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Co-phylogeny of a hyper-symbiotic system: Endosymbiotic bacteria (Gammaproteobacteria), chewing lice (Insecta: Phthiraptera) and birds (Passeriformes)

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ABSTRACT

Chewing lice are hosts to endosymbiotic bacteria as well as themselves being permanent parasites. This offers a unique opportunity to examine the cophylogenetic relationships between three ecologically interconnected organismal groups: birds, chewing lice, and bacteria. Here, we examine the cophylogenetic relationships between lice in the genus *Guimaraesiella* Eichler, 1949, their endosymbiotic *Sodalis*-allied bacteria, and a range of bird species from across South China. Both event and distance-based cophylogenetic analyses were explored to compare phylogenies of the three organismal groups. Pair-wise comparisons between lice-endosymbionts and bird-endosymbionts indicated that their evolutionary histories are not independent. However, comparisons between lice and birds, showed mixed results; the distance-based method of ParaFit indicated that their evolutionary histories are not independent, while the event-based method of Jane indicated that their phylogenies were no more congruent than expected by chance. Notably, louse host-switching does not seem to have affected bacterial strains, as conspecific lice sampled from distantly related hosts share bacteria belonging to the same clade.

1. Introduction

Lice (Insecta: Phthiraptera) are permanent ectoparasites with no free-living stages, and complete their entire life cycle upon their host, from which they also derive all nutrients (Marshall, 1981; Nelson and Murray, 1971). Lice have limited dispersal opportunities, meaning that they typically must rely on direct contact between hosts to *e.g.,* avoid inbreeding or disperse to new hosts. However, some louse species are known to be phoretic, and hitch rides between hosts on hippoboscid flies (Diptera: Hippoboscidae) (*e.g.,* Harbison et al., 2008; Lee et al., 2022). Most louse species are known from one or a few closely related host species, whereas others may infest hosts belonging to multiple orders (Price et al., 2003; Gustafsson and Najer, 2022). Due to these unique constraints on habitats and dispersal, parasitic lice are often used as a model system to study co-evolutionary processes (*e.g.,* Banks et al., 2006; Paterson et al., 1999; Sweet and Johnson, 2016). Similarly, these factors make lice an ideal system for examining the co-speciation of insects and their bacterial endosymbionts.

Insect endosymbionts are most often beneficial, allowing their hosts to inhabit environments or provision nutrients that would otherwise be unavailable (Cardoza et al., 2006; Dale et al., 2002; Douglas, 2009; Rajagopal, 2009). Endosymbiotic bacteria are often derived from free-living relatives (McCutcheon and Moran, 2012). As many gene functions are no longer required in bacteria that have transitioned to an endosymbiotic lifestyle, the genomes of symbiotic bacteria are often smaller than their free-living counterparts. Mutations may accumulate due to the loss of a DNA repair system, genetic drift, and lack of recombination (McCutcheon and Moran, 2012). This may lead to genome degeneration in the endosymbiont, which may ultimately result in symbiont extinction and replacement by a new free-living strain. However, endosymbiont replacement may also occur due to external ecological influences, such as a change in host diet or host plant shift

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(Conord et al., 2008). Unlike in free-living insect hosts, the obligatory parasitic lifestyle that lice experience likely minimizes the influence of the external environment on the processes of symbiont replacement.

Bacteriocyte-associated endosymbiotic bacteria have been known in chewing lice since the 1930s (Ries, 1931), but these have not been widely studied until recent decades. To date, only the endosymbionts of pigeon lice in the genus *Columbicola* Ewing, 1929, and the songbird louse genus *Brueelia* have been studied (Alickovic et al., 2021; Fukatsu et al., 2007; Smith et al., 2013; Sweet et al., 2023). Each louse individual harbours a single endosymbiont strain that is maternally transmitted (Fukatsu et al., 2007). In both louse genera, the endosymbionts belong to the class Gammaproteobacteria and are allied to *Sodalis glossinidius* found in tsetse flies (*Glossina* spp.).

Co-phylogenetic analyses has revealed that the degree of co-speciation between *Sodalis*-allied endosymbionts and their *Columbicola* hosts is not higher than expected by chance (Smith et al., 2013). This is contrary to the expectations for a vertically transmitted, maternally inherited symbiont, and unlike what has been found in other insect-symbiont systems (Baumann, 2005; Moran et al., 1993). Based on the topology of the *Sodalis*-allied clade there may have been repeated symbiont acquisition and replacement cycles from a common bacterial source that is ubiquitous in the environment. This repeated replacement may be why there is no evidence for long-term co-speciation between *Columbicola* and their *Sodalis*-allied symbionts.

How lice acquire new symbionts in a replacement event is currently unknown. Two free-living *Sodalis* spp. have been described; however, both are associated with wood (Clayton et al., 2012; Tláskal et al., 2021). Since lice never leave their host, opportunities for them to come into contact with wood are limited, and at the discretion of their bird hosts, *e.g.,* nesting, roosting, or foraging. Seeing that lice spend almost all their time on their bird host, the birds themselves may be the source of bacteria. The only other organism that lice of the genera *Columbicola* and *Brueelia* come into contact with are hippoboscid flies, which are known to harbour *Sodalis* endosymbionts (Nováková et al., 2015; Šochová et al., 2017). Hippoboscids could thus possibly be one source of symbiont replacement in lice.

It is also not clear whether the patterns seen in *Columbicola* are representative of all chewing louse-endosymbiont systems. No other chewing louse-endosymbiont systems have been comprehensively studied, but *Columbicola* is a somewhat aberrant louse. For instance, no close relatives of *Columbicola* are known (*e.g.,* de Moya et al., 2019). Moreover, unlike the majority of louse genera, species of *Columbicola* are known to be capable of phoresy on hippoboscid flies, which is known to have an impact on their population, *e.g.* the population genetic structure compared with non-phoretic lice occurring on the same hosts (DiBlasi et al., 2018). Potentially, the ability to move more freely between bird hosts through phoresy could also increase the rate of exposure of lice to sources of replacement endosymbionts.

Here, we examine the bacterial symbionts of the louse genus *Guimaraesiella* Eichler, 1949, from South China. In order to examine the potential for the bird hosts as sources for replacement strains of the endosymbionts, we examine the cophylogenetic relationships between all three organism groups: birds, chewing lice, and bacteria. The genus *Guimaraesiella* parasitizes a range of songbird hosts (Passeriformes; see Gustafsson and Bush, 2017), and includes both host specialists and host generalists (Bush et al., 2016), as well as at least some species that are capable of phoresy (Lee et al., 2022). Lee et al. (2022) constructed louse/fly/bird networks and found one species of *Guimaraesiella* [referred to as *Guimaraesiella* (*Guimaraesiella*) sp. 6 by Tian et al., 2022] that parasitizes over 50 bird species and has a geographical range that spans from New Guinea and Australia over China, Thailand and India to Malawi (Bush et al., 2016). Lee et al. (2022) hypothesized that part of the reason for this species success in dispersing to multiple hosts is its use of hippoboscid flies, which are known to feed on a wide variety of bird hosts (Bequaert, 1953).

