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Host generalists and specialists emerging side by side: an analysis of evolutionary patterns in the cosmopolitan chewing louse genus *Menacanthus* ☆

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ABSTRACT

Parasites with wide host spectra provide opportunities to study the ecological parameters of speciation, as well as the process of the evolution of host specificity. The speciose and cosmopolitan louse genus *Menacanthus* comprises both multi-host and specialised species, allowing exploration of the ecological and historical factors affecting the evolution of parasites using a comparative approach. We used phylogenetic analysis to reconstruct evolutionary relationships in 14 species of *Menacanthus* based on the sequences of one mitochondrial and one nuclear gene. The results allowed us to validate species identification based on morphology, as well as to explore host distribution by assumed generalist and specialist species. Our analyses confirmed a narrow host use for several species, however in some cases, the supposed host specialists had a wider host spectrum than anticipated. In one case a host generalist (*Menacanthus eurysternus*) was clustered terminally on a clade almost exclusively containing host specialists. Such a clade topology indicates that the process of host specialisation may not be irreversible in parasite evolution. Finally, we compared patterns of population genetic structure, geographic distribution and host spectra between two selected species, *M. eurysternus* and *Menacanthus camelinus*, using haplotype networks. *Menacanthus camelinus* showed limited geographical distribution in combination with monoxenous host use, whereas *M. eurysternus* showed a global distribution and lack of host specificity. It is suggested that frequent host switching maintains gene flow between *M. eurysternus* populations on unrelated hosts in local populations. However, gene flow between geographically distant localities was restricted, suggesting that geography rather than host-specificity is the main factor defining the global genetic diversity of *M. eurysternus*.

1. Introduction

The coevolutionary process in host–parasite systems may display a surprisingly high complexity: even in closely related lineages the genealogy and population structure may not reflect the most apparent biological features. Such a situation has been found, for example, in a genealogical study on the human associated lice of the genus *Pediculus* (i.e. Reed et al., 2004) and a similar pattern of "random" changes of ecological features has been confirmed on a phylogenetical/genealogical scale in two additional host–parasite associations, the lice of the genus *Polyplax* (Štefka and Hypša, 2008; du Toit et al., 2013) and the tapeworm *Ligula intestinalis* (Štefka et al., 2009). In host–parasite systems, the combination of geographical distribution and host specificity creates a complex background for genetic diversification and population structuring.

In chewing lice, long evolutionary periods of tight coexistence with their hosts and relatively few opportunities for dispersing among other host species were traditionally believed to constrain these parasites, causing them to show a high degree of codivergence and parallel evolution with their hosts (Eichler, 1941, 1942; Page and Hafner, 1996). Lice infesting multiple unrelated hosts were long thought to constitute cryptic species (Eichler, 1941), which resulted in the erection of new species, and even

☆ *Note:* Nucleotide sequence data reported in this paper are available in the GenBank database under accession numbers **KJ730527**–**KJ730843**.

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genera, based primarily on host relationships; many of these names have subsequently found little acceptance (see e.g., Price et al., 2003). A similar problem exists with many described species of *Menacanthus* (Price, 1975, 1977; Pilgrim and Palma, 1982; Palma et al., 1998; Krištofík, 2000).

When analysed genetically, euryxenous (broad host range) parasite species are frequently revealed to constitute an assemblage of cryptic species (e.g. Jousson et al., 2000; Demanche et al., 2001; Smith et al., 2006). On the other hand, an increasing number of studies on chewing lice at the lower taxonomic level have revealed that multi-host (generalist) louse species are quite common, especially in the Ischnocera (e.g. Johnson et al., 2002a, 2003; Clayton and Johnson, 2003; Gustafsson and Olsson, 2012). Host generalists evidently do occur and, more importantly, contrary to the presumed idea of continuous host specialisation in evolution (Fahrenholz, 1913; Eichler, 1941), generalist lice have been derived from host specialists several times independently (Johnson et al., 2009, 2011).

Dispersal capabilities are probably among the most important factors affecting the level of host specificity in lice, and in parasites in general. Bueter et al. (2009) compared general phylogenetic patterns in the ischnoceran genus *Brueelia* and the amblyceran *Myrsidea*, and found fewer host specialists in *Brueelia* than in *Myrsidea*. Similarly, when comparing levels of intraspecific genetic variability and codivergence with their hosts across the Galapagos archipelago, Štefka et al. (2011) found a tighter correlation with hosts in *Myrsidea* than in *Brueelia*. In both studies phoresy, or "hitchhiking", on hippoboscid flies was suggested to explain the differences, as it is relatively common in the ischnoceran lice (Keirans, 1975). However, some amblyceran lice, particularly the genus *Myrsidea*, are probably also able to switch between distantly related hosts that share similar habitats and geographic distributions (Bueter et al., 2009). Whether or not host switching occurs by phoresy is presently unknown, but phoresy is not unknown in amblyceran lice (Hopkins, 1946). Similar results have been arrived at in the case of *Myrsidea* elsewhere (Clay and Meinertzhagen, 1943; Kounek et al., 2011; Sychra et al., 2014).

Host specificity and dispersal abilities in multi-host amblyceran lice have not previously been explored using molecular methods. However, taxonomists have long cast doubts on the actual numbers of species in several multi-host genera, for example in the genus *Colpocephalum* from the Corvidae (Price and Beer, 1965) or *Trochiliphagus* from the Trochilidae (Rheinwald, 2007). In this study we focused on the phylogenetic patterns of the amblyceran genus *Menacanthus*, and in particular on the genetic variability of *Menacanthus eurysternus*. *Menacanthus* is a speciose and cosmopolitan louse genus, comprising 98 species parasitising approximately 460 species of birds belonging to seven orders of birds (Cicchino, 2003; Price et al., 2003; Palma and Price, 2005; Bansal et al., 2012); however, despite the wide host range, they are most numerous on wildfowl (Galliformes), woodpeckers (Piciformes) and passerines (Passeriformes).

In the case of *Menacanthus* from passerines, 10 of the 36 recognised species are monoxenous (a single host parasite), while 25 are stenoxenous (with a narrow host range) with 2–22 closely related host species that usually belong to the same family (Price et al., 2003). The most euryxenous and cosmopolitan species within the genus is *M. eurysternus*, which has been recorded from eight species of woodpeckers and 170 species of passerines belonging to 20 families (Price et al., 2003). *Menacanthus eurysternus* often shows a relatively high prevalence (e.g., 56.4%, Boyd (1951); 68.4%, Chandra et al. (1990)) and can reach high intensities of infestation. It is haematophagous and can thus impact the condition of its hosts (Agarwal et al., 1983), and its population dynamics are synchronised with the reproduction cycle of the host (Foster, 1969; Srivastava et al., 2003).

Opinions are divided on the complex of species represented by *M. eurysternus* sensu lato. While the checklist of Price et al. (2003) considered *M. eurysternus* to be one widely distributed species, some authors (for example Fedorenko, 1983) consider it to be a complex of several remarkably similar species (sensu Banks and Paterson, 2005). Mey (2003) considered the various proposed species to be subspecies of *M. eurysternus*. Only a few *M. eurysternus* sequences have been published to date, all of which are mitochondrial (mt)DNA sequences (hosts *Lybius torquatus*, Piciformes, from Africa, *Zosterops japonicus*, *Pycnonotus blanfordi*, *Pycnonotus finlaysoni*, Passeriformes, from Vietnam). These samples possess a relatively low level of differentiation, with sequences differing only in approximately 4–7% of nucleotide positions (Najer et al., 2014). Such low genetic differentiation is surprising, given the diverse geographic and host origin of the samples. If the same trend was confirmed using a larger sampling size, it would represent a unique situation among lice, which typically possess narrow host specificity limited to one or a few related hosts (see the checklist of Price et al., 2003) and show higher levels of divergence between louse lineages or species from distantly related hosts (Johnson et al., 2003; Bueter et al., 2009).

