Distribution and molecular phylogeny of biliary trematodes (Opisthorchiidae) infecting native *Lutra lutra* and alien *Neovison vison* across Europe

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ABSTRACT

The recent identification of *Pseudamphistomum truncatum*, (Rudolphi, 1819) (Trematoda: Opisthorchiidae) and *Metorchis bilis* (Braun, 1790) Odening, 1962 (synonymous with *Metorchis albidus* (Braun, 1893) Looss, 1899 and *Metorchis crassiusculus* (Rudolphi, 1809) Looss, 1899 (Trematoda: Opisthorchiidae)) in otters from Britain caused concern because of associated biliary damage, coupled with speculation over their alien status. Here, we investigate the presence, intensity and phylogeny of these trematodes in mustelids (principally otters) across Europe (Czech Republic, Denmark, France, Germany, Norway, Poland and Sweden and Britain). The trematodes were identified to species using the internal transcribed spacer II (ITS2) locus. Both parasites were found across Europe but at unequal frequency. In the German state of Saxony, eight out of eleven (73%) otters examined were infected with *P. truncatum* whilst this parasite was not found in either mink from Scotland (n = 40) or otters from Norway (n = 21). Differences in the phylogenies between the two species suggest divergent demographic histories possibly reflecting contrasting host diet or competitive exclusion, with *M. bilis* exhibiting greater mitochondrial diversity than *P. truncatum*. Shared haplotypes within the ranges of both parasite species probably reflect relatively unrestricted movements (both natural and anthropogenic) of intermediate and definitive hosts across Europe.

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1. Introduction

Although parasites play an integral role in ecosystem functioning [1–2], there is incomplete knowledge of their geographic and host ranges. This arises in part because of the morphologically cryptic nature of many parasitic taxa and, specifically, the challenge of detection and species determination [2–3]. This is particularly problematic for invading parasites, which often present serious risks to novel host populations, largely because of naïve host immune responses coupled with disruption of ecosystem equilibrium [4]. The recent identification of *Pseudamphistomum truncatum* (Rudolphi, 1819)

and *Metorchis albidus* (Braun, 1893) Loos, 1899 (Trematoda: Opisthorchiidae) in Britain [5–6] caused initial concern because of the biliary damage to otters that was associated with both digeneans, and speculation over their alien status in Britain [5–7].

P. truncatum occurs across Europe and was reported in the early 1900s [8] from mammals that are native to Britain. However, without details of host origin, the recorded hosts (red fox *Vulpes vulpes* [also see 7], grey seal *Halichoerus gryphus*, domestic cats *Felis domestica* and dogs *Canis familiaris*, common seal *Phoca vitulina* and the harp seal *Phoca groenlandica* [8]) may have been sampled in continental Europe. Therefore, it remains unclear whether *P. truncatum* is a recent invader in Britain.

Historically, the taxonomy of the *Metorchis* genera has been complicated because of variable parasite morphology in multiple vertebrate

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hosts (including man) across Eurasia [9–12]. Since the discovery of *M. albidus* in Britain [6], molecular analysis has revealed an identical ITS2 sequence across all specimens identified morphologically as *M. albidus* (collected from otters *Lutra lutra* and mink *Neovison vison* as part of the current study), as well as specimens identified as *M. bilis* (Braun, 1790) Odening, 1962, and *M. crassiusculus* (Rudolphi, 1809) Looss, 1899 (principally from fish-eating birds and opportunistic birds of prey; Heneberg, pers. Comm.). Consequently, we support the view that *Metorchis bilis* (Braun, 1890), *M. albidus* and *M. crassiusculus* are synonymous, and the name *bilis* should take priority [see also [12] and references therein]. There are only ambiguous historic records of *M. bilis*/*M. albidus* in Britain [8], whereas *M. crassiusculus* has been noted with more conviction from British birds but only using morphological identification methods [13]. Species determination of a morphologically challenging genus like *Metorchis* can now be addressed molecularly [e.g. [14], Heneberg, pers. Comm.]. The damage associated with host-parasite interactions makes the identification of parasitic species and their geographic ranges particularly crucial for successful conservation efforts [15–16].

The life-cycle of the Opisthorchiidae family has been elucidated only rarely [see [12]] but involves two intermediate and a definitive host. For both *P. truncatum* and *M. bilis*, the first intermediate hosts are freshwater snails. A free-living cercarial stage is then released and encysts in a freshwater fish intermediate host. The development of *P. truncatum* can be completed once consumed by a mammalian definitive host, whereas *M. bilis* seems more generalist in terms of suitable definitive hosts (piscivorous mammals and birds) [8–13].

Here, we aimed to explore the distribution, intensity and molecular phylogenies of *P. truncatum* and *M. bilis* in otters and mink from across Europe. To address this, we collected samples across Europe, confirmed species identification using internal transcribed spacer region II (ITS2) ribosomal DNA sequences, and compared genetic diversity between the parasite species and between populations using two mitochondrial DNA markers (COX1 and COX3).