Additionally, many of the hosts *Guimaraesiella* sp. 6 infests are members of mixed-species feeding flocks (Bush et al., 2006; Chen and Hsieh, 2002; Zou et al., 2018), which may facilitate host switches of lice by increasing opportunities for switching between non-conspecific hosts. Host participation in mixed-species feeding flocks has previously been suggested to influence host association patterns and co-evolutionary patterns of lice (Balakrishnan and Sorenson, 2007; Ren et al., 2023). While some *Columbicola* species are known for being phoretic, the louse/fly/bird networks in that system are more localized and does not spread over multiple continents, nor does it extend to assemblages of birds of such different ecology, size, and behaviour as in mixed-species flocks of passeriforms (Johnson et al., 2002; Lee et al., 2022; Price et al., 2003). Potentially, if replacement of symbiont strains originates in bacterial populations on different bird species, lice that can switch between host species may have less homogeneous bacterial faunas than those that cannot easily switch hosts. Alternatively, features of the symbiotic bacteria in a given louse species may be a factor that allows the transition from host generalist to specialist, in which case host generalist lice from different hosts (*e.g., Guimaraesiella* sp. 6) may be expected to have the same symbionts.

Thus, by examining bacteria from a group of lice that are genetically homogeneous over large geographical areas and host ranges, including many hosts that participate in mixed-species flocks, a more complete picture of co-evolution between lice and their symbionts may emerge. By comparing the phylogeny of chewing lice to that of their endosymbiotic bacteria we can see if they are more similar than expected by chance. This can then be compared to the relationship endosymbiotic bacteria have with the bird hosts their lice are infesting, while taking into account the relationship the lice themselves have with their bird hosts to see if chewing lice or their host birds have a stronger influence on the endosymbiotic bacteria present.

2. Methods

2.1. Taxon sampling

Birds were caught at 15 sampling locations across southern China (Fig. 1) between 2012 and 2019, using mist nets (2 m X 6 m, mesh size: 15 mm). Lice were removed by fumigation following the protocol outlined by Gustafsson et al., (2019a). Birds were identified using MacKinnon and Phillipps (2000) and Arlott (2017), and taxonomy follows Clements et al. (2022). Lice were stored in absolute ethanol at −80 °C, until DNA extraction.

2.2. DNA extraction and PCR

To prepare for DNA extraction each louse was cut halfway through the pterothorax with a scalpel. DNA was extracted using DNeasy™ Tissue Kit (Qiagen, Shanghai, China) following the manufacture's protocol except: lice were incubated in digestion buffer for 24 hrs at 55 °C, after Buffer AL was added samples were incubated at 70 °C and only 50 μl of Buffer AE was used for each elution. After DNA extraction the louse exoskeletons were retrieved and stored in ethanol, until they were slide mounted. Using polymerase chain reaction (PCR), we targeted 16S ribosomal RNA gene (16S) for bacteria, and cytochrome oxidase subunit 1 (COI), 16S, hypothetical protein (HYP) and elongation factor 1α (EF-1a) for lice. See Table 1 for primers used in this study.

For PCR reactions we used either Cytiva PureTaq Ready-To-Go beads (GE Healthcare, Vienna, Austria) or Qiagen Hot StarTaq Master Mix Kit (Qiagen, Shanghai, China), following the manufacture's protocol for 25 μl reactions. PCR products were screened using gel electrophoresis and those that had satisfactory bands were sent for sequencing at Tianyi Huiyuan Gene Technology, Co. Ltd. (Guangzhou, China).

2.3. Phylogenetic analysis

Sequences were assembled using the *de novo* assemble tool in

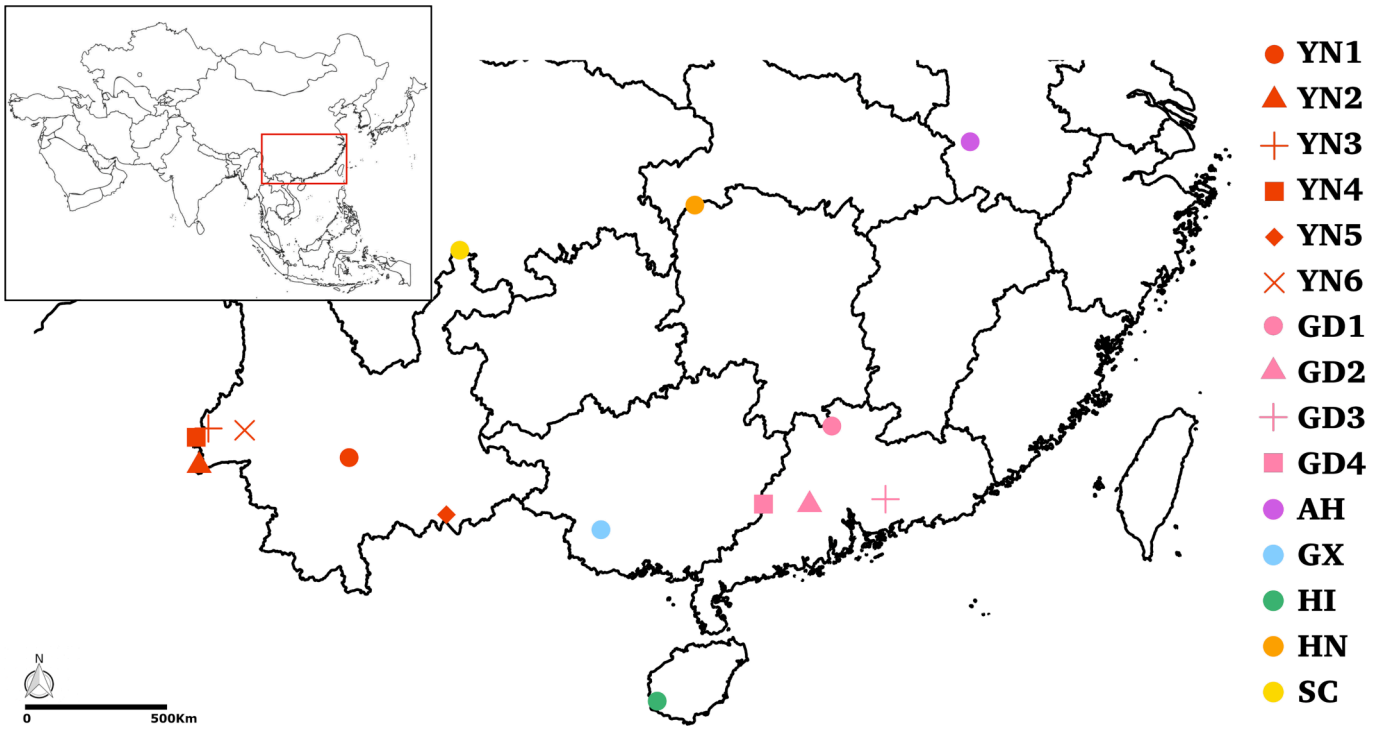

Fig. 1. Map of sampling sites in southern China. Sampling site abbreviations: YN1 - Ailaoshan National Nature Reserve Ecological Station, YN2 - Weijiao Village, YN3 - Hongbeng River, Yingjiang County, YN4 - Banyan Tree King in Daonong Village, YN5 - Dawei Mountain Nature Reserve, YN6 - Gaoligong Mountain National Nature Reserve, GD1 - Babaoshan Management Station, GD2 - Dinghushan National Nature Reserve, GD3 - Dinghushan National Nature Reserve, GD4 - Tongle Nature Reserve, AH - Baiguo Village, GX - Pairu Village, HI - Hainan Jianfengling National Nature Reserve, HN - Hunan Badagong Mountain Reserve Tea Station, SC - Laojun Temple in Laojunshan Nature Reserve.