However, even such low genetic divergence as seen in the six *M. eurysternus* samples does not a priori exclude the existence of distinct populations, where moderate levels of host specificity or geographic fragmentation have evolved. Thus, apart from presenting an interesting taxonomical problem, the lice of the genus *Menacanthus* (and *M. eurysternus* in particular) provide a rare opportunity to study the evolution of host specificity in parasites. Given the complicated taxonomy of the genus and somewhat ambiguous morphological determination of several species, we first reconstructed the phylogenetic relationships between *M. eurysternus* and 13 other species (10 species from passerines, one from a woodpecker and two from *Gallus gallus*) to validate species determinations and their relationships. Then selected lineages (euryxenous versus stenoxenous) were studied in more detail. We analysed the population genetic structure in two selected lineages of *Menacanthus* which differed in the width of their host spectrum and contrasted the patterns obtained through these analyses with the morphological traits and bionomy (ecology and physiology) of *Menacanthus* spp. to test the contribution of host generalist or specialist parasitic strategy on the formation of genetic structure and speciation in lice. Using the phylogenetic approach with wider taxon sampling, we were able to identify two more *Menacanthus* spp. with potential multi-host distribution (e.g. *Menacanthus obrteli*).

2. Materials and methods

2.1. Sampling of lice

Chewing lice of the genus *Menacanthus* were collected from 29 localities across a broad geographic range covering 12 countries (Supplementary Table S1). Samples were either collected by the authors or provided by collaborators listed in the Acknowledgments. Lice were collected from birds captured in mist nets using the fumigation chamber method (Clayton and Drown, 2001) with a visual examination of the head. Collected specimens were preserved in pure 95% or denatured 70% ethanol and stored in a refrigerator. Lice were cut between the thorax and the abdomen, and genomic DNA was extracted from individual specimens using the QIAamp DNA Micro Kit (Qiagen, Germany). Following DNA extraction, remaining exoskeletons were mounted in Canada balsam onto microscope slides and stored as vouchers at University of Veterinary and Pharmaceutical Sciences Brno, Czech Republic or

Yamashina Institute for Ornithology, Chiba, Japan (see Supplementary Table S1). Specimens were identified based on Price (1977).

2.2. PCR amplification and DNA sequencing

Partial sequences of the nuclear coding gene for elongation factor 1-α (EF-1α, 347 bp) and the mitochondrial gene for cytochrome oxidase subunit I (COI, 381 bp) were amplified using PCR. PCR products of EF-1α were obtained using primers EF1-For3 and Cho10 (Danforth and Ji, 1998). Primers L6625 and H7005 (Hafner et al., 1994) were used for COI amplification. PCRs were carried out in a 20 μl volume using 1 μl of extracted DNA, 5 pM of each primer, 15 mM $MgCl_2$, 10 mM dNTPs, 10× PCR buffer and 0.25 U of High Fidelity PCR Enzyme Mix (Fermentas, United Kingdom). The amplification protocol consisted of one denaturation step at 95 °C for 3 min, then 30 cycles of denaturation at 95 °C for 1 min, annealing at 50 °C (COI)/45 °C (EF-1α) for 45 s and an extension step at 72 °C for 1.5 min, followed by the last elongation step at 72 °C for 10 min. PCR products were cleaned up in a single-step enzymatic reaction using 0.2 μl of Exonuclease I (ExoI) and 0.2 μl of Calf Intestinal Alkaline Phosphatase (CIP) enzymes (New England Biolabs Inc., USA). Purified PCR products were sequenced using the PCR primers in a commercial laboratory (Macrogen Inc., Korea and the Netherlands). Obtained sequences were deposited in GenBank (GB) (see Supplementary Table S1).

2.3. Phylogenetic analysis

Datasets containing mitochondrial, nuclear and concatenated sequences of the two genes were aligned in BioEdit 7.05 (Hall, 1999). Sequences were collapsed to haplotypes using the Collapse 1.2 program (http://darwin.uvigo.es/software/collapse.html). Molecular phylogenies were reconstructed using Maximum Likelihood (ML) and Bayesian Inference (BI) approaches. The analyses were performed individually for each dataset (mitochondrial, nuclear and concatenated). PhyML software (Guindon et al., 2005) was used to obtain ML phylogenies with a TVM + I + G model for COI, TIM3ef + G for EF-1α and HKY85 + I + G for the concatenated alignment. Substitution models of the molecular evolution for each dataset were selected in jModeltest 2 using the Akaike Information Criterion (AIC) (Guindon and Gascuel, 2003; Posada, 2008; Darriba et al., 2012). Parameters were estimated from the data and bootstrap supports were obtained by 1,000 replications. BI analyses were conducted with Mr. Bayes version 3.2.2 (Ronquist and Huelsenbeck, 2003) for COI and EF-1α datasets separately and with the concatenated dataset divided into two partitions, using the same models as in the ML analysis, separately for each gene partition. For each BI analysis we ran two parallel runs for 10 million generations, each with four Markov chains (Huelsenbeck and Bollback, 2001). Markov chains were sampled every 1,000 generations, yielding 10,000 parameter point estimates. The first 2,500 trees (25%) were discarded as burn-in when summarising phylogenies and Bayesian posterior probabilities. Convergence between estimated values of model parameters obtained in independent BI runs and their effective sampling sizes were checked using Tracer 1.6 (http://tree.bio.ed.ac.uk/software/tracer/). Convergence of inferred BI topologies was inspected using the 'are we there yet' (AWTY) method (Nylander et al., 2008). COI and EF-1α sequences of the chewing lice *Dennyus hirundinis* (GenBank Accession Nos. **AF385013** and **AF385032**) and *Myrsidea marksi* (GenBank Accession Nos. **DQ366669** and **FJ171315**) were used as outgroups.

2.3.1. Analysis of intra-clade diversity and population history

Haplotype networks were constructed for two species clades, "*eurysternus*" and "*camelinus*" (*Menacanthus camelinus*), in the TCS 1.21 program (Clement et al., 2000) using COI data. Information about the biogeographic history of the *M. eurysternus* clade was inferred using Nested Clade Phylogeographic Analysis (NCPA) (Templeton et al., 1987; Templeton and Sing, 1993) and used as input data for the analysis of the geographical dependence of genetic variability in Geodis (Posada et al., 2000) in ANeCAv1.2 software (Panchal, 2007). The program implements TCS and GeoDis algorithms (Clement et al., 2000; Posada et al., 2000) for testing the congruence between the population genetic structure and geographic distribution of haplotypes. The inference key of Templeton (2004) was implemented to evaluate possible historical and geographical events. The probability of the null hypothesis (no association between genetic structure and geography) was estimated by 1 million permutations. According to the suggestions of Posada et al. (2000) and Panchal (2007), four regions with large gaps between sampled populations were indicated to prevent a false inference of isolation by distance. These regions cover unsampled areas in North America, central Africa, eastern Europe and China where *M. eurysternus* probably occurs.