2. Material and methods

2.1. Sample collection

Gall bladders of 723 Eurasian otters (*L. lutra*) and 144 American mink (*N. vison*) were sourced from across Europe. Samples were included from Britain, Czech Republic, Denmark, France, Germany, Norway, Poland, Scotland, and Sweden, and preserved in 95% ethanol (a list of samples and host locations is provided in Supplementary Information 1). Each gall bladder was opened along its length in a petri dish containing fresh ethanol. The bladders were examined thoroughly and then rinsed, everted and the mucosa was finely combed to ensure all parasites were found, morphologically identified using a dissecting microscope according to [17] and counted. A sub-sample of parasites (N = 65, see Table 1) was selected for molecular analysis. For British samples (positive cases were found within England and Wales), stratified random sampling was applied, to select trematodes broadly representative of the geographic distribution previously identified [6] (*P. truncatum* from the Counties of Somerset, Dorset, Gwent and Powys; *M. bilis* from Bedfordshire, Cambridgeshire, Essex, Hertfordshire and Suffolk). For continental samples DNA sequencing was performed on all samples.

A general linear model (GLM), with an associated binary error distribution, was used to compare the parasite prevalence (the infection status of an individual regardless of the number of parasites present; infected = 1, uninfected = 0) between European regions where the sample size was equal to or larger than 10. To investigate intensity (the number of parasites infecting each individual, excluding those without infection) differences across host populations in Europe, a GLM (with a negative binomial error distribution) was fitted to the intensity data for regions where sample size of infected hosts was greater than or equal to 4. This analysis of intensity was limited however because of the small sample size available (Table 1). All analyses were conducted in R, version 3.2.0 [18].

2.2. DNA analysis

The internal transcribed spacer II (ITS2 *P. truncatum*: GenBank Accession number: JF710315; *M. albidus* (synonymous with *M. bilis*): GenBank Accession number: JF710316) of the ribosomal DNA region was used for species discrimination because it has been shown to be relatively conserved in the Digenea and has been used previously for interspecific analyses [12,14,19–20] while fragments of the mtDNA COX1 and COX3 genes were used to examine genetic variation of *P. truncatum* and *M. bilis* populations across Europe.

DNA was extracted from whole individuals. The tissues were stored in 90% ethanol, which was evaporated by gentle heating (55 °C) prior to digestion of the sample. Whole individual trematodes were digested for 3 h at 55 °C in 15 μl TE buffer containing 0.45% Tween 20 and 2 μg Proteinase K, followed by 10 min at 95 °C to denature the proteinase K [adapted from 21]. This treatment was sufficient to extract the DNA in preparation for the polymerase chain reaction (PCR), the mixture was centrifuged prior to use in the PCR. The PCR was conducted using 2 μl of the DNA extract in a final volume of 10 μl, containing: $1 \times$ PCR buffer II (Applied Biosystems), 2 mM $MgCl_2$ (Applied Biosystems), dNTP (0.25 mM each), primers (1 μM each) (ITS2 rDNA: Ophet F1 5′-CTCG GCTCGTGTGTCGATGA-3′ and Ophet R1 5′-GCATGCARTTCAGCGGGTA-3′ see [22]; or COX1 mtDNA: ThaenCO1F 5′-CGGGTTTTGGAGCGTCATTC-3′ and ThaenCO1R 5′-ACAGGCCACCACCAAATCAT-3′; or COX3 mtDNA: CO3FTremat 5′-ATGAGWTGATTACCKTT-3′ and CO3RTremat 5′-ACAACC

Table 1
Summary of otter (*Lutra lutra*) and mink (*Neovison vison*) biliary parasites. The country of origin (location), total number of hosts examined (N), number of infected hosts and the corresponding percentage and intensities of the two species (*Pseudamphistomum truncatum* and *Metorchis bilis*) isolated from European piscivorous mammals.

Location	N (otters unless otherwise specified)	*Pseudamphistomum truncatum*		*Metorchis bilis*	
		Number of infected hosts (%)	Mean intensity[+] (N for DNA analysis)	Number of infected hosts (%)	Mean intensity (N for DNA analysis)
Czech Republic	*	–	– (1)	–	– (1)
Denmark	52	3 (5.8)	2.3 (4)	16 (30.8)	3.4 (8)
France, Brittany	22	0	-	4 (18.2)	2 (4)
France, Poitou-Charentes	19	1 (5.3)	4 (1)	0	–
Germany, Saxony	11	8 (72.7)	29 (8)	2 (18.2)	1 (1)
Norway	21	0	–	0	–
Poland	*	–	– (2)	–	–
Scotland	40 (mink)	0	–	0	–
Sweden	12	2 (16.7)	96 (3)	2 (16.7)	13.5 (2)
England and Wales	586	79 (13.5)	28.3 (17)	46 (7.8)	4.1 (12)
England and Wales	104 (mink)	9 (8.7)	222 (0)	0	–