Table 1
Primer pairs used for bacteria and louse PCR.

Locus	Primer name	Primer sequence	Source
Bacteria			
16S	SodF	5′ ACCGCATAACGTCGCAAGACC 3′	Nováková and Hypša, 2007
	R1060	5′ CTTAACCCAACATTTCTCAACACGAG 3′	
Lice			
CO1	L6625	5′ CCGGATCCTTYTGRTTYTTYGGNCAYCC 3′	Hafner et al., 1994
	H7005	5′ CCGGATCCACNACRTARTANGTRTCRTG 3′	
16S	16S03F	5′ CAATACTTGGCTTGCATGT 3′	Tian et al., 2022
	16S03R	5′ GATAGAAACTGACCTGACTTAC 3′	
HYP	BR50-181L	5′ CTTGARCAATTRCAGAAAAAAGC 3′	Sweet et al., 2014
	BR50-621R	5′ GGRTTTTCWGGAGAYCTCATCC 3′	
EF-1a	EF1-For3	5′ GGNGACAAYGTTGGYTTCAACG 3′	Danforth and Ji, 1998
	EF1-CH10	5′ ACRGCVACKGTYTGHCKCATGTC 3′	

Geneious Prime® v.2022.1.1. All bacteria sequences were run through the Basic Local Alignment Search Tool (BLAST, https://blast.ncbi.nlm.nih.gov) to confirm the identity of *Sodalis*-allied symbionts and exclude contaminants. Sequences were considered to be *Sodalis*-allied symbionts if the top hit had an E value of 0.0 and identity between 85 and 100 %. After sequences were assembled and the ends were trimmed some sequences still contained a high number of ambiguities, out of an abundance of caution these were removed before downstream analyses. Sequences were aligned using MUSCLE (Edgar, 2004) with the default settings and checked manually. Substitution models for each gene were evaluated in MEGA11 (Kumar et al., 2021), with the best models being: HKY for 16S (bacteria) and HYP, HKY + I for CO1 and 16S (louse), and JC for EF-1a. The four louse genes were concatenated, and this data set was used for all downstream analyses. As an outgroup for lice, we used *Campanulotes bidentatus compar*, *Quadraceps punctatus* and *Saemundssonia lari* and for a bacterial outgroup we used *Vibrio cholerae* (see Table 2 for GenBank accession numbers). We performed Bayesian analysis using BEAST v2.7.3 (Suchard et al., 2018), with the following settings: the appropriate model(s) for each gene or gene partition were selected (trees were linked for the louse data set), Gamma category count was set to 4, Yule Model was used, Markov chain Monte Carlo (MCMC) was run for 1 x 10^8 generations, and sampled every 1000 trees. The log file produced by BEAST was examined in Tracer (Rambaut et al., 2018) to assess MCMC for convergence. TreeAnnotator v2.7.3 was then used for tree integration, we discarded the first 10 000 trees (10 %) as burn-in and constructed a maximum clade credibility tree.

Many of the louse/bird host associations that were positive for bacteria were not unique; therefore, to avoid artificially inflating the number of co-speciation events, the louse tree was pruned to only contain one representative from each species.

Similar to the louse tree, the endosymbiont tree was pruned down to the appropriate number of operational taxonomic units (OTUs) to avoid artificially inflating the number of co-speciation events. To test for the number of OTUs, General Mixed Yule Coalescent (GMYC) was used (Fujisawa and Barraclough, 2013). The endosymbiont tree generated in BEAST was imported into R and the "gmyc" function in the *splits* packages was used to determine the number of OTUs (Joseph and Vakayil, 2022).

A bird host tree was generated from CO1, Cytochrome *b* (CytB) and NADH dehydrogenase subunit 2 (ND2) genes acquired from GenBank (Supplementary material Table S1); these sequences were processed the same way as described above (substitution models: GTR + G + I for CO1

Table 2
Collection and GenBank accession numbers for specimens included in this study. Voucher number, the first four digits refer to the host individual and the last two digits refer to a unique louse. Thus, J0314.01 and J0314.02 came from the same host bird but different lice. See Fig. 1 for locality abbreviations. Louse species that could not be positively identified as any described species are denoted as "sp. #' (sp. # are consistent with those in Tian et al., (2022)).

