

## Vicariance in a generalist fish parasite driven by climate and salinity tolerance of hosts

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## Abstract

Acanthocephalans are parasites with complex lifecycles that are important components of aquatic systems and are often model species for parasite-mediated host manipulation. Genetic characterisation has recently resurrected *Pomphorhynchus tereticollis* as a distinct species from *Pomphorhynchus laevis*, with potential implications for fisheries management and host manipulation research. Morphological and molecular examinations of parasites from 7 English rivers across 9 fish species revealed that *P. tereticollis* was the only *Pomphorhynchus* parasite present in Britain, rather than *P. laevis* as previously recorded. Molecular analyses included two non-overlapping regions of the mitochondrial gene- cytochrome oxidase and generated 62 sequences for the shorter fragment (295 bp) and 74 for the larger fragment (583 bp). These were combined with 61 and 13 sequences respectively, from Genbank. A phylogenetic analysis using the two genetic regions and all the DNA sequences available for *P. tereticollis* identified two distinct genetic lineages in Britain. One lineage, possibly associated with cold water tolerant fish, potentially spread to the northern parts of Britain from the Baltic region via a northern route across the estuarine area of what is now the North Sea during the last Glaciation. The other lineage, associated with temperate freshwater fish, may have arrived later via the Rhine/Thames fluvial connection during the last glaciation or early Holocene when sea levels were low. These results raise important questions on this generalist parasite and its variously environmentally adapted hosts, and especially in relation to the consequences for parasite vicariance.

## 1. Introduction

Modern-day species distributions in many regions are a consequence of complex dispersal events shaped by the last glaciation (Pedreschi et al., 2019). Complex colonisation histories include the water vole *Arvicola amphibius* in the British Isles, where there are two genetically distinct populations today, both of which were previously assumed to have all arrived during the Holocene. However, ancient DNA (aDNA) analysis of fossil water voles from Britain has suggested that one of the populations of the species had arrived earlier during the last glaciation (Brace et al., 2016). Similar patterns have been made apparent from aDNA analyses of brown bear *Ursus arctos* that is now believed to have persisted in NW Europe during the Last Glacial Maximum (LGM- c.20.000-c.16.000 BC), possibly including in Britain (Ersmark et al., 2019). This further raises the possibility of other species being present in northern regions during the last glaciation that were previously believed to have colonised in the Holocene. It also raises important questions about how co-evolutionary relationships can be maintained through such climate changes (Graham and Lundelius, 1984), including those between free-living host species and their parasites. It also prompts questions regarding how vicariance in such organisms takes place (Schlutter, 2000).

The issues of understanding dispersal and colonisation events for parasites with complex lifecycles is important given their frequent use in studies on their ecology and co-evolutionary relationships with free-living hosts (e.g. Lefevre et al., 2009). For example, *Pomphorhynchus laevis* (Zoega in Müller, 1776) is a generalist parasite with a complex lifecycle involving a wide range of definitive hosts and has a broad geographical distribution in the Palaearctic (Kennedy, 2006, Spakulova et al., 2011). It has been frequently used as a model for testing hypotheses on parasite manipulation, where its modification of the

behaviour of its intermediate Gammaridae hosts has been studied extensively (e.g. Fayard et al., 2020). However, the phylogenetic and phylogeographic affinities of *P. laevis* in relation to that of its various hosts have not been considered. Moreover, high morphological and physiological variability has been reported in *P. laevis* in both continental Europe (Perrot-Minnot, 2004) and the British Isles (Kennedy et al., 1989, O'Mahony et al., 2004). In combination, both of these aspects are important given that genetic and morphological studies have recently indicated the existence of a cryptic species within the *P. laevis* species complex (Kral'ova-Hromadova et al., 2003, Perrot-Minnot, 2004, Spakulova et al., 2011, Perrot-Minnot et al., 2018, Reier et al., 2019). The presence of two *Pomphorhynchus* species has also been supported through karyotype analysis (Bombarova et al., 2007) with *Pomphorhynchus tereticollis* (Rudolphi, 1809) recently re-described as a distinct species following genetic work that indicated its divergence from *P. laevis* (Perrot-Minnot et al., 2018, Spakulova et al., 2011).