*Parasites provided directly. [+] Mean intensity defined as the mean number of parasites per infected host.
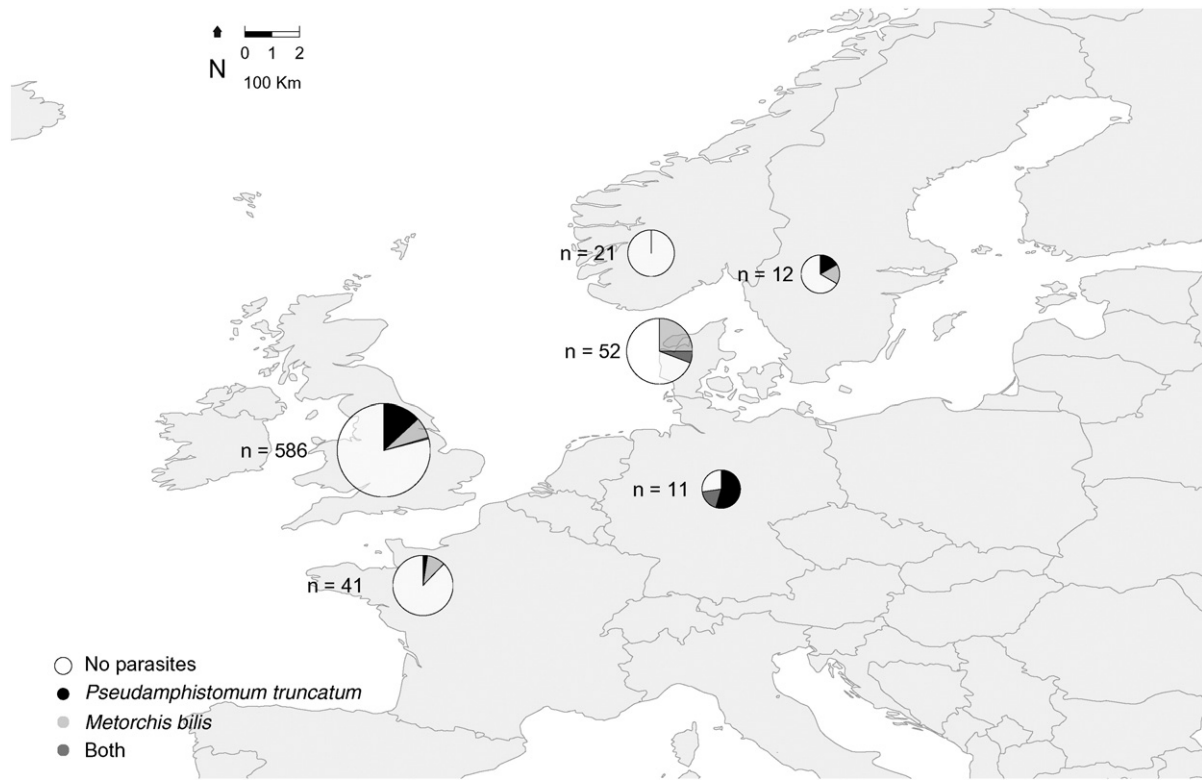
Fig. 1. Proportion of otters, *Lutra lutra*, that was positive for *Pseudamphistomum truncatum* and *Metorchis bilis*, Opisthorchiidae, across Europe. Size of the circles reflects sample size (see sample number, n). Please see supplementary information 1 for location details.

ACACATAATCCACAAAATG-3′ as appropriate) and 0.5 U Taq DNA polymerase (Invitrogen). The mitochondrial primers were designed in primer-BLAST [23]. PCR conditions were: 95 °C for 5 min, followed by 35 cycles of 94 °C for 30 s, 53 °C (ITS2 and COX3) or 55 °C (COX1 mtDNA) for 1 min and 72 °C for 1 min, with a final extension step of 72 °C for 7 min (GenAmp PCR System 9700, Applied Biosystems). The samples were run on a 1.5% agarose gel and produced 300 bp and 333 bp (ITS2, *P. truncatum* and *M. bilis*, respectively), 435 bp (COX1) and 394 bp (COX3) amplicons. Sequencing was conducted by Macrogen (Macrogen Inc., Seoul, South Korea). For *P. truncatum*, 27 individuals were sequenced for ITS2 rDNA, 16 for COX1 and 22 for COX3 mtDNA while for *M. bilis* 22 individuals were sequenced for ITS2 rDNA, 12 for COX1 and 11 for COX3 mtDNA. Alignment of forward and reverse sequences was performed in Sequencher™ (version 4.9, Gene Codes Corporation, USA).

The species present were identified using the ITS2 sequences. Unique COX1 and COX3 haplotypes were identified as follows, and have been assigned GenBank Accession numbers KP869069–KP869078, KP869080–KP869096 (Supplementary information 2): seven *P. truncatum* COX1 (unique haplotypes from the Czech Republic, France, Germany, Poland, Sweden, 1 unique haplotype from England and Wales, and 1 haplotype common to England and Wales, Denmark and Sweden); eight *P. truncatum* COX3 (unique haplotypes from the Czech Republic, Denmark, England and Wales, Poland, Sweden, two unique sequences from Germany, and one haplotype common to Denmark, England and Wales, Germany and Sweden); nine *M. bilis* COX1 (unique haplotypes from the Czech Republic, Denmark, England and Wales, and Germany, three unique haplotypes from France, one haplotype common to Denmark and England and Wales, and one haplotype common to Denmark and Sweden); three *M. bilis* COX3 (a unique haplotype to England and Wales, one common to Denmark and France, and one common to Denmark and Germany). Bayesian inference (BI) methods were used to reconstruct the phylogenetic relationships among the mtDNA haplotypes for COX1 and COX3

separately for each species using MrBayes version 3.2 [24]. One million Markov Chain Monte Carlo (mcmc) generations were carried out with the initial 25% discarded as burn in. Convergence was assessed through effective sample size values and correlation plots. MrModeltest version 2 [25] was used to estimate the adequate model of sequence evolution of these datasets. For both loci in each species the inferred model of evolution was Hasegawa, Kishino and Yano. We used a gamma-shaped rate variation with a proportion of invariable sites (Invgamma). The human liver fluke *Clonorchis sinensis* (Trematoda: Opisthorchiidae) was used as an outgroup for both datasets. To complement the phylogenies, and to further explore mtDNA structure, we also constructed haplotype