				Sequence data				
Louse species	Bird host species	Voucher no.	Loc.	Bacteria	Louse			
				16S	CO1	16S	HYP	EF-1a
***Guimaraesiella* s. str.**								
G. (G.) sp. 1	*Turdus hortulorum*	J2542.42	GD2	OR076897	OL514094 *	OL527874 *	OR068096	OR068129
G. (G.) sp. 6	*Parus minor*	J2834.01	AH	OR076900	OR162104	OR076929	OR068078	OR068111
G. (G.) sp. 6	*Niltava sundara*	J4214.01	YN1	OR076913	OR162110	OR076940	OR068089	OR068122
G. (G.) sp. 6	*Rhipidura albicollis*	J0528.15	YN4	OR076887	OL514053 *	OL527833 *	OR068097	OR068130
G. (G.) sp. 6	*Rhipidura albicollis*	J4170.01	YN6	OR076912	OR162109	OR076939	OR068088	OR068121
G. (G.) sp. 6	*Pomatorhinus ruficollis*	J3646.01	GD2	OR076908	OR162108	OR076935	OR068084	OR068117
G. (G.) sp. 6	*Erpornis zantholeuca*	J3309.07	GD3	OR076906	OR162106	OR166350	OR159782	OR159794
G. (G.) sp. 6	*Erpornis zantholeuca*	J3309.08	GD3	OR076907	OR162107	OR166351	OR159783	OR159795
G. (G.) sp. 6	*Erpornis zantholeuca*	J3309.3B	GD3	OR076905	OR162105	OR076934	OR068083	OR068116
***Guimaraesiella (Cicchinella) sehri* species group**								
G. (Ci.) falcifrons	*Minla ignotincta*	J2239.01	SC	OR076892	OR162103	OR076925	OR068074	OR068107
G. (Ci.) falcifrons	*Minla ignotincta*	J0175.1B	YN5	OR076877	OR162097	OR076917	OR068066	OR068099
G. (Ci.) falcifrons	*Actinodura cyanouroptera*	J0212.06	YN5	OR076878	OL514046 *	OL527826 *	OR068090	OR068123
***Guimaraesiella (Cicchinella) gombakensis* species group**								
G. (Ci.) sp. 1	*Alcippe hueti hueti*	J2839.01	AN	OR076901	OR161090	OR076930	OR068079	OR068112
G. (Ci.) sp. 1	*Alcippe hueti hueti*	J2841.01	AN	OR076902	OR161091	OR076931	OR068080	OR068113
G. (Ci.) sp. 1	*Alcippe davidi davidi*	J0095.01	HN	OR076874	OL514041 *	OL527821 *	OR068095	OR068128
G. (Ci.) sp. 1	*Alcippe fratercula*	J4159.11	YN6	OR076911	OR161096	OR076938	OR068087	OR068120
G. (Ci.) petilorica	*Alcippe nipalensis*[+]	J1220.01	YN2	OR076890	OR162102	OR076924	OR068073	OR068106
G. (Ci.) petilorica	*Alcippe nipalensis*[+]	J0525.01	YN4	OR076884	OR162098	OR166352	OR159784	OR159791
G. (Ci.) petilorica	*Alcippe nipalensis*[+]	J0525.1B	YN4	OR076885	OR162099	OR076921	OR068070	OR068103
G. (Ci.) petilorica	*Alcippe nipalensis*[+]	J0525.02	YN4	OR076886	OR162100	OR166353	OR159785	OR159792
G. (Ci.) petilorica	*Alcippe poioicephala*	J1062.01	YN3	OR076889	OR162111	OR076923	OR068072	OR068105
G. (Ci.) petilorica	*Niltava grandis*	J0578.01	YN4	OR076888	OR162101	OR076922	OR068071	OR068104
G. (Ci.) mcgrewi	*Alcippe hueti hueti*	J0365.02	GD1	OR076883	OR161085	OR166354	OR159786	OR159790
G. (Ci.) mcgrewi	*Alcippe hueti hueti*	J0365.01	GD1	OR076882	OR161084	OR076920	OR068069	OR068102
G. (Ci.) mcgrewi	*Alcippe hueti hueti*	J0137.1B	GD1	OR076876	OR161080	OR076916	OR068065	OR068098
G. (Ci.) mcgrewi	*Alcippe hueti hueti*	J0314.01	GD1	OR076879	OR161081	OR076918	OR068067	OR068100
G. (Ci.) mcgrewi	*Alcippe hueti hueti*	J0314.02	GD1	OR076880	OR161082	OR166355	OR159787	OR159789
G. (Ci.) mcgrewi	*Alcippe hueti hueti*	J0315.01	GD1	OR076881	OR161083	OR076919	OR068068	OR068101
G. (Ci.) mcgrewi	*Alcippe hueti hueti*	J2513.12	GD2	OR076896	OL514037 *	OL527817 *	OR068094	OR068127
G. (Ci.) mcgrewi	*Alcippe hueti hueti*	J2691.01	GD2	OR076898	OR161089	OR076928	OR068077	OR068110
G. (Ci.) mcgrewi	*Alcippe hueti hueti*	J2718.04	GD2	OR076899	OL514031 *	OL527811 *	OR068091	OR068124
G. (Ci.) mcgrewi	*Alcippe hueti hueti*	J2383.01	GD4	OR076894	OR161087	OR076927	OR068076	OR068109
G. (Ci.) mcgrewi	*Cyornis brunneatus*	J2089.33	GD4	OR076891	OL514085 *	OL527866 *	OR068093	OR068126
G. (Ci.) mcgrewi	*Alcippe hueti hueti*	J3909.01	GD4	OR076910	OR161095	OR076937	OR068086	OR068119
G. (Ci.) mcgrewi	*Alcippe hueti hueti*	J2383.02	GD4	OR076895	OR161088	OR166356	OR159788	OR159793
G. (Ci.) mcgrewi	*Alcippe hueti hueti*	J3904.01	GD4	OR076909	OR161094	OR076936	OR068085	OR068118
G. (Ci.) mcgrewi	*Alcippe hueti hueti*	J2283.01	HI	OR076893	OR161086	OR076926	OR068075	OR068108
G. (Ci.) mcgrewi	*Alcippe davidi schaefferi*	J2980.01	GX	OR076904	OR161093	OR076933	OR068082	OR068115
G. (Ci.) mcgrewi	*Alcippe davidi schaefferi*	J2890.01	GX	OR076903	OR161092	OR076932	OR068081	OR068114
G. (Ci.) mcgrewi	*Pomatorhinus ruficollis*	J0136.02	GD1	OR076875	OR161097	OL527822 *	OR068092	OR068125
Outgroups								
Campanulotes bidentatus compar				–	AF384997.1	AY139934.1	KF841392.1	HQ332855.1
Quadraceps punctatus				–	ON643966.1	ON643969.1	KF841401.1	AF447209.1
Saemundssonia lari				–	AY149406.1	AY139931.1	KF841403.1	AY149435.1
Vibrio cholerae				LC487865.1	–	–	–	–

* Denotes sequences that came from Tian et al., (2022).
[+] Arlott (2017) and Clements et al., (2022) do not list *A. nipalensis* as breeding in China, but it is listed by Zheng, (2017). The collection locality (Yingjiang County, Dehong Prefecture, Yunnan Province) is right at the border of the range of *A. nipalensis* outlined by Arlott (2017). None of the present authors participated in the collection trip to Dehong when these samples were collected, and this host association would need to be verified by future collection trips.

and CytB, and TN93 + G + I for ND2).

2.4. Louse identification

After DNA extraction the gut was removed, and the louse exoskeleton was placed into clove oil for 30 min up to 24 h before it was slide mounted in Canada balsam and allowed to cure at room temperature for 30 days. All slides were deposited at the Institute of Zoology, Guangdong Academy of Sciences, Guangzhou, China. Specimens were examined through a Nikon Eclipse Ni microscope (Nikon Corporation, Tokyo Japan) and identified using Gustafsson et al., (2019b, 2021). Genetic distances for within and between groups for CO1 were computed with MEGA11. Additionally, genetic distances for our specimens were compared to those from Tian et al., (2022). It should be noted that some of the lice in this study are the same as those in Tian et al., (2022) (Table 2); the numbers added to undescribed species are the same here as in Tian et al., (2022).

2.5. Cophylogenetic analysis

We conducted both event-based and distance based co-phylogenetic analyses. For an event-based approach, we used Jane 4.0 to map out potential evolutionary events of the symbiont in relation to the host phylogeny (Conow et al., 2010). Jane was chosen over its successor eMPRess, as it allows for symbionts to be associated with multiple hosts, which is not possible in eMPRess. Event costs were set to: co-speciation:

0, duplication: 1, duplication and host switch: 2, loss: 1, and failure to diverge: 1 The cost of co-speciation is set to zero because it is considered a "null event". This cost scheme is commonly used in phylogenetic studies (*e.g.,* Johnson et al., 2021; Sweet and Johnson, 2016), and thus allows for comparisons with other studies. Default settings for the Genetic Algorithm parameters of 100 generations, and population size of 100 were used. After finding the most optimal solution, we determined if the overall cost of reconstruction was significantly lower than expected by chance, by randomizing the tip mappings 100 times. If the randomization procedure indicated our optimal solution was lower than expected by chance, this would indicate some level of congruence between the host and symbiont phylogenies.

For a distance based approached we used ParaFit (Legendre et al., 2002), which assesses the overall congruence between host and symbiont phylogenies and the significance of each host/symbiont association. ParaFit was implemented in RStudio v2021.09.0 (R Core Team, 2018), host and symbiont phylogenies were converted into patristic distance matrices using the "cophenetic" command in the *ape* package (Paradis and Schliep, 2019), and sorted each distance matrix according to the host-symbiont association matrix. We ran ParaFit for 10 000 permutations with the Cailliez correction for negative eigenvalues and tested for the contribution of each individual link using the ParaFitLink1 and ParaFitLink2 tests. Tanglegrams were generated in RStudio using the "cophylo" function in the *phytools* package (Revell, 2012).