3. Results

In total 168 sequences of the COI gene and 151 sequences of EF-1α were obtained from lice of the genus *Menacanthus*. Amplification of either COI or, more commonly, EF-1α failed in some of the samples stored in denatured 70% ethanol. Such samples were removed from the concatenated dataset, resulting in 129 combined mitochondrial and nuclear sequences, which thus only includes samples which were sequenced for both markers. The sequences were collapsed into 61 haplotypes in COI, 28 haplotypes in EF-1α and 51 concatenated haplotypes. The list of sequenced specimens with their geographical origin, morphological identification and associated haplotype numbers are available in Supplementary Table S1.

3.1. Phylogenetic analysis

3.1.1. COI

The topology of the resulting ML tree was compatible with the topology obtained with BI analysis. The most basal division is between one clade ("*cornutus*" in Fig. 1) containing *Menacanthus cornutus* from domestic chickens clustered together with GenBank sequences of *Menacanthus* sp. from Cracidae (Figs. 1 and 3), and the rest of the ingroup; however the division received poor support. The larger clade, in turn, was split into two major clades, each with relatively high bootstrap support and posterior probabilities. We provisionally named these clades the "*curuccae*" and "*alaudae*" clades (see Fig. 1), after the earliest described species in each clade: *Menacanthus curuccae* and *Menacanthus alaudae*, respectively.

The distribution of haplotypes between the two major clades was mostly consistent with morphological identification with, however, some exceptions. A well-supported "*obrteli*" lineage was recovered (see Figs. 1 and 2) which contained lice from four families of hosts from central Europe, identified as *M. obrteli* and *Menacanthus* sp. (morphological identification of *Menacanthus* sp. specimens was not possible due to the excessive damage to those vouchers during DNA extraction or because they were represented only by nymphs). According to the literature (Sychra et al., 2008), *M. obrteli* is specific to *Locustella luscinioides* from the family Locustellidae. In our analyses the samples from *Locustella* clustered with samples from Turdidae, Laniidae and Paridae.

Another conflict between the morphological and genetic data occurred in a group of specimens identified on a morphological basis as *Menacanthus takayamai*. Although these specimens were morphologically homogeneous, they did not form a monophyletic

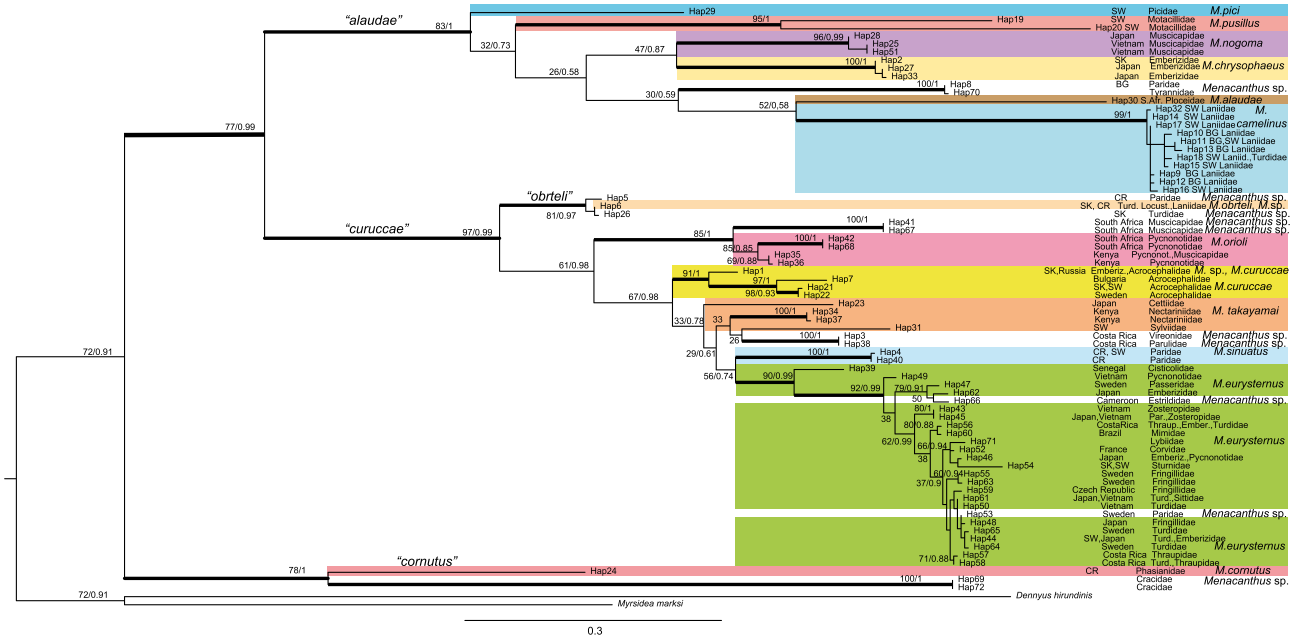

Fig. 1. Maximum Likelihood tree topology of *Menacanthus* based on cytochrome oxidase subunit I sequence data. Maximum Likelihood bootstrap values for 1,000 replicates and Bayesian Inference posterior probabilities are provided. Clades highlighted in bold indicate bootstrap values >70% and posterior probabilities >0.95. The tree was rooted with *Dennyus hirundinis* and *Myrsidea marksi* sequences from GenBank. Colours (shades of grey) mark morphological determinations of the species. BG, Bulgaria; CR, Costa Rica; Ember, Emberizidae; Hap, haplotype; Laniid, Laniidae; Locust, Locustelidae; SK, Slovakia; S. Afr, South Africa; SW, Sweden; Turd, Turdidae; Par, Paridae; Pycnonot, Pycnonotidae; Thraup, Thraupidae.