Table 2
Summary of the data on the genetic diversity of *Pseudamphistomum truncatum* and *Metorchis bilis* across Europe: Samples size (number of parasite sequences), the gene sequenced, the population (region of origin), the haplotype diversity, the nucleotide diversity (π), and the number of haplotypes within the population.

| Parasite	Sample size	Gene	Population (region of origin)	Haplotype diversity/nucleotide diversity (π)	Number of haplotypes
P. truncatum	6	COX1	England and Wales	0.5333/0.0049	2
	5	COX1	Scandinavia	0.400/0.0009	2
	3	COX1	Other Europe	0.667/0.0031	4
	5	COX3	England and Wales	0.400/0.0011	2
	6	COX3	Scandinavia	0.600/0.0017	3
	10	COX3	Other Europe	0.756/0.0035	5
M. bilis	2	COX1	England and Wales	1.000/0.0322	2
	4	COX1	Scandinavia	0.833/0.0207	3
	6	COX1	Other Europe	0.933/0.0210	5
	1	COX3	England and Wales	n/a	1
	6	COX3	Scandinavia	0.600/0.0152	2
	4	COX3	Other Europe	0.500/0.0127	2
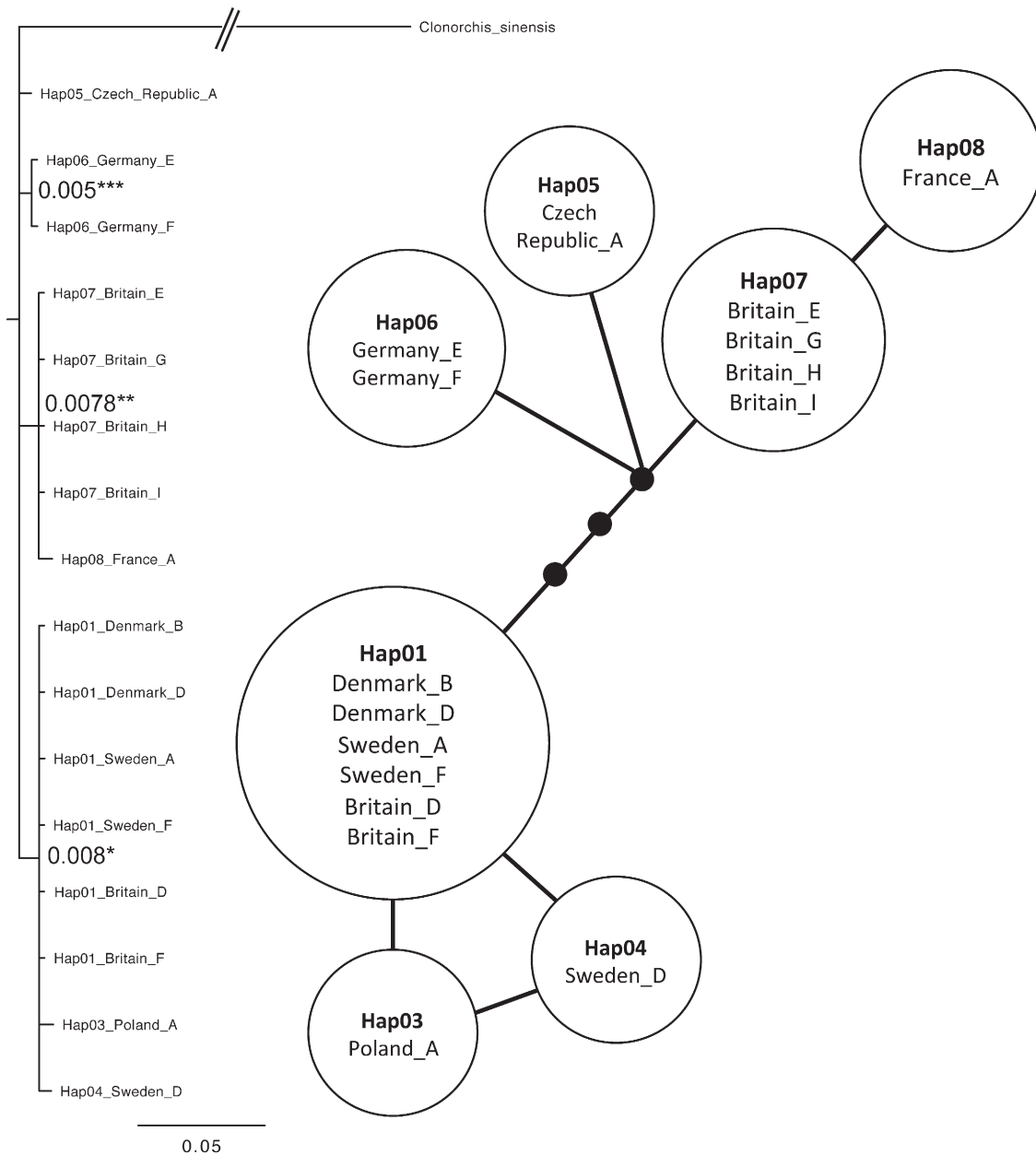
Fig. 2. MrBayes phylogeny and TCS network for *Pseudamphistomum truncatum* based on the cytochrome c oxidase sub-unit I (COX1) mitochondrial DNA region (435 base pairs) across Europe. Phylogeny node labels show raw branch lengths stars indicate Bayesian posterior probabilities (* >0.5, ** >0.65, *** >0.85). Each black dot on the network corresponds to a single mutational change.

networks for both *P. truncatum* and *M. bilis* using TCS v1.21 [26]. Genetic diversity statistics (haplotype and nucleotide diversity) were calculated using DnaSP v5 [27].