Within the British Isles, *P. laevis* was originally recorded from three river systems, the Thames, the Severn and the Hampshire Avon, with these populations comprising the 'British strain'. A 'marine strain' was also believed to exist in the Baltic and North Sea (Kennedy et al., 1989), which was genetically similar to that found in Ireland and Scotland (O'Mahony et al., 2004). This discontinuous distribution was explained by natural post-glacial events and subsequent dispersal by anthropogenic activities, such as the translocation of infected fish, especially European barbel *Barbus barbus*. In the introduced range, the parasite utilised locally available species of Gammaridae as intermediate hosts and cyprinid fishes as final hosts (Kennedy and Rumpus, 1977). Whilst the only genetic investigation into *P. laevis* in the British Isles gave support for two major British strains that were associated with different host typologies, this was based on a short fragment of subunit I of cytochrome c oxidase and

used only a limited number of samples from specific hosts (O'Mahony et al., 2004), thus limiting the inferences that can be drawn from these data.

More recent work on *Pomphorhynchus* spp. across a wide European range used a longer fragment of subunit I of cytochrome c oxidase and included 13 samples from the UK and Ireland, with all samples having been identified as *P. tereticollis* (Perrot-Minnot et al., 2018). The samples from Great Britain had sequences that belonged to the two main clades, PtL1 and PtL2, which have different phylogeographic histories (Perrot-Minnot et al., 2018). Perrot-Minnot et al., (2018) demonstrated that *P. tereticollis* has differentiated in Western and Central Europe and then expanded to form two main lineages; PtL1 (Central and European lineage) which subsequently underwent dispersal, vicariance and admixture between the Baltic Sea and the British Isles, and PtL2 (Western and Ponto-Caspian lineage) whose dispersal, vicariance and admixture occurred within the Rhone and Rhine rivers, the Carpathians and the British Isles. In light of these recent results, it could be hypothesized that the 'marine strain' of the British Isles (Kennedy et al., 1989) belong to the PtL1 lineage whereas the 'British strain' (Kennedy et al., 1989) would be of the PtL2 lineage.

Consequently, following this new information on the phylogeography of *P. tereticollis* (Perrot-Minnot et al., 2018) and the non-species specificity of the genetic region amplified by O'Mahony et al., (2004), the aims here were to (a) investigate the genetic and morphological identity of British *Pomphorhynchus* parasites by increasing the number of *Pomphorhynchus* parasites tested; and (b) extending the work of O'Mahony et al., (2004) and Perrot-Minnot et al., (2018) by further testing the role of host and vicariance in driving strain formation in *Pomphorhynchus* spp. in Britain through using newly generated genetic sequences combined with publicly available sequences.

## 2. Materials and Methods

### 2.1 Sample collection

Adult *Pomphorhynchus* spp. samples were obtained from their fish hosts by electric fishing six rivers in England (Table 1). The sampled fish species, comprising of both definitive and paratenic hosts, were *Cottus gobio*, *Squalius cephalus*, *Barnbatula barbatula*, *Phoxinus phoxinus*, *Anguilla anguilla*, *Salmo trutta*, *Gobio gobio*, *Rutilus rutilus* and *Cyprinus carpio*. The fish were identified to species, euthanized (anaesthetic overdose; Tricaine methanesulfonate) and dissected in the laboratory, with any *Pomphorhynchus* spp. present in the gastrointestinal tract removed. These adult *Pomphorhynchus* samples were supplemented by the collection of larval samples from five rivers, four for which adult samples were already available (Table 1). The larval samples were collected from their amphipod intermediate hosts. This required the hosts to be collected by kick-sampling in the rivers, sorting for infected amphipods (identified by an orange spot on their back; Sheath et al. 2016), followed by dissection of the larval parasites from the amphipod host in the laboratory. All *Pomphorhynchus* spp. samples were then preserved in 98 % ethanol.