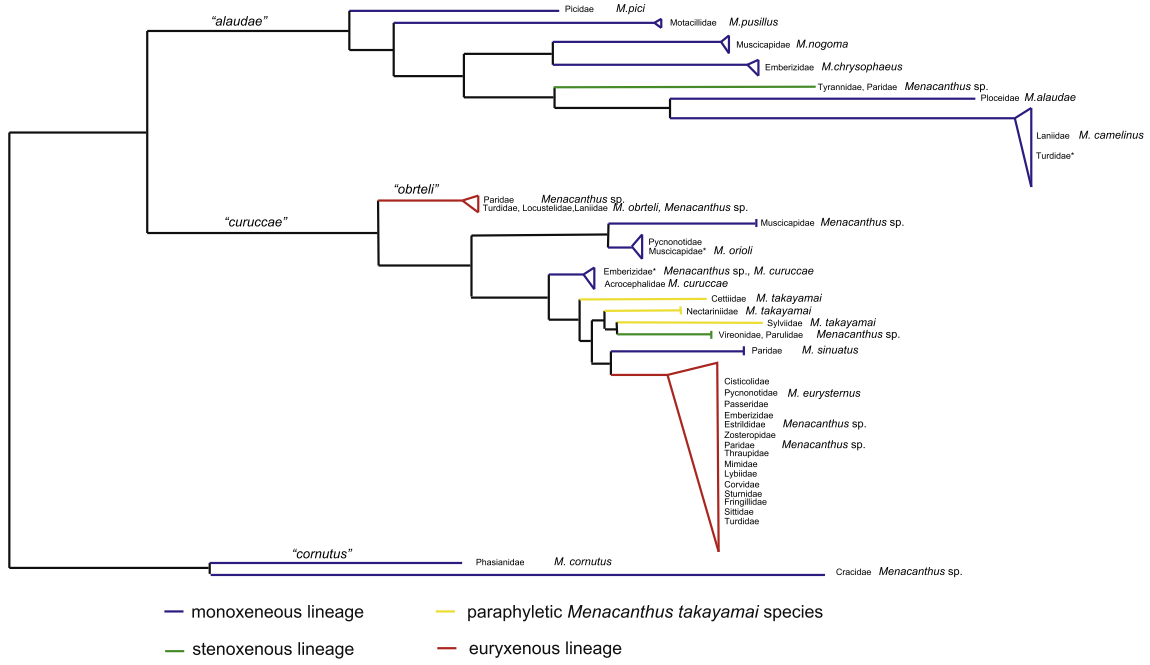

Fig. 2. Scheme of the evolution of the *Menacanthus* lineages. The topology was adapted from the cytochrome oxidase subunit I phylogeny in Fig. 1. Blue (dark grey) colour marks monoxeneous lineages (single host family), green colour (dashed line) stenoxenous lineages (two host families) and red colour (dotted line) euryxenous lineages (multi-host). Yellow (light grey) clades mark the paraphyletic *Menacanthus takayamai* species. *A record of a non-specific straggler. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

group in either the BI or ML analyses (see Figs. 1 and 2), but were paraphyletic with respect to *M. eurysternus* and *Menacanthus sinuatus*, as well as an unidentified lineage from two host families from Costa Rica. However, the topology of *M. takayamai* and the lineages related to it did not receive robust clade support and thus is not stable enough to draw further conclusions.

Most of the supported "species" lineages (clades where morphological determination of species correlated with genetic lineages) consisted of lice sampled from only one (blue clades in Fig. 2), or less commonly two (green clades in Fig. 2), host families. However, the "*eurysternus*" and "*obrteli*" lineages differed from the other lineages in their wide variety of hosts (red clades in Fig. 2)

and geographic locations (Fig. 1). The "*eurysternus*" lineage represented the most diverse monophyletic species lineage, comprising specimens from 63 host species in 13 families of passerine birds sampled from 15 localities in Europe, Asia the Neotropics and Africa, including 13 new louse-host associations (Supplementary Table S1).

3.1.2. EF-1α

The EF-1α sequences provided a weaker phylogenetic signal, resulting in lower topological resolution (Supplementary Fig. S1). The EF-1α topology recovered two highly supported sister clades. One contained *Menacanthus stramineus* from domestic chickens and *Menacanthus* sp. from picid and turdid hosts, both collected at the same locality (Supplementary Table S1), whereas the other contained the rest of the ingroup.

Despite generally lower clade support for the EF-1α topology and fewer samples analysed compared with the COI dataset (due to occasional amplification failures), in most cases the lice that were morphologically identified as a single species formed monophyletic groups in the EF-1α phylogeny, and the "*curuccae*" and "*alaudae*" clades of the COI phylogeny were also obtained in the EF-1α analysis (Supplementary Fig. S1). However, the "*cornutus*" group was placed as sister to the "*alaudae*" clade, although this placement received no support and may be spurious.

As in the COI analysis, the "*obrteli*" clade is sister to the "*curuccae*" clade, and the specimens identified as *M. takayamai* are paraphyletic with regards to *M. sinuatus* and *M. eurysternus*; however in the EF-1α phylogeny, these three morphological groups form a polytomy which also includes *M. curuccae* (Supplementary Fig. S1).

3.1.3. Concatenated alignment

The results of the phylogenetic analysis of the concatenated data set were largely similar to those obtained from the COI data set, but with some differences (Fig. 3). In both datasets the most basal division is between "*cornutus*" and the rest of the ingroup. Also, the topology of the "*curuccae*" clade is more or less the same as in the COI phylogeny, except for clades included in the COI data set but not included in the concatenated data set (due to failure to amplify EF-1α for these individuals). The topology within the "*alaudae*" clade differs between the COI and concatenated data sets. However, in both phylogenies much of the structure within this clade received no or little support. Furthermore, despite these conflicts the terminal clades formed the same "species" lineages with high support in both datasets (Figs. 1 and 3).

3.2. Analysis of intra-clade diversity and population history

mtDNA haplotype networks were prepared for the two most prevalent clades among the sampled lice (*M. camelinus* and *M. eurysternus*). Notable differences in the character of population structure between the two clades were found in the diversity of host spectra and geographic distribution of the haplotypes (Fig. 4).

The "*camelinus*" clade network showed little sequence variation (Fig. 4A), and the majority of haplotypes belonged to lice from only one host species, *Lanius collurio*, sampled in two European countries, Sweden and Bulgaria. The "*camelinus*" network contained several haplotypes without clear correlation between their genetic relationship and geographic origin in the two countries (Fig. 4C). The only host species other than *L. collurio* found in the network was *Turdus merula*. The single specimen from this host shared its haplotype with three other lice from *L. collurio* from the same locality.

By comparison, the "*eurysternus*" network (Fig. 4B) contained two strongly differentiated lineages. One lineage comprised a single specimen from *Prinia subflava* sampled in Africa, whereas the second lineage was almost global in its distribution and contained samples from the rest of the world (Europe, Asia, central America) as well as samples from two other African hosts (*L. torquatus* and *Lagonosticta rara*, Fig. 4D). Despite its wide geographical