3. Results

In total, 723 otters and 144 mink gall bladders were dissected from samples taken across 8 European countries (see Table 1, Fig. 1). The highest prevalence of *P. truncatum* was detected in otters from Germany (73%, although the sample size was low; 8 out of 11 otters were infected) (GLM binomial error distribution: $\chi^2_{769,757} = 4.188$, SE = 0.901, p < 0.001) while *M. bilis* was most prevalent in otters from Denmark (16 out of 52, 31%) (GLM with a binomial error distribution: $\chi^2_{769,757} = 2.333$, SE = 0.6061, p < 0.05). There were, in contrast, no parasites in any of the gall bladders from Norwegian or Scottish

samples, whereas otter populations examined from all other countries had biliary trematodes (Table 1). Only mink samples were examined from Scotland and none were infected whilst mink in England had biliary trematodes.

There was no significant difference in the intensity of *P. truncatum* among infected otters from England and Wales or Germany (the only countries with large enough sample sizes to compare statistically, GLM negative binomial error distribution: F = 0.3167, df = 86, p = 0.57). Equally, the intensity of *M. bilis* infection did not differ between comparable data sets from France, Sweden, and England and Wales (GLM negative binomial error distribution: F = 2.42, df = 62, p = 0.097).

Only a single ITS2 haplotype was identified across Europe for each parasite species. Nucleotide diversity between the two species was 0.01347 across 300 bp. Analysis of mitochondrial DNA markers COX1
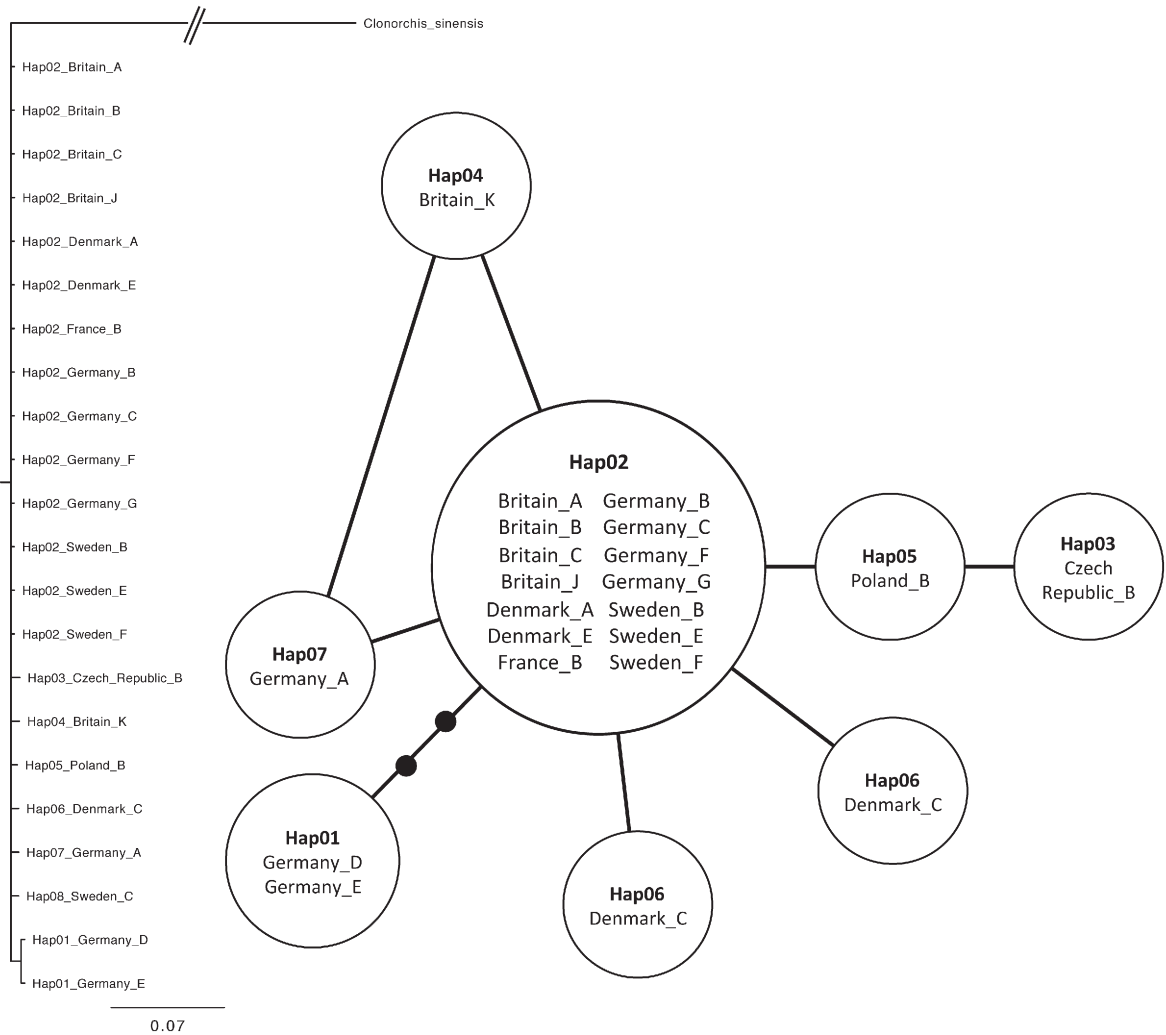
Fig. 3. MrBayes phylogeny and TCS network for *Pseudamphistomum truncatum* based on the cytochrome c oxidase sub-unit III (COX3) mitochondrial DNA region (394 base pairs) across Europe. Phylogeny node labels show raw branch lengths stars indicate Bayesian posterior probabilities (*>0.5, **>0.65, ***>0.85). Each black dot on the network corresponds to a single mutational change. There were no meaningful branch lengths for the phylogeny due to low genetic variation at this locus for *P. truncatum* COX3.