3. Results

3.1. Phylogenetic analysis

Of the 257 *Guimaraesiella* specimens that were screened for *Sodalis*-allied symbionts, 80 (31 %) were positive and of those 40 had sequences of high enough quality to be included in further analysis. For *Guimaraesiella* spp. we sequenced 446 bp of the COI locus (132 variable sites, 115 parsimony-informative sites), 533 bp of the 16S locus (133 variable sites, 117 parsimony-informative sites), 364 bp of HYP (31 variable sites, 28 parsimony-informative sites), and 366 bp of EF-1a (19 variable sites, 18 parsimony-informative sites). The *Guimaraesiella* spp. tree (Fig. 2) shows six monophyletic clades each having very little structure within them. This further supports that the undescribed species of *Guimaraesiella* (*Guimaraesiella*) sp.1, *Guimaraesiella* (*Guimaraesiella*) sp.6, *Guimaraesiella* (*Cicchinella*) sp.6, proposed by Tian et al., (2022) are valid species.

For *Sodalis,* we sequenced 829 bp of the 16S locus (61 variable sites, 51 parsimony-informative sites). The *Sodalis*-allied symbiont tree (Fig. 3) depicts six monophyletic clades. These clades correspond to the results from the OTU test.

3.2. Co-phylogenetic analysis

When the phylogeny of *Guimaraesiella* spp. is compared to that of its *Sodalis*-allied symbionts, both Jane and ParaFit indicated that they are more congruent than expected by chance. Jane recovered 6 sorting events: 4 co-speciation, 1 duplication and host switch, 1 loss and 0 failures to diverge (p-value = 0.01) (Table 3, Fig. 4, supplementary material Fig. S1). The ParaFit analysis was significant across the entire data set (p-value = 0.03, ParaFitGlobal = 0.00031) thus rejecting the independence of the louse and symbiont phylogenies. The ParaFitLink1 test recovered 2 louse-symbiont links as significantly contributing to the global score (Fig. 4).

When *Guimaraesiella* spp. lice are compared to their bird hosts, Jane recovered 38 sorting events: 0 co-speciation, 2 duplication, 3 duplication and host-switch, 21 losses and 12 failures to diverge (Table 3), however the cost for reconstruction was not significantly lower than expected by chance (p-value = 0.09, supplementary material Fig. S1). The ParaFit analysis indicated congruence between the host and parasite trees (p-value = 0.002, ParaFitGlobal = 0.103) and the ParaFitLink1 test

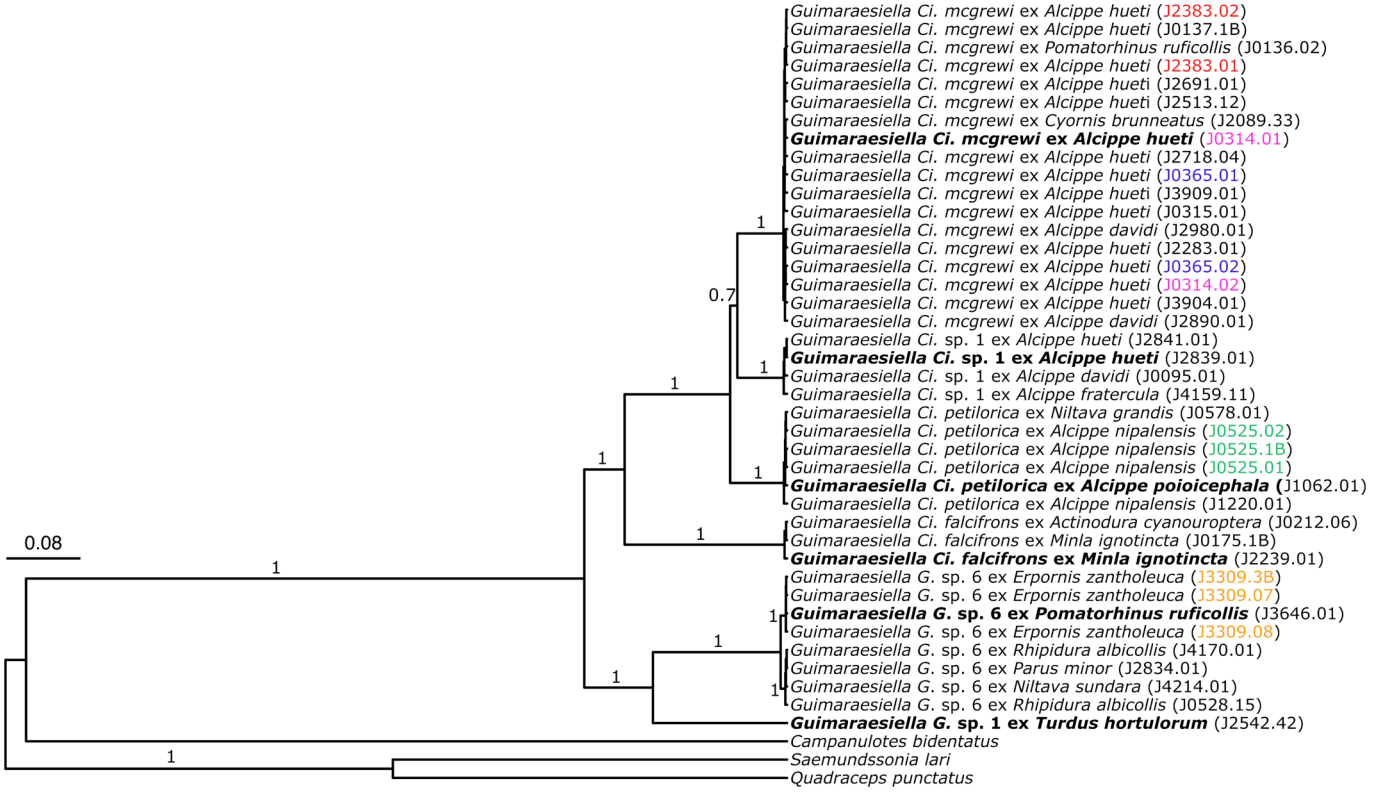

Fig. 2. Phylogenetic reconstruction of *Guimaraesiella* spp. inferred by BEAST, with node posterior probabilities greater than 0.9 displayed. Corresponding coloured voucher numbers indicate lice infesting the same bird individual. Bolded names indicate sequences that were used in co-phylogenetic analysis. Abbreviations used: *Ci.* = *Cicchinella*; *G.* = *Guimaraesiella*.