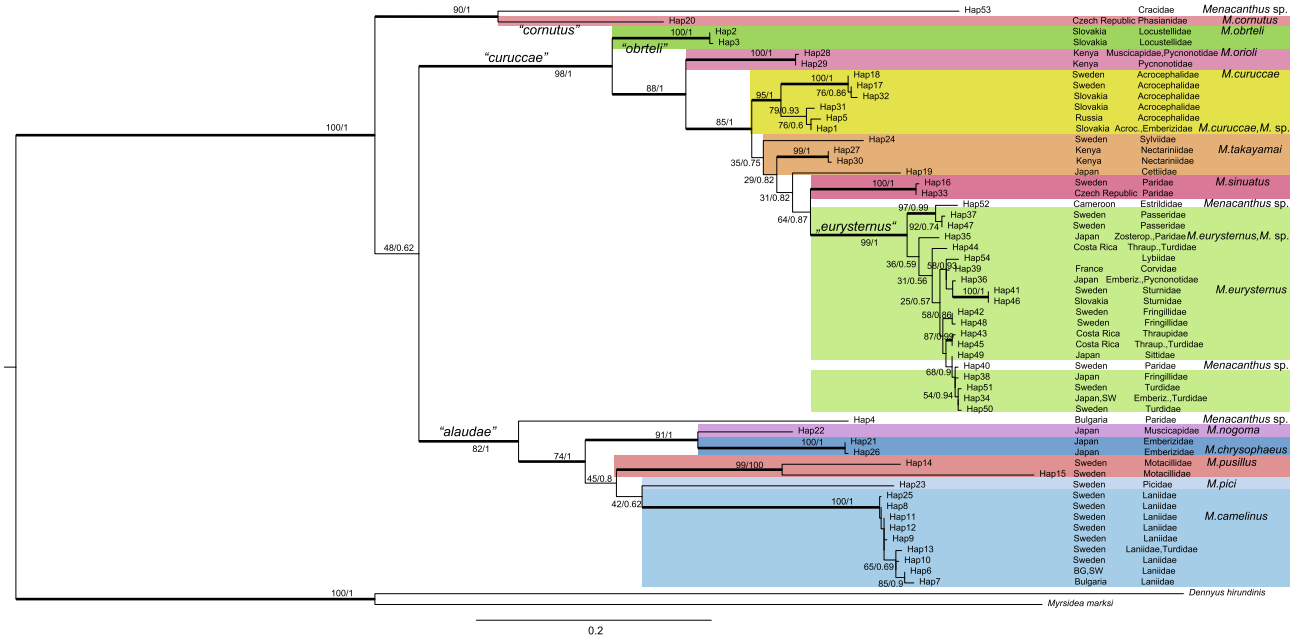

Fig. 3. Maximum Likelihood tree topology of *Menacanthus* based on concatenated sequence data of nuclear elongation factor 1-α and mitochondrial DNA cytochrome oxidase subunit I genes. Maximum Likelihood bootstrap values for 1,000 replicates and Bayesian Inference posterior probabilities are provided. Clades highlighted in bold indicate bootstrap values >70% and posterior probabilities >0.95. The tree was rooted with *Dennyus hirundinis* and *Myrsidea marksi*. Colours (shades of grey) mark morphological determinations of the species. Acroc, Acrocephalidae; BG, Bulgaria; Emberiz, Emberizidae; Hap, haplotype; SW, Sweden; Thraup, Thraupidae; Zosterop, Zosteropidae.

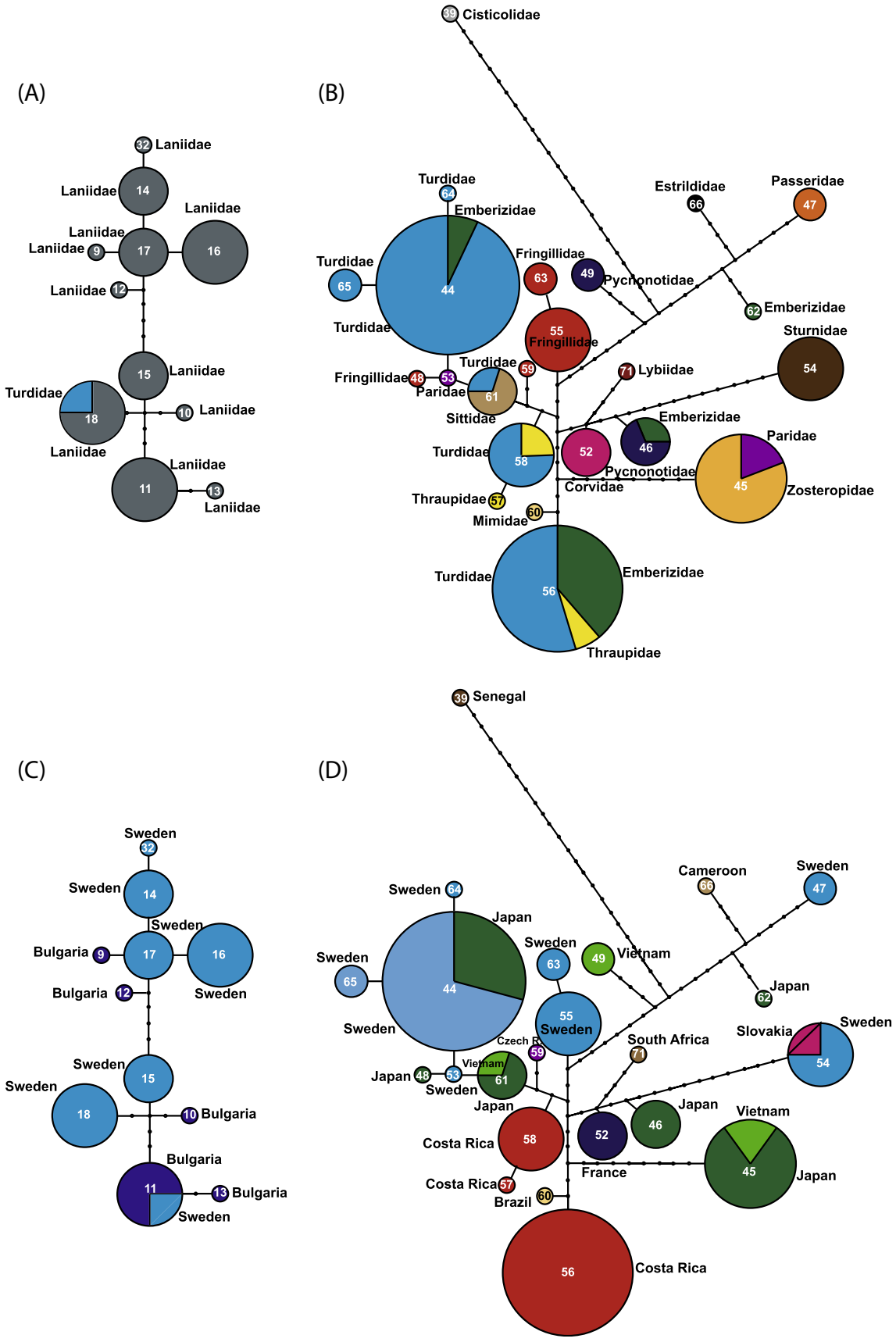

Fig. 4. *Menacanthus* cytochrome oxidase subunit I haplotype networks of "*camelinus*" and "*eurysternus*" clades generated with TCS software. Colours of the haplotypes refer to (A, B) host families or (C, D) the geographic origins of the specimens. Circle sizes reflect the numbers of specimens and show the haplotype number. Mutational steps between haplotypes are shown as dots.

distribution, the sequence diversity of this global "*eurysternus*" lineage was (in terms of the number of mutation steps between haplotypes) only moderately higher than that of the "*camelinus*" lineage.

In contrast to the rather straightforward "*camelinus*" clade, the widespread "*eurysternus*" clade showed a complex structure. It comprised lice from 13 passeriform host families. In approximately two-thirds of the host records, the lice from one host family formed unique haplotypes, while the remaining haplotypes were shared between two host families. Haplotypes did not cluster into specific lineages that would reflect evolutionary relationships between the hosts (Fig. 4B). For instance, haplotypes from turdid and emberizid birds were dispersed throughout the whole network. The genetic pattern was rather influenced by the geographic origin of the sequenced specimens (Fig. 4D). Except for one haplotype that was shared by lice from two distant parts of the Palearctic (Sweden and Japan), all haplotypes contained lice exclusively from one or two countries located on the same subcontinent. In concordance with this fact, the results of NCPA analysis performed for the "*eurysternus*" clade showed several instances of geography-determined evolutionary events (Fig. 5). In several cases allopatric fragmentation was identified (levels 2-20, 3-10, 4-4, 5-2). In addition, restricted gene flow with isolation by distance was suggested for levels 1-5, 3-3, 4-2 and 4-3. No pattern was found for the highest nesting level (6), probably due to the fact that the two lower categories (5-1 and 5-2) both contained samples from all biogeographic areas.