and COX3 showed greater diversity within *M. bilis* than *P. truncatum* (Table 2).

For *P. truncatum* and *M. bilis* respectively, COX1 haplotype diversity (Hd) was 0.767 and 0.955, while COX3 Hd was 0.541 and 0.618; COX1 nucleotide diversity (π) was 0.007 and 0.019, and COX3 π was 0.002 and 0.018. These differences corresponded to 16 segregating sites in *P. truncatum* (9/435 for COX1, 7/394 for COX3) and 35 in *M. bilis* (19/435 for COX1 and 16/394 for COX3). The majority of haplotypes (for both species, across both COX1 and COX3) were unique to a single source country. For *P. truncatum*, 13/15 were unique; exceptions were one haplotype for COX1 (Hap01) common to England and Wales, Denmark and Sweden (Fig. 2), and one for COX3 (Hap02) widespread throughout Europe (there were no meaningful branches) (Fig. 3). For *M. bilis* a smaller proportion (8/12) were unique to a single source country. Exceptions for COX1 were Hap03 (Denmark and Sweden) and Hap08 (Denmark, and England and Wales) (Fig. 4), and for COX3, Hap01 (Denmark and Germany) and Hap02 (Denmark and France) (Fig. 5).

The *M. bilis* phylogeny showed two monophyletic clades, for both COX1 and COX3. Branch lengths of the *P. truncatum* clades were 0.005–0.008 for COX1 (Fig. 2; genetic diversity of COX3 was too low to produce meaningful branch lengths, Fig. 3), whereas branch lengths for the two *M. bilis* clades were 0.023 and 0.036 for COX1 (Fig. 4) and 0.022 and 0.023 for COX3 (Fig. 5).

4. Discussion

The discoveries of both *P. truncatum* and *M. bilis* in British mammals occurred as a direct result of systematic screening [5–6]. It is only with systematic and widespread screening (e.g. [28,29]) combined with molecular analysis that taxonomic confusion, such as that surrounding *Metorchis* [9–12], can be elucidated, providing a clearer understanding of parasite fauna and disease.

The *P. truncatum* samples showed low mitochondrial genetic diversity relative to *M. bilis*. This lower genetic diversity is observed in the shorter branch lengths of the *P. truncatum* phylogeny, lower haplotype diversity and shorter distance between the haplotypes in the genetic network. These differences indicate considerable differences in the demographic and evolutionary history of these species in Europe. Shared haplotypes across multiple European countries suggests genetic mixing of both parasite species, across Europe. There is insufficient evidence to attempt to date an initial introduction to Britain for either species, but the presence of more than one haplotype (for both species) may indicate more than one introduction event or long-term residency. Shared haplotypes between Scandinavian and British samples (*P. truncatum*: Hap01, COX1; *M. bilis*, Hap08, COX1) implicates Scandinavia as a potential origin for both species into Britain.