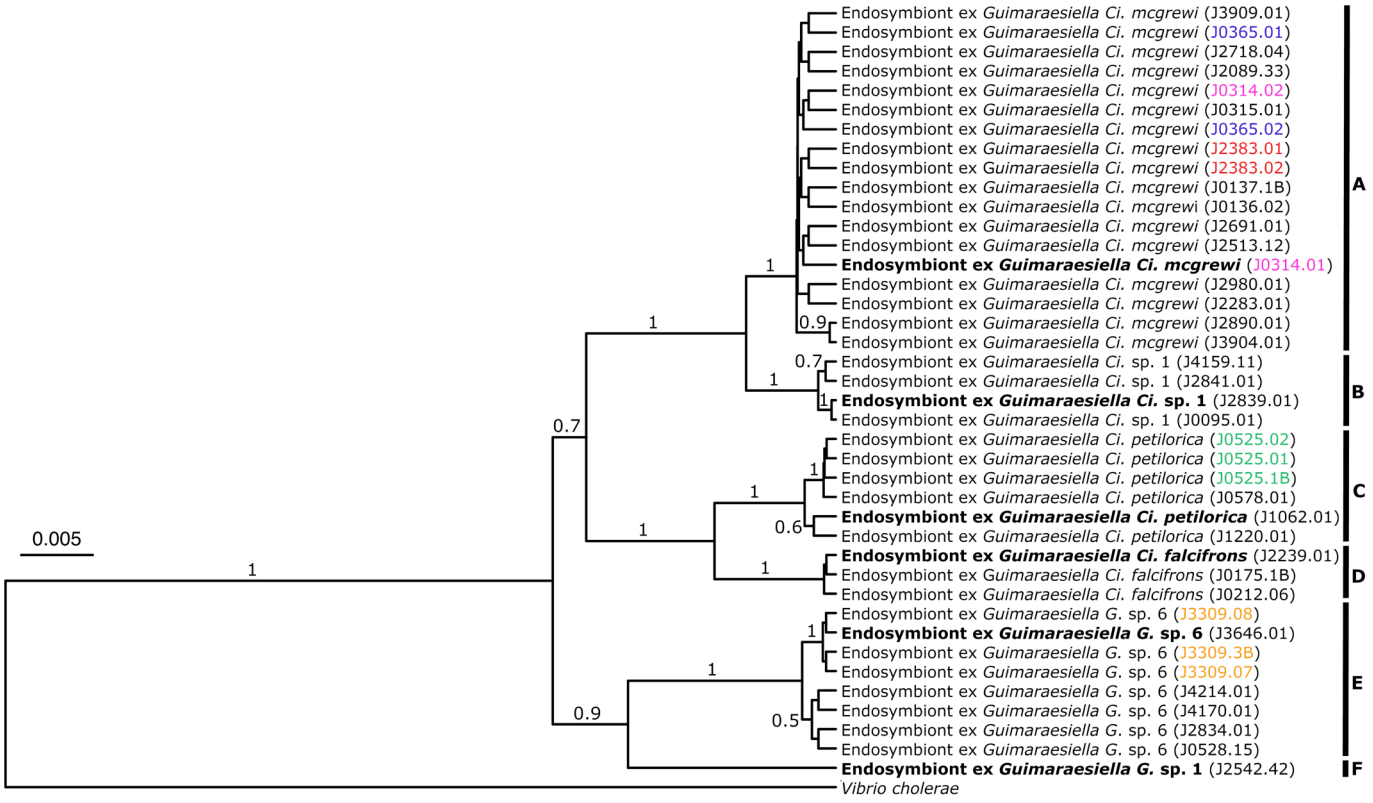

Fig. 3. Phylogenetic reconstruction of *Sodalis*-allied symbionts found in *Guimaraesiella* spp. inferred by BEAST, with node posterior probabilities greater than 0.9 displayed. Corresponding coloured voucher numbers indicate endosymbionts that came from lice sampled from the same bird individual. Bolded names indicate sequences that were used in co-phylogenetic analysis. Clades indicated on the right side are the OTUs from GMYC. Abbreviations used: *Ci.* = *Cicchinella*; *G.* = *Guimaraesiella*.

Table 3
Summary of Jane results for each comparison (cost scheme: co-speciation 0, duplication 1, duplication and host switch 2, loss 1, and failure to diverge 1).

Phylogenetic Comparison	Sorting Events					Cost	p-value
	Co-speciation	Duplication	Duplication & Host Switch	Loss	Failure to Diverge		
Guimaraesiella spp. & *Sodalis*-allied symbiont	4	0	1	1	0	3	0.01
Bird hosts & *Guimaraesiella* spp.	0	2	3	21	12	41	0.09
Bird hosts & *Sodalis*-allied symbiont	1	1	3	18	12	37	0.02

recovered 14 host - parasite links as significantly contributing to the global score (Fig. 5).

For the comparison of *Sodalis*-allied symbionts and bird hosts, Jane found that the cost of reconstruction was significantly lower than expected by chance (p-value = 0.02, Table 3, supplementary material Fig. S1), and recovered 35 sorting events: 1 co-speciation, 1 duplication, 3 duplication and host-switch, 18 losses and 12 failures to diverge (Fig. 6). ParaFit also indicated congruence between the host and symbiont trees (p-value = 0.004, ParaFitGlobal = 0.00072). The ParaFitLink1 test recovered 9 bird - symbiont links as significantly contributing to the global score (Fig. 6).

4. Discussion

The distance- and event-based analyses both indicate that the evolutionary histories of two of the pair-wise comparisons (lice-endosymbiont and bird-endosymbiont) are not independent, whereas the comparisons between the louse and bird phylogenies produced mixed results. The distance-based method of ParaFit indicated that the bird/louse phylogenies were more similar than expected by chance while the event-based method of Jane found the bird/louse phylogenies to be independent. As the symbionts are located in bacteriocytes on the inside of the lice (Smith et al., 2013), and the endosymbionts show strong evidence of co-speciation with the lice, patterns in the bird-endosymbiont comparison were expected to be similar to those of the louse-bird comparisons. However, this was not the case; while the louse-bird comparisons had mixed results of phylogenies being independent of each other, the bird-endosymbiont comparisons were more similar then expected by chance. No co-speciation events were inferred for the louse-bird comparison, but one co-speciation event was inferred between the bird-endosymbiont phylogenies (Table 3, Fig. 6).

Notably, each species of louse in our study was associated with a single endosymbiont clade (Figs. 2–3), even in cases where conspecific lice were derived from different bird host species or families. There is thus no evidence that *e.g.*, *Guimaraesiella* (*G.*) sp. 6 has significantly different endosymbionts on different hosts, or that lice switching from one bird host to another – even between *e.g.*, Corvides to Passerida hosts – exposes the lice to novel bacterial strains that have replaced established strains, at least not over short time periods. This would imply that novel strains of *Sodalis*-allied bacteria that could replace extant endosymbiont populations may not be derived from the birds, but this requires further testing.

