed northward to the North and Baltic Sea, and the other one eastward to the Rhine and the Carpathians. Dispersal from a Rhone ancestral population to Rhine river could have occurred when the contemporary Alpine Rhine was diverted northward to flow into the Rhine drainage during Middle Pleistocene (Dubut et al., 2012, and ref. therein). Occasional inter-basin exchanges within the Rhine/Rhone corridor prevented Pleistocene differentiation between these basins. On the other hand, considering repeated and transient connections between the Rhine, Rhone and Danube drainages during Pleistocene (op. cit.), the restricted Ponto-Caspian distribution of *P. tereticollis* to the Carpathians within this lineage is puzzling, and raises issues about either sample representativeness, ecological constraints, or Pleistocene extinction in Ponto-Caspian Europe.

4.4 | *Pomphorhynchus tereticollis* and the biogeographical history of the British Isles

In *P. tereticollis*, several waves of Pleistocene colonization from continental Europe left their genetic imprint in the British Isles, with the presence of the two COI lineages Pt_L1 and Pt_L2. Two routes of dispersal from continental Europe accounted for the introduction of haplotypes of both lineages into the British Isles during the past 0.42 Myr, a northern route across the North Sea, and a southern route through the English Channel. The northern route across the Baltic-North Seas allowed the introduction of Pt_L1 haplotypes. The lack of geographical structure within Pt_L1 suggests a recent colonization or recurrent dispersal opportunities as the Middle Pleistocene between the Northern British Isles and Northwestern Europe, despite prolonged periods of glaciation. At the time, the

Fennoscandinavian ice sheet (FIS) could have forced northern rivers from the current Baltic Sea drainage to flow into the North Sea and connect with Scottish continental waters. The southern route through the English Channel allowed the introduction of Pt_L2 haplotypes from continental Rhine-Rhone populations. Here again, the weak genetic structure within this lineage suggests that introduction into Southern England has a recent origin or, alternatively, has occurred repeatedly since the Middle Pleistocene. This latter interpretation is congruent with the activity of the 'Fleuve Manche' palaeoriver over the last 0.35 Myr, connecting rivers from southern England and Western Europe, including the Thames, Seine and Rhine, via the English Channel (Toucanne et al., 2009). The presence of the two lineages of *P. tereticollis* validates the description of distinct strains of *P. laevis s.l.* in the British Isles (Kennedy, 2006; O'Mahony, Bradley, Kennedy, & Holland, 2004), here identified as *P. tereticollis*.

4.5 | Host-related biogeographical history

Overall, the phylogeographical pattern of these parasites is not directly comparable to that of host species, with respect to the geographical distribution and timing of diversification. *Pomphorhynchus laevis* shows higher co-occurrence of divergent genetic lineages across Western Europe and Ponto-Caspian area, and more recent divergence during Pleistocene, compared to freshwater amphipod hosts (Hou et al., 2011; Mamos et al., 2016), and to rheophilic fish hosts (Costedoat et al., 2006; Kotlík & Berrebi, 2002; Šedivá et al., 2008). Compared to its contemporary main fish hosts, *P. laevis* shows more ancient differentiation than its common host, *Barbus barbus* (Kotlík & Berrebi, 2001; Kotlík et al., 2004), and a distinct Pleistocene history compared to the European chub, *Squalius cephalus* (Seifertová et al., 2012), but with a similar biogeographical pattern (Durand, Ünlü, Doadrio, Pipoyan, & Templeton, 2000). Interestingly, lowland warm-adapted fish species responded differently to Pleistocene glaciation cycles compared to cold adapted species from headwaters, with higher dispersal and/or extinction events (Dubut et al., 2012; Šedivá et al., 2008; Seifertová et al., 2012). The use of a range of final hosts with different ecology may thus explain the mixed pattern of the timing and migration route observed in *P. laevis*. The most striking difference compared to all freshwater fish studied in the Western Palaearctic is the co-occurrence, within the Danubian system, of two lineages, one in the Danube itself, the other in its tributaries. The ichthyofauna and amphipod fauna from the Danube have been profoundly changed with the introduction of Ponto-Caspian species in recent times. Replacement by the Ponto-Caspian *P. laevis* may have occurred with the expansion of the barbel *B. barbus* from Black Sea refugium into the Danube around the end of the Pleistocene (Kotlík et al., 2004), along with Ponto-Caspian amphipod hosts. The use of distinct intermediate hosts in the Danube and its tributaries could further constitute an ecological barrier to lineage mixture within the Danube basin, a hypothesis that deserves further investigation.

In *P. tereticollis*, host strains have been reported in the British Isles (Kennedy, 2006; O'Mahony et al., 2004), that could match the two phylogeographical lineages identified here. The Central and Western Europe lineage likely correspond to the marine-estuarine strain infecting flatfish, plaice and founder and the Irish strain infecting trout, and the Western and Ponto-Caspian Europe lineage to the strain found in cyprinids. We hypothesize that only the Irish marine strain, parasitizing anadromous cold-adapted fish hosts with long-distance dispersal, could have reached the Northern part of the British Isles from the Baltic and North Seas during Pleistocene. This scenario is further supported by the literature records of this strain infecting a salinity-tolerant (*Gammarus duebeni*) and an estuarine (*Gammarus zadacchi*) gammarid species as intermediate hosts (Guillen-Hernandez & Whitfield, 2001, and ref. herein).

5 | CONCLUSION

Our phylogeographical study provides unique insights into the contrasted histories and distributions of two species of acanthocephalan parasites of fish. The phylogeographical patterns of *P. laevis* and *P. tereticollis* are concordant with known palaeogeographical events within the Western Palaearctic. They are also weakly to moderately concordant with the phylogeographical pattern of their amphipod and fish hosts, as expected for multihost parasites with a complex life cycle. A more extensive sampling scheme, with respect to host range, within-population variation, and biogeographical area, should be implemented in the future, to allow full understanding of the relative contribution of historical and ecological factors to the biogeographical history of *P. laevis* and *P. tereticollis*. The possible existence of cryptic species within *P. laevis*, as suggested by the extent of genetic divergence between lineages and divergence time estimates, should also be addressed. We expect that future research on these subjects will further enhance the value of the acanthocephalan parasites as models for studying the historical biogeography of host–parasite interactions in continental freshwaters.

DATA ACCESSIBILITY

All new COI and ITS sequences were deposited in the EMBL database 'European Nucleotide Archive' under the accession numbers LN994840 to LN995000 and LN995001 to LN995058, respectively, and are available at http://www.ebi.ac.uk/ena/data/view/LN994840-LN995058.

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BIOSKETCH

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SUPPORTING INFORMATION

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