The branch lengths for the *M. bilis* phylogeny are an order of magnitude longer than for the *P. truncatum* phylogeny. This, coupled with the
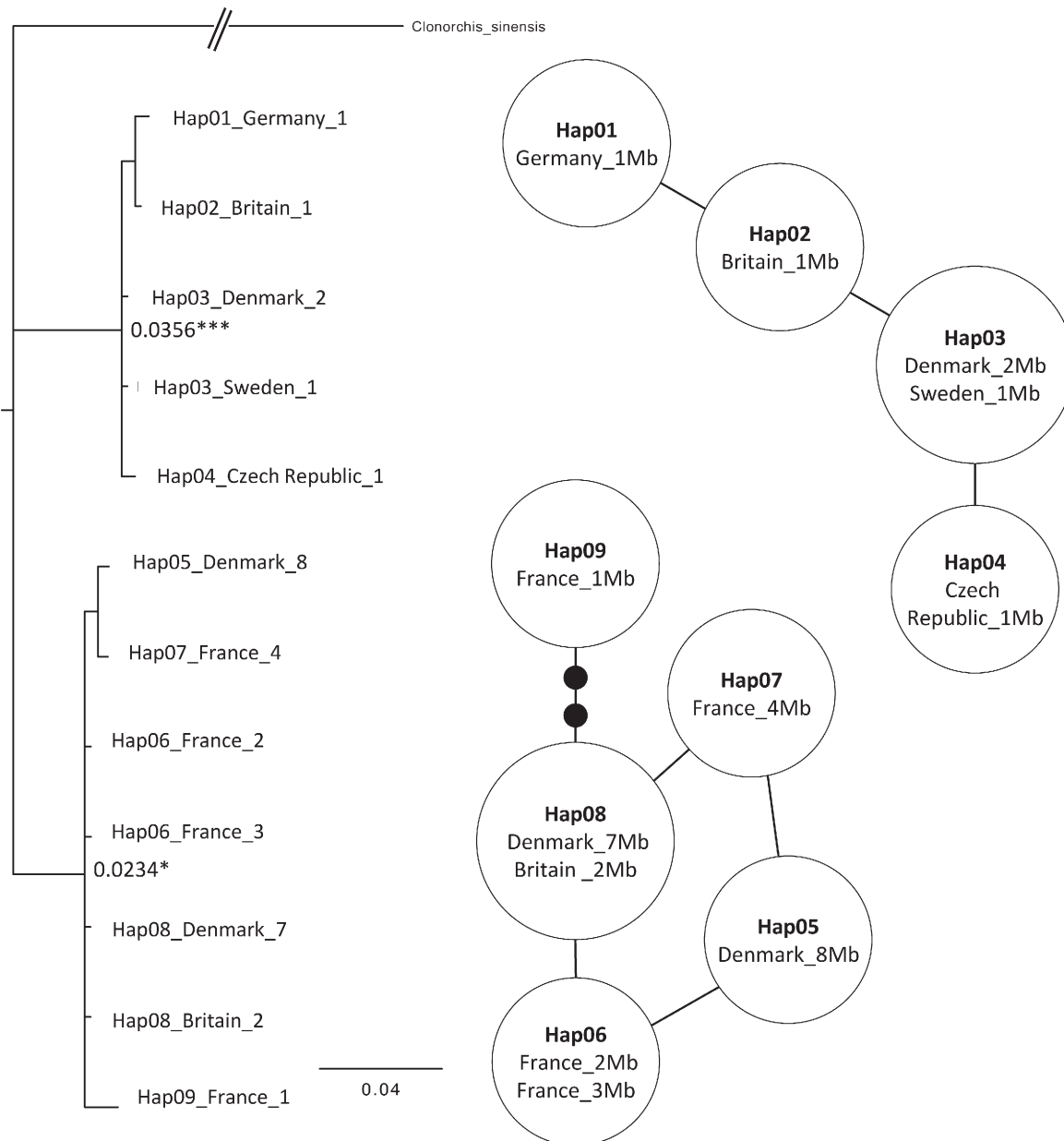
Fig. 4. MrBayes phylogeny and TCS network for *Metorchis bilis* based on the cytochrome c oxidase sub-unit I (COX1) mitochondrial DNA region (435 base pairs) across Europe. Phylogeny node labels show raw branch lengths stars indicate Bayesian posterior probabilities (* >0.5, ** >0.65, *** >0.85). Each black dot on the network corresponds to a single mutational change.

difference in the distributions of the haplotypes between the two species implies differing demographic histories, which may correspond to differences in their definitive hosts [e.g.,5,6,8, 12,13,30]. *Pseudamphistomum truncatum* is predominantly reported from mammals [5–6,8] whilst *M. bilis* (and more specifically synonym *M. crassiusculus*) are also noted from migratory birds [[13], Heneberg, pers. Comm.], perhaps increasing the potential for genetic mixing for *M. bilis* across Europe. Legislation is operative to protect fish from disease (e.g. EU Council Directive 2006/88/EC) but does not apply to most digeneans. In part, this relaxed approach to screening fish for digeneans stems from their reported low level impact on fish [but see 31–32] and it is therefore deemed unnecessary to restrict fish movements on this basis. Consequently, the widespread translocation of fish stocks, natural migration of definitive hosts and fish, alongside movement of snails and parasite eggs with plants, gravel or water, across Europe almost certainly contributes to a widespread distribution of digenean species. For example, *P. truncatum* is found in Ireland [33] where cyprinid fish (the second intermediate host

for both *P. truncatum* and *M. bilis*) are not native but were introduced in the 17th Century and have been continually relocated to new habitats across Ireland ever since [34].