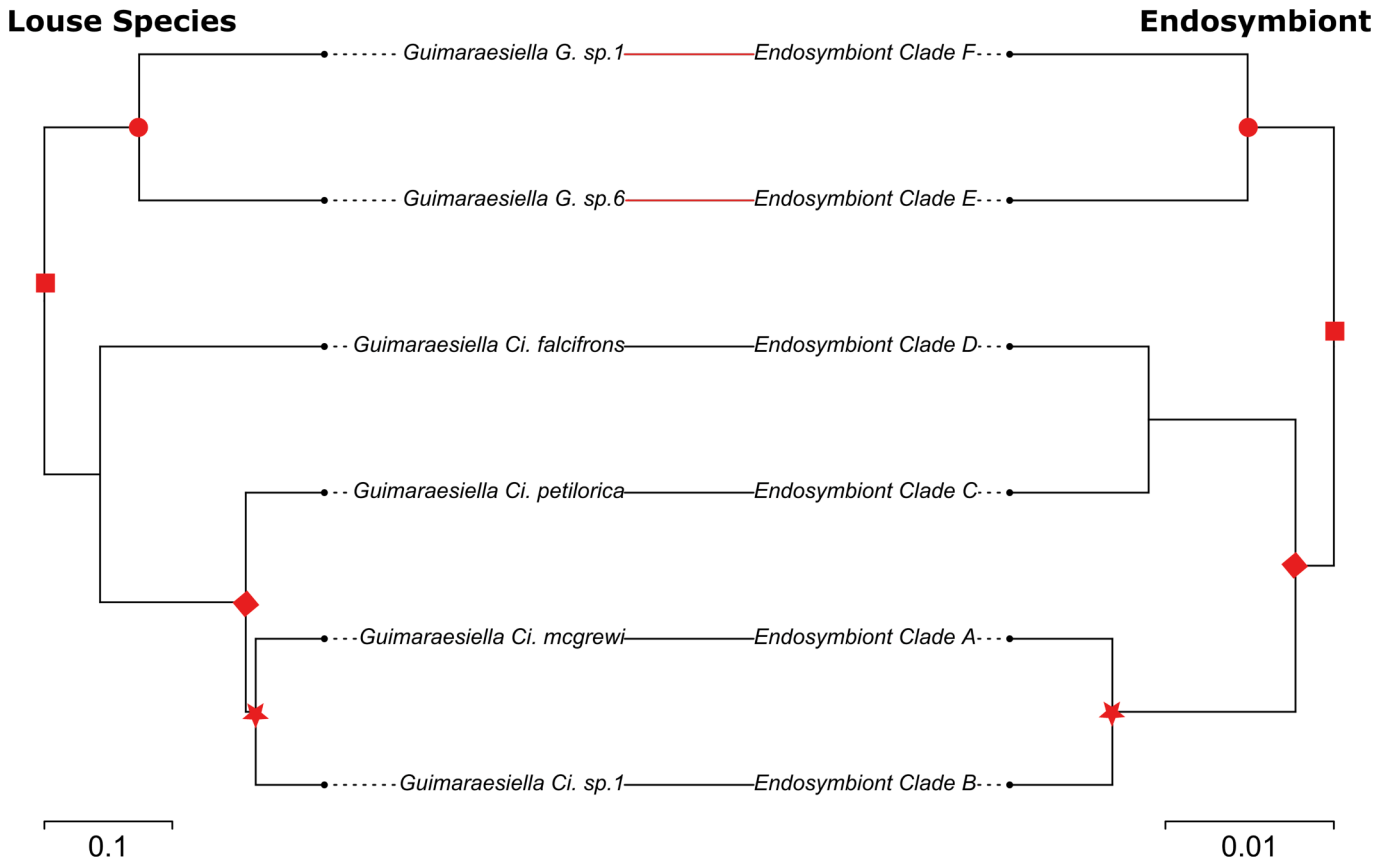

Fig. 4. Tanglegram of *Guimaraesiella* spp. and their *Sodalis*-allied symbionts. Connecting lines indicate host/symbiont association; red lines indicate significant host-parasite links estimated by the ParaFitLink1. Corresponding red shapes indicate co-speciation events recovered by Jane. Abbreviations used: *Ci.* = *Cicchinella*; *G.* = *Guimaraesiella*. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

The event-based results from Jane inferred no co-speciation events between the louse and bird phylogenies (Table 3; Fig. 5), which may be due to the presence of both host generalists and host specialists in the genus *Guimaraesiella* (*e.g.,* Bush et al., 2016). The *Guimaraesiella* spp. in this study have all been recorded on more than one host with the exception of *Guimaraesiella* (*G.*) sp. 1 which has only been recorded from *Turdus hortulorum* (Gustafsson et al., 2019b, Tian et al., 2022). However, the louse hosts of endosymbiont clades A–B are mostly from closely related, allopatric host species that have until recently been considered conspecific (Zou et al., 2007, Song et al., 2009). Moreover, these birds are frequent pilot species in mixed species flocks, and it cannot be excluded that the specimens from *e.g. Cyornis brunneatus* derive from accidental stragglers rather than established populations (Tian et al., 2022). If so, the lack of bird host diversity among the lice from which symbiont clades A–C were derived from may indicate that symbiont strain may have an influence on the ability of lice to become generalists. Potentially, some feature of symbiont clade E may allow for their lice to survive more easily on novel hosts than lice with symbionts from other clades; however, much more data from diverse groups of *Guimaraesiella* are needed to test this more exhaustively.

Moreover, host participation in mixed-species feeding flocks has been documented for most of the bird species included here (Chen and Hsieh, 2002; Zhang et al., 2013, Zou et al., 2018), and has previously been suggested to influence host association patterns and co-evolutionary patterns of lice (*e.g.,* Balakrishnan and Sorenson, 2007; Ren et al., 2023). Close association of different bird species for prolonged periods of time may contribute to dispersal opportunities even in the absence of phoresy; if hippoboscid flies are also present in these flocks, these two factors may work synergistically. Of the louse species examined here, at least *Guimaraesiella* (*G.*) sp. 6 is known to be phoretic on hippoboscid flies (Lee et al., 2022), and is known from a wide range of bird hosts from Australia, South Asia, and Africa (Bush et al., 2016; Lee et al., 2022; Tian et al., 2022). This ability to disperse opportunistically from one bird host to another would naturally erode coevolutionary patterns between the lice and their hosts. Notably, *Guimaraesiella* sp. 6 occurs on hosts in both the Corvides and Passerida radiations (Fig. 5). Although our sample size of this species is small, this is consistent with the overall patterns of distribution in *Guimaraesiella* (Gustafsson and Bush, 2017). This louse genus is generally more speciose and diverse on Corvides hosts than on Passerida hosts, but transfers between the two major songbird radiations must have been frequent (Bush et al., 2016; Sweet et al., 2018).

The louse genus *Columbicola* also contains both host specialist and host generalist, some of which are known to be phoretic; moreover, phylogenies between *Columbicola* and their hosts are more congruent than expected by chance (Clayton and Johnson, 2003; Sweet and Johnson, 2016). Even though *Columbicola* and *Guimaraesiella* share these ecological traits, the relationships between these lice and their respective *Sodalis*-allied symbiont strains differ. The *Sodalis*-allied symbionts in *Columbicola* show repeated extinction and acquisition, resulting in little congruence between their phylogenies (Smith et al., 2013). In contrast, *Guimaraesiella* and its *Sodalis*-allied symbionts share more co-speciation events than expected by chance (Fig. 4, Table 3), with the Jane analysis inferring four co-speciation events of a possible five based on tree topology. Notably, louse host-switching does not seem to have affected symbiont strains, as conspecific lice sampled from distantly related hosts share symbionts belonging to the same clade (Fig. 3).