### 2.2 Genetic analyses

Two genetic regions were amplified for each *Pomphorhynchus* spp. sample (adult and juvenile): (1) subunit I of cytochrome c oxidase (a mitochondrial gene) (Kral'ova-Hromadova et al., 2003, Spakulova et al., 2011); and, (2) the small fragment of subunit I of cytochrome oxidase I used by O'Mahony (2004) (with the latter analysed to provide consistency across all the studies, as it represents the only genetic region from historical British *Pomphorhynchus* samples).

Total genomic DNA was extracted from adult and larval parasites using the Qiagen DNeasy extraction kit (Qiagen), and genetic analyses were performed on the cytochrome oxidase subunit I (COI) gene using primers LCOI1490 and HCO2198, and yielding 583 bp DNA fragments which from here on are referred to as COI-long (primer details in Perrot-Minnot et al., 2018). Strain formation in the British Isles was further investigated by amplifying a separate region of the COI gene using the primers p4 and p3, as per O'Mahony et al., (2004), yielding a DNA fragment of 295 bp, hereafter referred to as 'COI-short'. Polymerase chain reactions were performed using the standard protocol of the Multiplex PCR kit (Qiagen) in 10 µl reaction volume with 10 ng of template DNA and annealing temperature of 50 °C for all primer pairs.

### 2.3 Phylogenetic analyses

All sequences were manually aligned using BioEdit ver. 5.0.9 (Hall, 1999), with haplotype number determined using DnaSP v.5 (Librado and Rozas, 2009). The haplotype sequences were deposited in the GenBank database (accession numbers KY075791-KY075800). For phylogenetic analyses, sequences available from GenBank (which included information on the host from which the parasite was extracted from) were added to the dataset (Supplementary material Table 1). Phylogenetic relationships for the COI-long region were reconstructed using MrBayes (Pronquist et al., 2012) using sequences from the present study, plus all the sequences of *P. tereticollis* available from GenBank, with *P. laevis* used as an outgroup (LN994840, LN994890, LN994940, LN994943, LN994930 and LN994924). The substitution model, GTR + I, was chosen using jmodeltest (Darriba et al., 2012). Robustness of the nodes was estimated using posterior probability with MrBayes (Ronquist et al., 2012). Two independent runs of four heated Markov chains Monte Carlo (MCMC) were used. The

generation number was set to 1,000,000 MCMC replications with one tree sampled every 500 generations. The first 250,000 trees were discarded from the analysis (25% burn-in). The tree was visualized using figtree (<http://tree.bio.ed.ac.uk/software/figtree/>). The median-joining haplotype network was calculated using PopART (Leigh and Bryant, 2015).

#### 2.4 Testing the influence of host salinity tolerance, country and biogeographical region on strain formation

The influence of host, host salinity tolerance and biogeographical region in explaining the genetic variation with the *Pomphorhynchus* spp. samples was investigated using all of the COI-long sequences generated in this study, plus the available *P. tereticollis* sequences available in Genbank (see Supplementary material Table 1); this resulted in 87 sequences for analysis in AMOVA (Arlequin ver 3.5.2.2; Excoffier and Lischer, 2010). Using the COI-short region, the data generated in this study were combined with those of O'Mahony et al., (2004), and used in AMOVA to test which variable (country [England, Scotland and Ireland] and host salinity tolerance) explained the most variation within Britain and Ireland. This provided a more balanced design (sequences from 75 stenohaline vs. 48 euryhaline hosts) when compared to O'Mahony et al., (2004) (20 stenohaline vs. 41 euryhaline sequences).