4. Discussion

In this study we analysed genetic relationships and variability in 14 species of a globally distributed ectoparasite genus, and showed that it contains species with strikingly different levels of host spectra and geographic distributions. Phylogenetic reconstructions based on mitochondrial COI and nuclear EF-1α sequences were used to infer inter-specific relationships. COI haplotype networks were then used to describe the patterns of intraspecific structure in the two most sampled species, which revealed striking differences between the two species.

Phylogenetic analyses mostly provided well-supported lineages with minor topological differences between genes. The differences between single gene analyses were partially caused by slightly different taxon sampling (due to amplification failure in some samples fixed in denatured alcohol) and by the lower genetic diversity of the nuclear EF-1α gene providing less information. These differences, however, did not affect the general picture of relationships between *Menacanthus* spp., which formed two major clusters termed "*alaudae*" and "*curuccae*" (Figs. 1 and 3, Supplementary Fig. S1).

The species with the widest host spectrum, *M. eurysternus*, was positioned within the "*curuccae*" clade, together with a series of host-specific or stenoxenous species clades (*Menacanthus orioli*, *M. curuccae*, *M. takayamai* group, *M. sinuatus* and unidentified *Menacanthus* sp. from Costa Rica and South Africa, Fig. 1). The terminal position of the euryxenous *M. eurysternus* within this host-specific

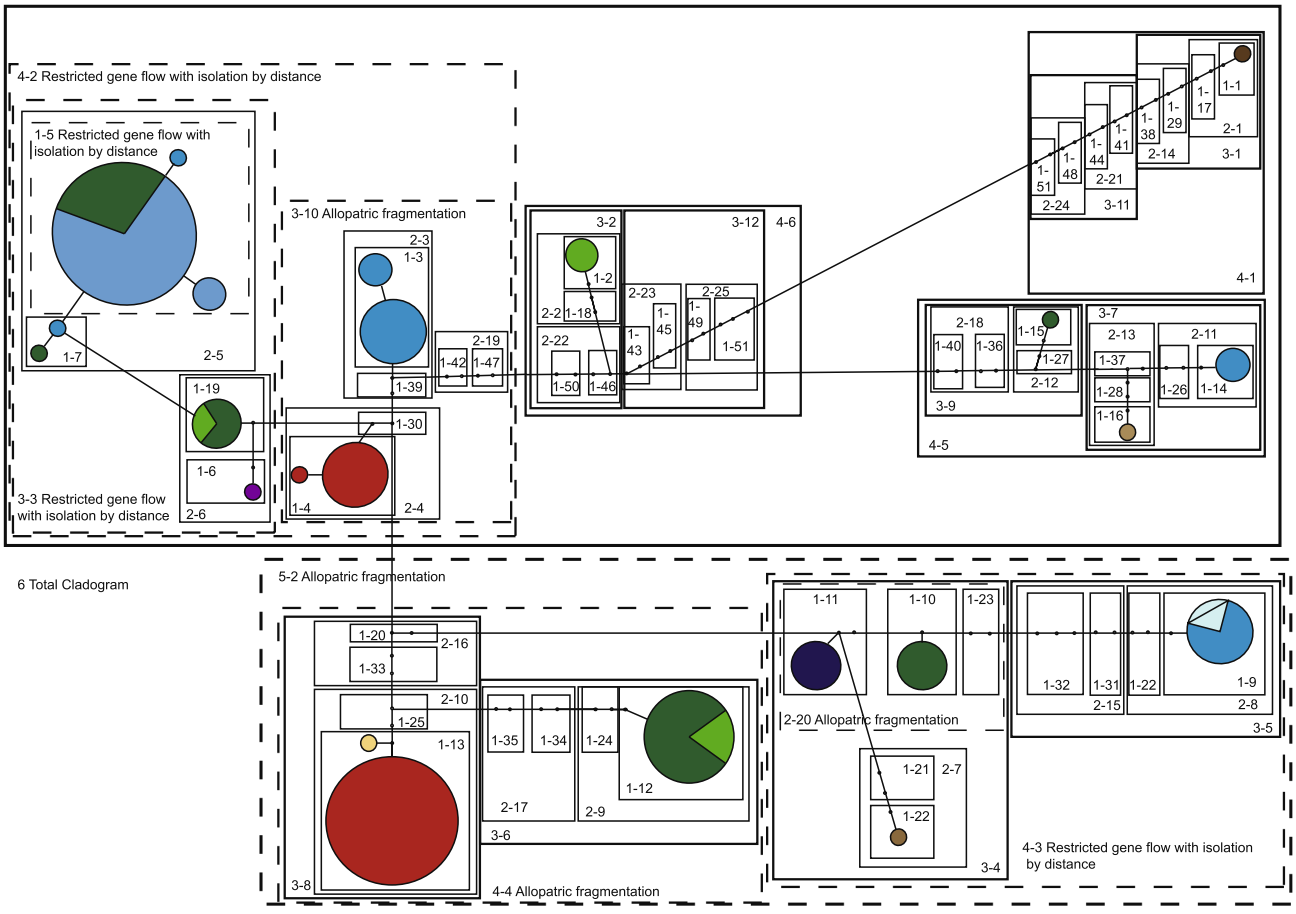

Fig. 5. Nested clade diagram obtained from *Menacanthus* cytochrome oxidase subunit I haplotypes of the "*eurysternus*" clade. Neighbouring haplotypes are connected by a single mutation; dots represent missing haplotypes along mutational pathways. The colours (shades of grey) of the haplotypes refer to the geographic origins of the specimens and are the same as in Fig. 4D. Nested clade levels are indicated by the numbers within particular nested clades. Dashed lines highlight nesting categories where population events were recognised using the nested clade phylogeographic analysis.

clade (Fig. 2) suggests that its ancestor underwent a life strategy reversal from a lineage of host specialists to a host generalist. An alternative interpretation would involve the independent evolution of host specialisation in each species clade from a generalist ancestor, which is less parsimonious (five changes rather than one). Despite containing many host-specific lineages, the *Menacanthus* tree topology does not reflect the evolutionary tree of their passerine bird hosts in any clear manner, not even for the clades containing related stenoxenous lineages (e.g. *M. curuccae* to *M. sinuatus* on Figs. 1 and 2). The evolution of the group probably did not progress through a co-speciation process known in other louse groups that infect less diverse ranges of hosts (e.g. Hafner et al., 1994; Clayton et al., 2003; Page et al., 2004; Hughes et al., 2007). Instead, host switching between different bird host families must have occurred.

The evolutionary patterns of *Menacanthus* lice sampled across multiple bird hosts presented here contribute new data that contradicts the traditional hypotheses that (i) parasites tend to evolve from host generalists into host specialists (Eichler, 1941), and (ii) parasites tend to co-speciate with their hosts (Fahrenholz, 1913; Eichler, 1948; Hafner and Nadler, 1988). Patterns revealing multi-host parasite species have often been considered artefacts caused by incomplete sampling (Dowling et al., 2003; Taylor and Purvis, 2003; Brooks et al., 2004) or cryptic speciation (Eichler, 1941). In contrast, we have shown that *Menacanthus* lice create complex patterns with post-speciation colonisation of new hosts (i.e. host switching), and that they tend toward switching from host specialists to host generalists in some lineages (*M. eurysternus* and possibly *M. obrteli*, Fig. 2). Similar results have recently been arrived at in other ectoparasitic insects such as fleas and ischnoceran body and wing lice (Poulin, 2006; Johnson et al., 2009, 2011).