One difference between Smith et al. (2013) and this study is the scale at which sampling was conducted. *Guimaraesiella* was sampled from birds caught in southern China, while *Columbicola* was sampled from

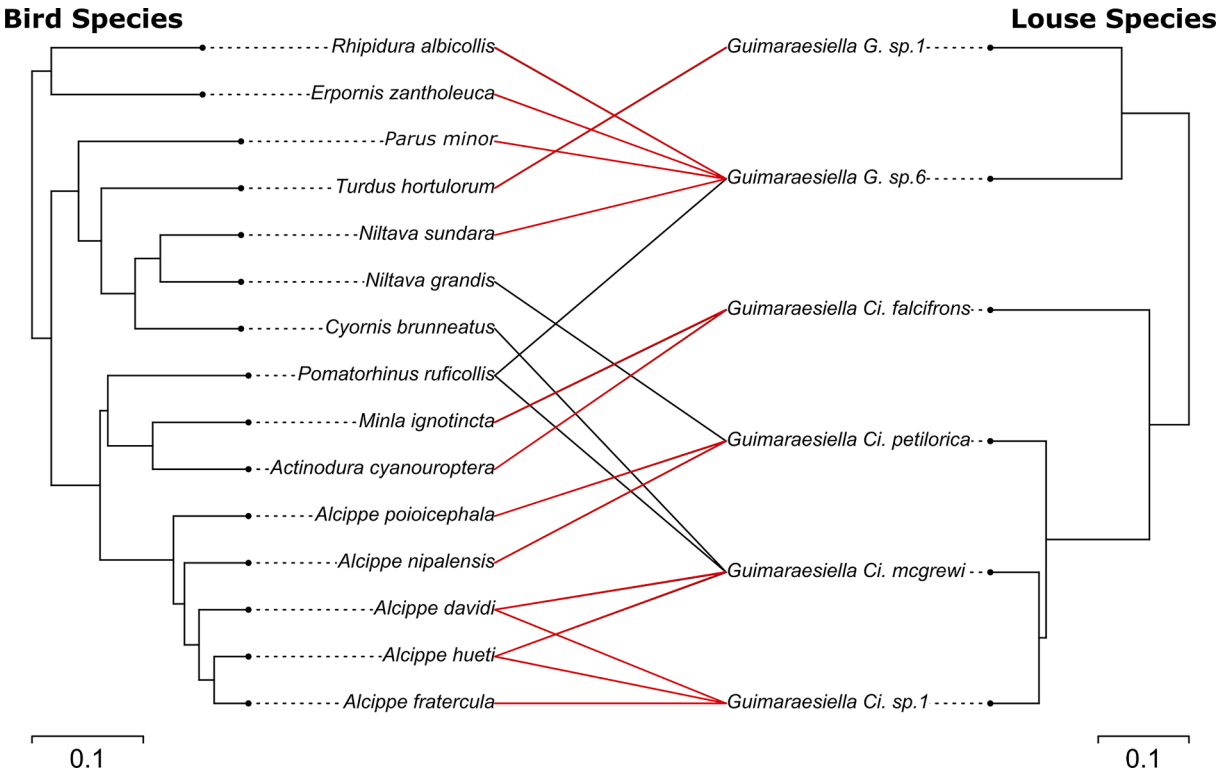

Fig. 5. Tanglegram of host bird species and their *Guimaraesiella* spp. lice. Connecting lines indicate host/parasite association; red lines indicate significant host-parasite links estimated by the ParaFitLink1. Abbreviations used: *Ci.* = *Cicchinella*; *G.* = *Guimaraesiella*. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

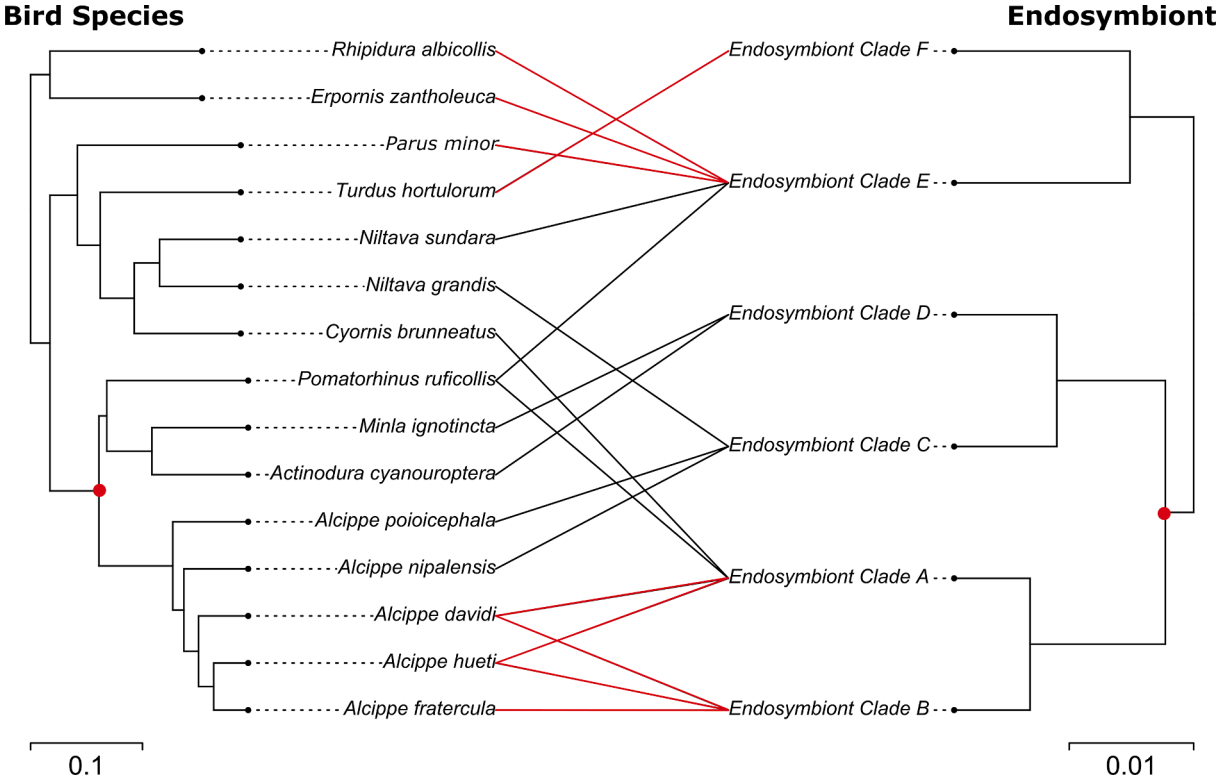

Fig. 6. Tanglegram of host bird species and *Sodalis*-allied symbionts. Connecting lines indicate host/symbiont association; red lines indicate significant host-parasite links estimated by the ParaFitLink1. The red circle indicates the co-speciation event recovered by Jane. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

birds over multiple continents. Much is still unknown about the relationships between both louse genera and their respective *Sodalis* symbiont lineages, especially with regards to acquisition of symbionts and the potential for replacement of one symbiont strain by another. Conceivably, the differences in geographical scale may influence the results of these studies, with our study reflecting a more local situation. As more samples from different regions become available, a more thorough comparison between the *Sodalis*-allied symbionts in *Columbicola* and *Guimaraesiella* may be necessary. The difference in prevalence of *Sodalis* between *Columbicola* (83 %; Smith et al., 2013) and *Guimaraesiella* (31 %) may also indicate that the louse-symbiont relationships work differently in different groups of lice. It should be noted that even more extreme differences in prevalence have been reported from tsetse flies: 93.7 % in *Glossina brevipalpis,* 17.5 % in *Glossina morsitans* and 1.4 % in *Glossina pallidipes* (Dennis et al., 2014).

The differences between the *Columbicola*/symbiont system and the *Guimaraesiella*/symbiont system highlights how little is known about the differences in ecology between different groups of lice. These two systems almost represent opposites with regards to their coevolutionary history, despite both being similar in *e.g.,* global range and their ability for phoretic dispersal. Based on current knowledge, it would appear that characters of a louse/symbiont system cannot necessarily be predicted based on ecological traits of the lice, but more systems need to be examined before larger patterns can be found. In particular, lice that are incapable of phoresy or that are associated with *e.g.*, water-living birds would provide good contrasts to the two systems studied to date.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ympev.2023.107957.

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