The distribution of *P. truncatum* across Europe appears to be focal, although more extensive sampling would be required to examine this further. The German state of Saxony has a relatively high proportion of *P. truncatum* infections (8/11) but only 11 otters were examined, whilst the parasite was not found in mink from Scotland (n = 40) or otters from Norway (n = 21). Previously, no biliary parasites were found in another eleven otters screened in Scotland [7]. There are no apparent barriers to spread according to the underlying distribution of some of the potential host species: both the first and second intermediate hosts (gastropod families Lymnaeidae and Bithyniidae, and freshwater fish family Cyprinidae, respectively [[30], Sherrard-Smith et al., unpublished data]), occur throughout Europe. The predicted warmer conditions across Europe with climate change may suit both species and encourage a Northerly movement of their current distributions [22].
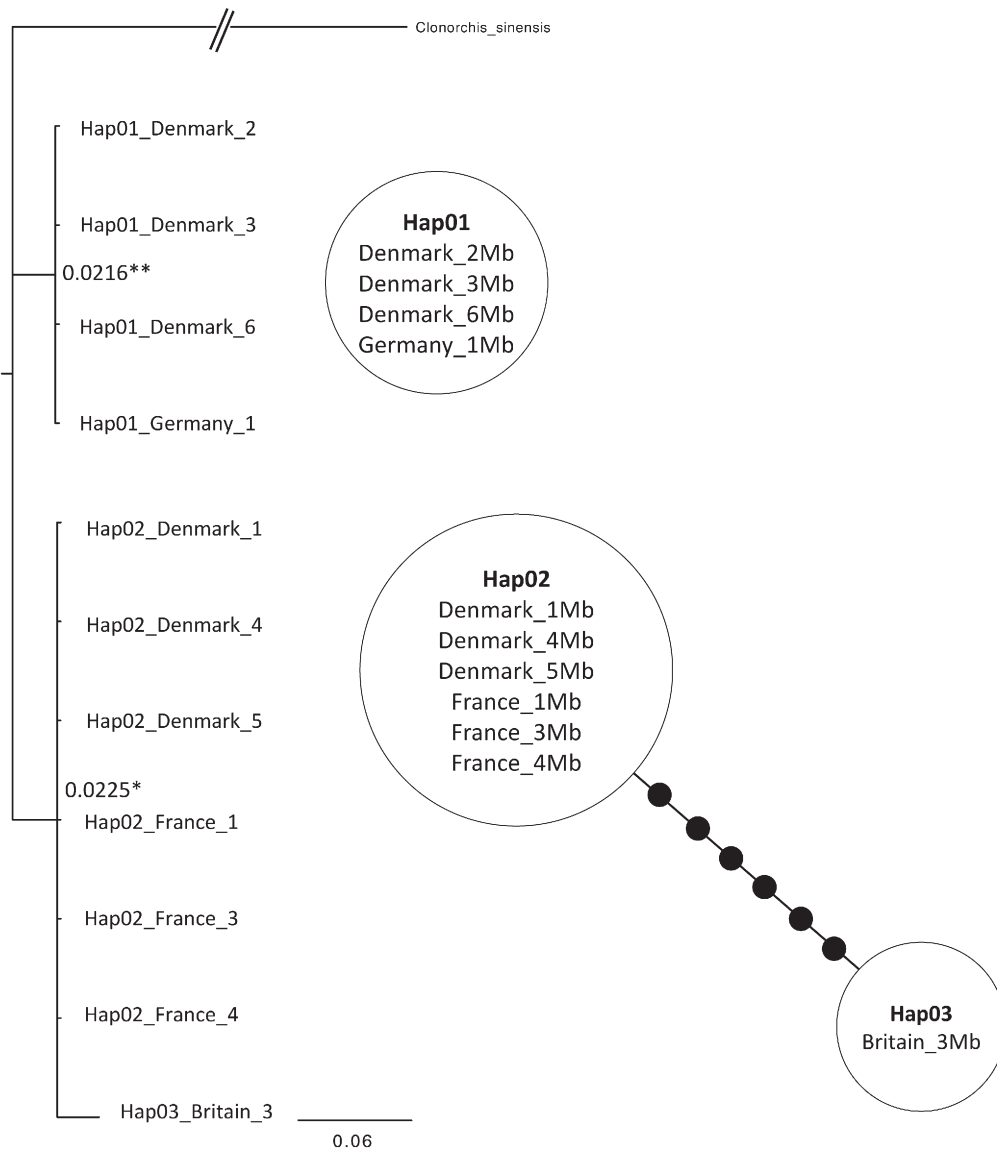
Fig. 5. MrBayes phylogeny and TCS network for *Metorchis bilis* based on the cytochrome c oxidase sub-unit III (COX3) mitochondrial DNA region (394 base pairs) across Europe. Phylogeny node labels show raw branch lengths stars indicate Bayesian posterior probabilities (* >0.5, ** >0.65, *** >0.85). Each black dot on the network corresponds to a single mutational change.

The density-dependent processes that determine the distribution of host populations can result in spatial aggregation of parasite populations [35] and have been used to explain the co-existence of species [e.g. 36–37]. The presence of two biliary parasites in the otter population across Europe may contribute to the observed patchiness in the distribution of each digenean. Speculatively, this may indicate that interspecific competition or some level of acquired host immunity is acting to separate *P. truncatum* and *M. bilis* and it is noteworthy that across the entire study, only 5 co-infections (where 8 would be expected by chance; 70/723 *P. truncatum* infections among 79 *M. bilis* positive hosts) were observed; 2 from Britain (out of 586 otters) and 3 in Denmark (out of 52 otters), but none elsewhere, of 229 otters). Specifically, a distinction between the geographic distributions of *M. bilis* and *P. truncatum* was observed in England and Wales [6], and France, with *P. truncatum* only found in the Poitou-Charentes Region (a single specimen), and *M. bilis* only in Brittany (although our sample size in France is relatively small: N = 22 Brittany, N = 19 Poitou-Charentes Region). Regional differences in host diet [e.g. 38] may contribute to geographic variation in parasite exposure. Co-existence of *P. truncatum* and *M. bilis* in the same host is rare despite geographic overlap across continental Europe but also in England and Wales

where sample size is large enough (n = 586) to make stronger conclusions [6].

The presence of widespread COX1 and COX3 haplotypes, particularly for *M. bilis*, indicates population mixing throughout Europe. The current study provides an insight into the genetic structure, but also geographic heterogeneity, of two widespread digeneans of threatened wild mammals.

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