Resolving inter-specific relationships in the phylogenetic analysis allowed us to explore genealogical differences between individual species, which revealed interesting facts about the ecology and evolution of host specialist and generalist parasites. The patterns revealed in the mtDNA haplotype networks of the "*camelinus*" and "*eurysternus*" species lineages nicely demonstrate the differences in life strategies between two species within a single parasite genus (Fig. 4).

The "*eurysternus*" network represents a host-generalist parasite that was recovered from 13 families of passeriform birds captured on four continents (Fig. 4B, D), which confirms the results of the taxonomic-morphological revision performed by Price (1975), and consequently adopted by Price et al. (2003) that have long been considered controversial by some authors (for example Fedorenko, 1983).

The "*camelinus*" network (Fig. 4A, C) represents a host-specific species where most specimens parasitise one host, *L. collurio*, and only one louse was found on an atypical host species, *T. merula* (Fig. 4A). That particular louse shared its COI haplotype with three other specimens from *Lanius* from the same locality in Sweden. We assume that it represents a straggler after an accidental host switch, rather than having established an independent long-term population on this host; otherwise, we would expect to recover *M. camelinus* from *T. merula* more frequently. The occurrence of other species of *Menacanthus* on atypical hosts (Supplementary Table S1) suggests that such accidental host switching can occur and may do so more often than expected.

Geographical patterns differed markedly between the network analyses of the two species (Fig. 4). The distribution of "*camelinus*" COI haplotypes was limited to Europe and thus they did not create clusters according to the geographic origin of their hosts (Sweden and Bulgaria, Fig. 4C). The geographic distribution may be connected to the migration patterns of the host, as all European populations of *L. collurio* migrate on a narrow front through Libya and Egypt during autumn migration and share relatively small wintering grounds in southern and eastern Africa (Harris and Franklin, 2000). Even if the populations are widely separated spatially during the breeding season, there may thus be ample opportunities for homogenisation of the louse populations on the hosts on their wintering grounds. A similar scenario has previously been suggested for cuckoo lice (Brooke and Nakamura, 1998) and shorebirds (Gustafsson and Olsson, 2012).

By contrast, a geography-dependent structure is apparent in the "*eurysternus*" network. Population structure is emerging in several parts of the network with many COI haplotypes specific to certain areas or localities but only rarely specific to particular host species (Fig. 4B, D). The overlapping distributions and habitat preferences of the hosts of *M. eurysternus* seem to be the most important factors maintaining genetic connectivity within geographic areas, as demonstrated by the six COI haplotypes (Nos. 44, 45, 46, 56, 58, 61), each being found on two to three unrelated families of birds (Fig. 4B and Supplementary Table S1). The best example is the Costa Rican haplotype, with 10 lice found on turdid, thraupid and emberizid birds caught in two nearby localities. Similar results have previously been arrived at for another widespread amblyceran genus, *Myrsidea*, where the sympatry of hosts may provide an opportunity for host switching between a turdid host and an ovenbird (Bueter et al., 2009). The sympatry and syntopy of host species was also found to be an important factor in the evolution of ischnoceran toucan lice of the genus *Austrophilopterus* (Weckstein, 2004). In addition, the "*eurysternus*" network contains both migrant and non-migrant hosts, as well as migrant hosts that follow very different migration routes.

The importance of geography rather than host specificity in driving the local genetic structure of *M. eurysternus* is also suggested by the results of the NCPA (Fig. 5). The analysis found several instances of statistically significant association between the COI haplotypes and their geographic distribution. The use of NCPA as a tool to analyse phylogeographic patterns has been challenged and model-based methods have been proposed as a replacement (Beaumont et al., 2010). However, in the present case, we think that the use of NCPA is valid. Inferring specific historical migrations or demographic events is beyond the scope of this study and would require more densely sampled sequence data or more genetic loci in order to draw any conclusions. Instead, we used NCPA to simply demonstrate the effect of geography on the distribution of "*eurysternus*" haplotypes. The results of the analysis imply that even if the haplotypes are closely related, many of them are unique to certain geographic units and/or must have dispersed across long distances.

The wide geographic distribution and emerging population structure in "*eurysternus*" populations provide opportunities for a future allopatric speciation and the evolution of new taxa. On the contrary, the narrow distribution of "*camelinus*" populations connected by the migration of their single bird host (*Lanius*) provides very little room for evolutionary changes other than host switching, which is a largely random and unpredictable process, probably with little success. Unless the parasite finds an unoccupied niche, which sometimes happens in depleted communities such as in island species (Whiteman et al., 2004) or in cases where the original parasite became extinct (Rozsa, 1993), straggling on atypical hosts is connected with high mortality rates and competition with established parasites. It is likely that there was probably an accidental host switch of the *camelinus* haplotype onto *Turdus merula* (COI Hap18 in Figs. 1 and 4A), which we consider a straggler rather than a representative of a new population. However, accidental host switching is probably not rare. Examining the diversity of hosts seen in the less densely sampled *Menacanthus* lineages revealed several other cases of stragglers on atypical hosts.

In the EF-1α phylogeny, *M. stramineus* from domestic chickens clustered together with *Menacanthus* sp. from two other host

orders and created a sister lineage to the rest of the *Menacanthus* samples (Supplementary Fig. S1). *Menacanthus stramineus* is presently known only from phasianid birds (Price et al., 2003), but our results indicate that this parasite may occasionally occur on hosts from other orders (Piciformes and Passeriformes). The sequences of *Menacanthus* recovered from the domestic chicken (EF-1α Hap19 and Hap20 in Supplementary Fig. S1) and from *Turdus* and *Dendrocopos* (EF-1α Hap16) were not identical, and their genetic distances are comparable with inter-specific levels seen elsewhere in the tree.

Several scenarios may explain such an unexpected distribution of the haplotypes. *Menacanthus stramineus* may comprise several lineages with complex population histories, potentially involving several species (represented here by the differentiated EF-1α haplotypes). A broader sampling of the domestic chicken could thus reveal the existence of EF-1α Hap16 (Supplementary Fig. S1) on this host as well, and the samples from the thrush and the woodpecker may be either genuine, but atypical, host associations, or serendipitous collections of rare stragglers. The overlapping microhabitats of the birds have probably played a major role in the establishment of naturally occurring populations of the same louse species on two or more distantly related hosts.

Similar host switching patterns were described by Clayton (1990) and Johnson et al. (2011) for ischnoceran lice, and by Bueter et al. (2009) for the amblyceran genus *Myrsidea*. Amblycerans (including *Menacanthus*) are in general more mobile than ischnocerans (Price et al., 2003), and under the conditions of poultry farming, where a food supply is also accessible to wild birds, opportunities for parasites to come into contact with new hosts may be common. Such host switching events from captive birds to distantly related birds have previously been reported for the poultry louse *Menopon gallinae* and *M. stramineus* on captive *Columba livia* (Dranzoa et al., 1999; Musa et al., 2011) and wild house sparrows, *Passer domesticus* (Hoyle, 1938). While this scenario is plausible for the EF-1α Hap16 (Supplementary Fig. S1) *Menacanthus* taken from *T. merula*, it seems less likely to be valid for that taken from *Dendrocopos major*, which is less likely to be feeding on seeds on the ground.