The biogeographical region used followed the work of Perrot-Minnot et al., (2018). As Perrot-Minnot et al., (2018) did not assign a biogeographical region to samples from Scotland and Ireland then in the AMOVA analysis performed here, two possible biogeographical groupings for Scottish and Irish samples were tested for: (a) grouped within the Western Europe–English Channel, and (b) in a proposed Northern Europe group (encompassing samples from Central Europe-Baltic Sea, Ireland and Scotland).



## 2.4 Morphological analysis

The genetic analyses were then complemented by morphological characterisation (as per Špakulová et al., (2011)) of 10 individual *Pomphorhynchus* parasites collected from 10 fish (*Anguila anguila* and *Oncorhynchus mykiss*) from the rivers Thames and Hampshire Avon (Supplementary material Table S1). Archived specimens from the rivers Kennet, Thames and Hampshire Avon were obtained from the Environment Agency (n=6, England) and the Natural History Museum (n=4 London, England) and were also assigned to species through morphological identification. These samples originated from English rivers between 1951 and 2015, and all had been previously recorded as *P. laevis* (as *P. tereticollis* was considered a junior synonym at that time). For the characterisation of the contemporary parasites, live adults were dissected from their fish hosts, relaxed in tap water to promote eversion of the proboscis and fixed in 100% ethanol, before they were cleared using creosote and examined microscopically (x100 to x400) to obtain hook measurements using Image J (Schneider et al., 2012). Small regions of the body from fixed parasites were removed and stored for molecular confirmation.

## 3. Results

### 3.1 Phylogenetic analyses and strain formation hypothesis

The COI-long and COI-short gene fragments were amplified from 74 and 62 samples respectively (Supplementary material Table S2). All sequences were deposited in Genbank (KY075791-KY075817).

The phylogenetic reconstruction using the COI-long used the 74 sequences from the present study and 13 sequences from Perrot-Minnot et al., (2018). The analysis identified that

haplotypes belonging to the two established *P. tereticollis* lineages are present in Britain, with individuals from Scotland and Ireland grouping within lineage PtL1 and all samples from England (collected in the present study) grouping within lineage PtL2 (Figure 1). The effect of country, biogeographical region and host salinity tolerance as explanatory variables for the genetic differentiation between lineages PtL1 and PtL2 for the COI-long gene revealed that host salinity tolerance and a potentially northern European biogeographical grouping explained the largest proportion of genetic variation (54.33 % and 63.24 % respectively (COI-long; Table 2, Figure 1). The influence of host and host-salinity tolerance was further tested using the COI-short region using the data generated in the current study (62 sequences) and the 61 sequences generated by O'Mahony et al., (2004), and revealed host salinity tolerance was the most significant explanatory variable (Table 2, Figures 2, 3). These results also demonstrated that the parasites present in western England grouped with those in the lineage present in the River Thames basin (Figure 1).

### 3.2 Morphological analysis

Morphological analysis of all adult parasites had characteristics consistent with recent descriptions of *P. tereticollis* (Spakulova et al., 2011). These included presence of medial hook projections, notably stouter hooks in rows 5-6, presence of hooks on the base of the proboscis bulb and hook morphometrics consistent with the ones reported by Špakulová et al., (2011) (Supplementary Figure 1, Supplementary Table S1). Furthermore, the re-examination of archived material also confirmed that all specimens from English rivers, previously identified as *P. laevis* since 1951, were morphologically consistent with recent descriptions of *P. tereticollis*.

## 4. Discussion

The results here indicate that *P. tereticollis* is present and established in British freshwaters, with no evidence that *P. laevis* is also present. This absence of *P. laevis* included rivers that have previously been reported to harbour historic *P. laevis* infections. The re-examination of archived material supported the misidentification of *P. tereticollis* as *P. laevis*. As such, it is proposed that *P. laevis* is absent from British freshwaters and that the name *P. tereticollis* should replace all previous literature pertaining to *P. laevis* in Britain (e.g Kennedy et al., 1989). This includes the only genetic information purporting to be of British *P. laevis*, which used the short COI gene that is not species specific (O'Mahony et al., 2004). It seems likely that this record also represents *P. tereticollis*, despite it being reported as *P. laevis* due to the more precise COI-long sequences reported here from four populations (O'Mahony et al., 2004, Perrot-Minnot et al., 2018).

The analysis of the genetic data collected in the present study, in combination with all *P. tereticollis* sequences deposited in Genbank with their reported host, revealed two distinct clades within *P. tereticollis*, which corresponded to those reported in Perrot-Minnot et al. (2018). Furthermore, the analysis provides support for the hypothesis put forward by Perrot-Minnot et al., (2018) that parasites belonging to clade PtL1, possibly associated with cold adapted fish, may have spread to the northern parts of Britain from the Baltic region via a northern route across the estuarine area in what is now the North Sea during the last glaciation. The second lineage, PtL2, may have arrived later via the Rhine/Thames fluvial connection during the last Glaciation or early Holocene when sea levels were low (as proposed by Perrot-Minnot et al., 2018). The present data supports this, as the English *P. tereticollis* populations cluster with *P. tereticollis* from the River Rhine which is one of the main rivers which connected to East flowing English rivers such as the Thames at that time.

Subsequently, *P. tereticollis* has invaded numerous western flowing rivers through the translocation of its main definitive hosts, particularly *B. barbus*, and has been documented to expand its range soon after its introduction (Kennedy et al., 1989).

The presence of a morphologically and genetically distinct *P. tereticollis* in Scotland and Ireland (Evans et al., 2001, O'Mahony et al., 2004) had been initially linked to a different strain, which possibly arose due to adaptation to salinity tolerant host species. In the current study, analyses with both COI gene segments (long and short) strongly support the effect of host salinity tolerance in explaining the genetic differentiation between the *P. tereticollis* lineages PtL1 and PtL2, as proposed by Perrot-Minnot et al., (2018). The analyses of variance conducted here, where samples from Ireland and Scotland were grouped within the Central European-Baltic sea biogeographic region (as per Perrot-Minnot et al., 2018), suggest the possibility that the PtL1 lineage spread from that region in the past. It appears to have co-evolved with cold and salinity tolerant fish hosts such as brown trout *Salmo trutta*, but it can still infect cyprinid species (Figure 1). Phylogeographic studies of *S. trutta* have suggested they survived as a species through the Late Pleistocene in northern refugia in Europe and that British populations cannot be distinguished from the continental populations, suggesting a relatively recent dispersal or connection (Bernatchez, 2001). This may imply that a movement of *S. trutta* with the PtL1 lineage of the *P. tereticollis* parasite spread since the end of the Last Glacial Maximum (post-LGM; circa 16 kyrs ago) from the Baltic region to Britain. This in turn may have been enabled by the low lying northern Doggerland area, which would have been dominated by deltaic/estuarine environments that possibly provided a link between the Baltic and northern Britain, including Scotland, during low sea levels (Sturt et al., 2013). This scenario is consistent with the phylogeographic analyses of other cold

tolerant fish, such as *C. gobio* (Hanfling et al., 2002), which suggest the survival of fish throughout the LGM in northern European rivers.

A second dispersal route is suggested for the PtL2 parasite lineage, with its association with warmer, freshwater adapted fish hosts. This was likely to have occurred later, during the Early Holocene, or perhaps the Late Glacial Interstadials, due to the warmer association of the specific fish hosts. However, it had to occur prior to the inundation by rising seas levels of the fluvial connection between the Rhine and the Thames (and other East flowing Southern English river catchments) at ca. 7 kyrs (Sturt et al., 2013).