Alternatively, the host switch between domestic fowl and the two other bird groups may be older, with EF-1α Hap16 (Supplementary Fig. S1) representing a previously unknown species of *Menacanthus* that parasitises piciform and passeriform hosts. However, unless the passeriform louse population is very localised, we would also expect to recover this lineage at other localities where piciforms and passeriforms were sampled. A denser sampling would provide more data to resolve whether *M. stramineus* is a parasite with a complex population structure and capable of straggling to atypical hosts or whether a host switching event occurred in the past.

Whether these atypical host associations are well established or the result of straggling or contamination is presently unknown, as the atypical host populations have only been sampled once. However, while all three host species were sampled at the same locality, collection took place at different time periods: *G. gallus* samples were collected in May 2005, *T. merula* samples in January 2006 and *D. major* samples in February 2006. Chewing lice are not able to survive periods longer than a few days without their host (Mullen and Durden, 2002; Price et al., 2003), thus the contamination of birds by non-specific parasites can be excluded as a mode of transfer between unrelated hosts. However, only further sampling can establish whether there are continuous populations of *M. stramineus* on the atypical hosts.

The division of *Menacanthus* spp. into two major clades, here named "*curuccae*" and "*alaudae*", almost precisely follows the morphological division of *Menacanthus* spp. from passeriform birds according to Price (1977). The major division in his key is couplet 5, which separates the species here included in "*alaudae*" (*M. alaudae*, *M. camelinus*, *Menacanthus chrysophaeus*, *Menacanthus nogoma* and *Menacanthus pusillus*) from those included in "*curuccae*" (*M. curuccae, M. eurysternus*, *M. orioli*, *M. sinuatus* and *M. takayamai*). Within these two major clades ("*curuccae*" and "*alaudae*"), morphologically identified species typically created monophyletic lineages occurring on only one or two host families (Fig. 2). A notable exception is *M. eurysternus*, which has been discussed separately above. Price's (1977) key only includes the species on passeriforms and thus does not include *Menacathus pici*. This species is here placed as sister to "*alaudae*" in the COI phylogeny (Fig. 1), but nested within "*alaudae*" in the EF-1α and concatenated phylogenies (Fig. 3 and Supplementary Fig. S1). This placement in phylogenies is consistent with the morphology, as *M. pici* has the same number of lateroanterior metanotal setae, shape of the female subgenital plate, and shape of the male genitalia as those of the "*alaudae*" clade (Price and Emerson, 1975). An extended morphological revision, based on that of Price (1977), is thus likely to confirm the placement of *M. pici* in our phylogenies.

Menacanthus obrteli Balat, 1981, was described after the construction of Price's (1977) key, and was therefore not included in it, however material from hosts closely related to the type host of *M. obrteli* (*L. luscinioides*) was included under *M. takayamai*. Palma et al. (1998) considered *M. obrteli* indistinguishable from *M. takayamai*, and formally synonymised the two, while Mey (2003) recognised it as a subspecies of *M. takayamai*. Sychra et al. (2008) re-examined the type material of *M. obrteli*, as well as fresh material including males, and resurrected *M. obrteli* from synonymy. Both genes analysed here show that *M. obrteli* is well separated from the paraphyletic *M. takayamai*, and placed as a sister group (group "*obrteli*") to the rest of group "*curuccae*" (Figs. 1–3 and Supplementary Fig. S1).

Menacanthus obrteli has previously been recorded only from *L. luscinioides* (Sychra et al., 2008), however in our COI data set (see Fig. 1) material from this host is identical to specimens collected from *T. merula* and *L. collurio*. In addition, it forms a monophyletic lineage together with *Menacanthus* specimens from turdid and parid hosts (COI Hap 5, 6, 26). The fact that all specimens were collected in one area (central Europe) and on distantly related host taxa requires further explanation. Similar habitat preferences (as in the case of *M. stramineus*), shared nest holes (Johnson et al., 2002b; Weckstein, 2004), phoresy (Keirans, 1975; Harbison et al., 2009) or the overlapping migration routes and wintering ranges of hosts (e.g. Gustafsson and Olsson, 2012) are inapplicable, or insufficient, to completely explain the high level of louse dispersal between the hosts of *M. obrteli*. The hosts of *M. obrteli* do not share habitats or wintering routes and phoresy on vectors is not common in Amblycera (Price et al., 2003). Additional data collected from more host individuals and localities are needed to explore the level of host specificity in *M. obrteli* and the mechanism of dispersal between distantly related hosts.

The morphological homogeneity of *M. takayamai* samples may also be questioned, and the material determined to belong to this species on morphological grounds may be a cryptic assemblage of species. In all three data sets (Figs. 1 and 3, Supplementary Fig. S1), *M. takayamai* formed several lineages that are paraphyletic with respect to *M. eurysternus* and *M. sinuatus*, as well as other species in the EF-1α data set. The paraphyly of *M. takayamai* may have been caused by uneven sampling, as every lineage consisted of only a few individuals, each from one host family and each from a different continent or subcontinent (Fig. 1). As support is weak for these clades, the species as presently circumscribed morphologically is probably not a valid monophyletic taxon. Additional sampling focused on this taxon would provide more information to prove or disprove the polyphyly of *M. takayamai* and its dissolution to several species.

On a higher taxonomic level, it should be noted that several previously erected genera are here monophyletic and may warrant acceptance at least as subgenera. An important couplet in the key of Price (1977) is couplet 5, which divides most of the *Menacanthus* spp. on passerines into two groups; these groups are here referred to as the "*curuccae*" and "*alaudae*" groups. The latter of these groups contains *M. camelinus*, which is the type species of *Lanicanthus* Zlotorzycka, 1965. The type species of *Menacanthus*, *Menacanthus robustus* (Kellogg, 1896), was not included in the present study, but was placed by Price (1977) in the other large group, which corresponds to our "*curuccae*". The two groups can be differentiated by the number of lateroanterior setae of the pronotum, the margins of the female subgenital plate, the shape of the male genitalia (Price, 1977), and, supposedly, by the relative size of the facial hooks (Zlotorzycka, 1965). Further taxonomic considerations will be the subject of a future study.

In this study we demonstrated the importance of geography in forming population structure in multi-host parasites and discussed ecological factors facilitating host switches and maintenance of gene flow between unrelated host taxa. The differences in host specificity in *Menacanthus* spp. lineages were only partially congruent with the ecology of their hosts. *Menacanthus eurysternus* is typically found on hosts that allow for inter-specific transmission such as colonial nesters, cavity nesters and birds that form mixed-species feeding flocks, either during the breeding season or during the wintering season (Clayton, 1990; Price et al., 2003). However, there is no common biological pattern apparent for all hosts of this extremely euryxenous louse. The ecological proximity of hosts has been suggested to explain the transmission of lice through active dispersal to a new host after escaping preening (Johnson et al., 2011). Similar mechanisms might possibly facilitate the dispersal of *Menacanthus* lice between phylogenetically unrelated hosts.

Moreover, some intrinsic features of *M. eurysternus* may predispose it to maintain a wider host spectrum. *Menacanthus eurysternus* is an agile louse capable of moving quickly across the skin of its host (Price et al., 2003), and it can leave its host and survive for up to a few days without it (Mullen and Durden, 2002; Price et al., 2003). Finally, haematophagy may also play a role through interaction with endosymbiotic bacteria (Ries, 1931), providing a competitive advantage to some *Menacanthus* spp. or lineages. Additional sampling and experimental work may provide clues to help us distinguish between alternative mechanisms allowing louse dispersal and survival on new hosts.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ijpara.2014.09.001.

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