The majority of the hosts sampled in this study were fish and the intermediate hosts were not as widely sampled. Therefore, the influence of the intermediate hosts and their respective vicariance in explaining the phylogeographic patterns of *P. tereticollis* remains largely unexplored. The parasite has a complex lifecycle and would have required the presence of both intermediate and final hosts in its colonization of the British Isles. Future work could therefore focus on the intermediate hosts, especially for the PtL1 lineage, with exploration of the role that they have played in the colonization route of the northern British Isles. Sampling should include both fresh and estuarine waters.

In conclusion, the present study provides further support for the proposed hypotheses of Perrot-Minnot et al., (2018) to explain the phylogeography of *P. tereticollis*. It also updates the only other British Isles genetic work on *Pomphorhynchus* parasites of O'Mahony et al., (2004). The results confirm that *P. tereticollis* is the only *Pomphorhynchus* parasite species present in British freshwaters, raising a series of evolutionary and ecological questions that require further investigation. Of particular interest is the potential to use *P. tereticollis* as a

model species to address the role of host-specialisation driving speciation in generalist parasites, especially as a comparator to *P. laevis*. In particular, *P. tereticollis* can be used to better understand the relationship between a generalist parasite and its variously environmentally adapted hosts, and the consequences of this to vicariance in that parasite.

#### Ethical Standards

Not applicable.

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#### Conflicts of Interest

The authors declare no conflicts of interest.

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Table 1: Sampling location and haplotype distribution by species for all samples collected in the present study.

River	Host	Haplotype distribution										
		COI-Long					COI-short					
		C1	C2	C3	C4	C5	C6	C7	L1 <sup>**</sup>	L2 <sup>**</sup>	L3 <sup>**</sup>	
Hampshire Avon	<i>Cottus gobio</i>		13							3	10	
	<i>Squalius cephalus</i>		3							1	2	
	<i>Barbatula barbatula</i>		3				1			2	1	1
	<i>Phoxinus phoxinus</i>		2							2		
	<i>Gammarus</i>		1						1			
	<i>Anguila anguila*</i>		1									
	<i>Anguila anguila</i>		3									3
	<i>Salmo trutta</i>		5									5
Darent	<i>Cottus gobio</i>		3							1	2	
	<i>Squalius cephalus</i>	1	2									3
	<i>Gobio gobio</i>		3									3
	<i>Phoxinus phoxinus</i>		3									3
	<i>Rutilus rutilus</i>		1									1
	<i>Barbatula barbatula</i>		3									3
Kennet	<i>Phoxinus phoxinus</i>		2							1	1	
Lodden	<i>Cottus gobio</i>		1	1								2
	<i>Phoxinus phoxinus</i>		2									2
	<i>Gammarus</i>		2									2
Teme	<i>Cottus gobio</i>		6		1					1	6	
	<i>Barbatula barbatula</i>		1							1		
	<i>Gammarus sp.</i>											
Thames	<i>Cyprinus carpio*</i>								1			
	<i>Gammarus sp.</i>							2	1			
Cain	<i>Gammarus sp.</i>		1									

\*Old samples; \*\*L1 = AY390511; L2 = AY390509; L3 = AY390512; Haplotypes C2 and C6 correspond to haplotypes Pt22 and Pt23 in Perrot-Minnot et al., (2018) respectively.

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Table 2: Results of AMOVA analyses where populations were grouped by country, biogeographic region (as defined by Perrot-Minnot et al., 2018), species and host salinity tolerance. Analyses were performed using sequences generated in this study plus sequences from Perrot-Minnot et al., (2018) for the long COI and O'Mahony et al., (2004).

	Groups	d.f	Among groups (%)	Among populations within groups (%)	Within populations (%)	F <sub>CT</sub>	P
COI long	Country	5	22.62	43.67	33.70	0.22624	0.09
	Biogeographic region (IRL&SCO included in Western Europe)	2	41.43	32.79	25.78	0.41427	0.02
	Biogeographic region (IRL&SCO as a distinct group)	3	52.82	22.15	25.03	0.52821	<b>0.003</b>
	Biogeographic region (IRL, SCO combined within the Central Europe group)	2	54.33	21.12	24.55	0.54327	<b>0.0009</b>
	Host salinity tolerance	1	63.24	8.59	28.17	0.63242	<b>0.009</b>
COI short	Country (IRE vs UK)	1	51.42	36.61	11.97	0.51417	0.05
	Country (IRE, ENG, SCO)	2	60.20	27.98	11.82	0.60197	0.02
	Host salinity tolerance	1	60.88	11.21	27.91	0.60878	<b>0.01</b>

For the long COI, a number of different groupings were tested for Ireland (IRL), Scotland (SCO) and England (ENG): (a) grouped together in the Western Europe biogeographic region; (b) IRL and SCO as distinct biogeographic region; (c) IRL, SCO grouped within central European group.

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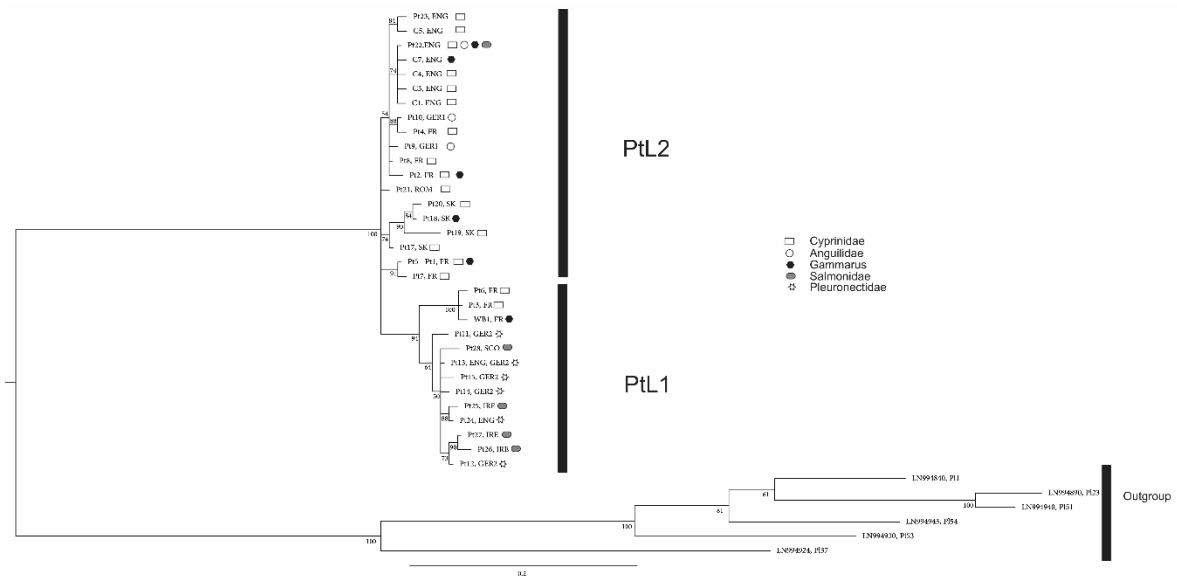


Figure 1 Bayesian tree reconstructed from COI-long sequences of *Pomphorhynchus tereticollis*. *P. laevis* was used as an outgroup. Branch support values are posterior probabilities. Haplotypes are indicated in Table S1. Geographical locations are denoted next to the haplotype and are: ENG-Englang; FR-France; GER1- Germany(Rhine); GER2- Germany (Baltic); IRE-Ireland; ROM-Romania; SK-Slovakia.

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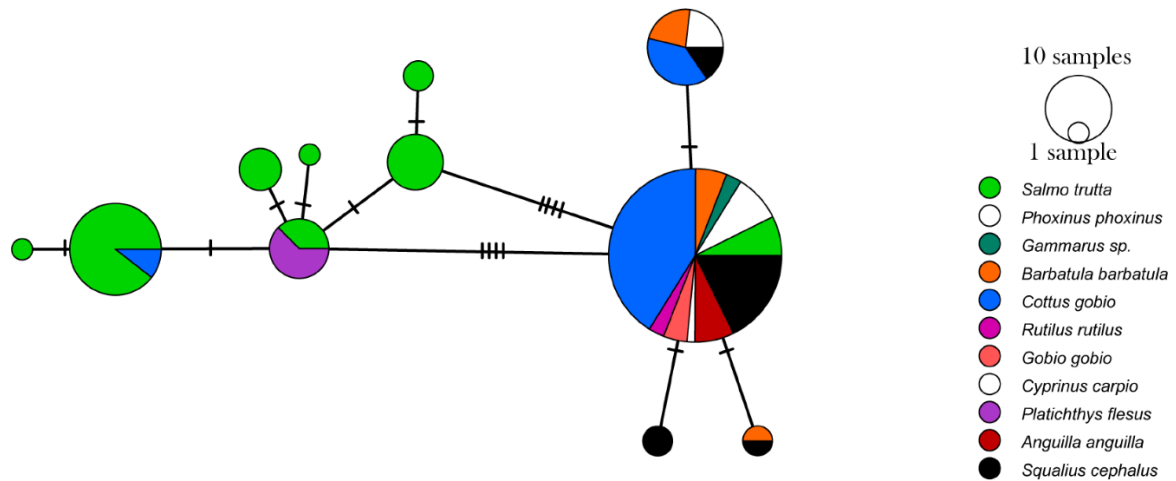


Figure 2 Network of *Pomphorhynchus tereticollis*, COI-short (295 bp) from Ireland and Britain. Samples were categorised by host species. Data from the current study (n=62) and O'Mahony et al., (2004) (n=56) were combined (detailed description of data in Supplementary Table 2). Each line represents 1 base difference between samples.

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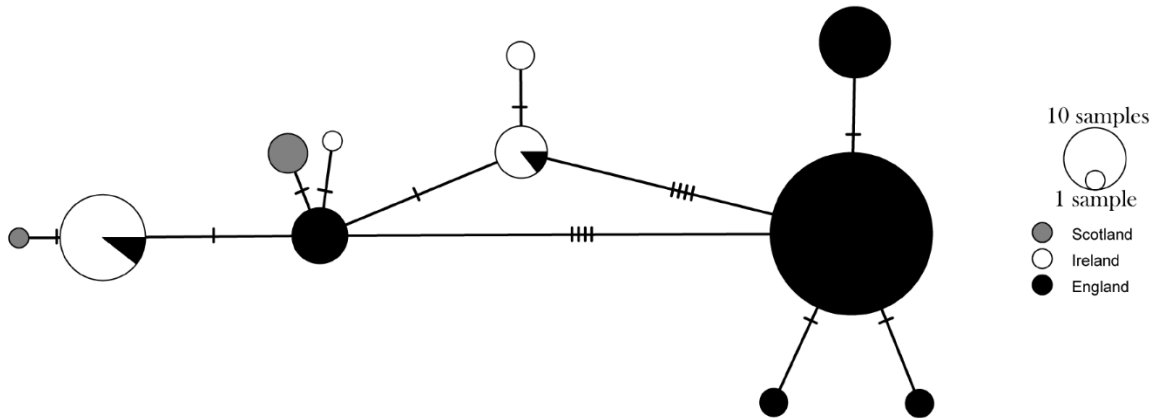


Figure 3 Network of *Pomphorhynchus tereticollis*, COI-short (295 bp) from Ireland and Britain. Data were categorised by country of origin. Data from the current study (n=62) and O'Mahony et al., (2004) (n=56) were combined (detailed description of data in Supplementary Table 2). Each line represent 1 base difference between samples

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