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Flyway homogenisation or differentiation? Insights from the phylogeny of the sandpiper (Charadriiformes: Scolopacidae: Calidrinae) wing louse genus *Lunaceps* (Phthiraptera: Ischnocera) ☆

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ABSTRACT

The wing louse genus *Lunaceps*, is the most speciose chewing louse (Phthiraptera) genus inhabiting sandpipers (Charadriiformes: Calidrinae) and is known from almost all sandpiper species. The hosts follow specific flyways from the Arctic breeding grounds to wintering locations in the southern hemisphere, and often form large mixed-species flocks during migration and wintering. We estimated a phylogeny of *Lunaceps* based on three mitochondrial loci, supporting monophyly of the genus but revealing extensive paraphyly at the species level. We also evaluated the relative importance of flyway differentiation (same host species having different lice along different flyways) and flyway homogenisation (different host species having the same lice along the same flyway). We found that while the lice of smaller sandpipers and stints show some evidence of flyway homogenisation, those of larger sandpipers do not. No investigated host species migrating along more than one flyway showed any evidence of flyway differentiation. The host–parasite associations within *Lunaceps* are in no case monophyletic, rejecting strict cospeciation.

1. Introduction

The Scolopacidae (sandpipers, snipes, curlews and allies; Charadriiformes) are hosts to a diverse louse fauna with several small, but morphologically distinct, genera that are typically found parasitising several host genera (Price et al., 2003). However, there is little correspondence between louse distribution and host phylogeny, and none of these louse genera are limited to a monophyletic group of hosts (e.g., Thomas et al., 2004a; Gibson, 2010). Phylogenetic relationships among the Scolopaci (Aves: Charadriiformes): implications for the study of behavioural evolution (M.Sc. Thesis, Department of Ecology and Evolutionary Biology, University of Toronto; (hereafter: Gibson, 2010)). A good example of this is *Lunaceps*, the most speciose and most widely spread (31 hosts in 12 genera; Price et al., 2003) of the Scolopacidae-specific louse genera. This genus preferentially occurs on the flight feathers and is characterised by certain head and genital characters (Clay and Meinertzhagen, 1939) as well as the long and slender body typical of wing lice (Clay, 1949). The 15 species (Price et al., 2003) have variously been lumped together in a few taxa with wide host distributions (e.g., Waterston, 1915; Emerson, 1972) or divided into several more host-specific species (e.g., Timmermann, 1954; Price et al., 2003). They mainly parasitise the sandpipers and stints (Calidrinae), but can also be found on all species of curlews (*Numenius*) and godwits (*Limosa*). Here we focus on those species living on the Calidrinae.

The Calidrinae hosts of *Lunaceps* form a monophyletic group within the Scolopacidae. However, until recently no comprehensive and stable phylogeny for this group has been available and many genera have been erected based on single aberrant taxa (e.g., *Philomachus*, *Eurynorhynchus*). Gibson (2010) constructed the first complete phylogeny of the subfamily and clarified most of the relations within it but no formal revision has been made. The other two host groups of *Lunaceps* (*Limosa* and *Numenius*) are not closely related to the Calidrinae (Thomas et al., 2004a; Gibson, 2010). Close relatives of the Calidrinae that are not hosts to *Lunaceps* include the turnstones (*Arenaria*), the shanks (*Tringa* and allies) and the phalaropes (*Phalaropus*) (Gibson, 2010), all of which are instead hosts to the genus *Quadraceps*, a widely spread louse genus on shorebirds (Price et al., 2003). Both of these genera, as well as several of the other Scolopacidae-specific lice and the shorebird head louse genus *Saemundssonia*, were placed in the subfamily Quadraceptinae by Eichler (1963).

Ischnoceran lice have no free-living stage and normally require that two host individuals come into direct contact to disperse. The traditional view of chewing louse evolution has been that dispersal to new hosts typically occurs either during mating (horizontal

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transmission; Hillgarth, 1996) or in the nest (vertical transmission; Clayton and Tompkins, 1994), leading to cospeciation with their hosts (“Fahrenholz’ rule”; Eichler, 1942; Klassen, 1992). However, over recent years it has become increasingly evident that while such mechanisms could explain the distribution and phylogeny of some groups of lice (Paterson et al., 2000; Page et al., 2004; Hughes et al., 2007), other distribution patterns can only be explained by more complex sets of mechanisms (Johnson et al., 2002a,b; Weckstein, 2004). In many cases, host ecology and behaviour have been invoked, such as shared nest holes (Johnson et al., 2002a; Weckstein, 2004), mass feeding aggregations (Brooke and Nakamura, 1998), dust baths (Hoyle, 1938; Clay, 1949) and shared nesting islands (Banks et al., 2006). Scenarios such as these may provide the most important circumstances facilitating lateral dispersal in cases where a group of lice parasitises more than one host order (Johnson et al., 2011).

Most sandpipers follow population-specific flyways (Fig. 1) from the breeding grounds in the Arctic or Subarctic to wintering grounds in the tropics (e.g., Wilson and Barter, 1998; Message and Taylor, 2005; Tjørve and Tjørve, 2007; Lopes et al., 2008), and often form large mixed-species flocks on stop-over and wintering sites, in contrast to the often low densities on breeding grounds. The structuring of the host populations by different flyways, and their multiple-species flock during migration and wintering could have two important consequences for the louse populations. First, a low migration rate from one flyway to another could lead to the louse populations along each flyway being effectively isolated from each other, resulting in local speciation on the same host species, a situation we here term “flyway differentiation”. Secondly, if communal roosts and the tendency to form large flocks during migration provide sufficient opportunity for lateral transmission to new host species, this could lead to the louse populations being structured geographically, rather than mirroring the host phylogeny. Different host species following the same flyway and using the same stop-over and wintering grounds could come to have the same species of lice, regardless of the phylogenetic relationships of the host species involved. This pattern is here termed “flyway homogenisation” and is essentially the opposite of “Fahrenholz’ rule” in that the phylogenetic patterns of the hosts have little or no influence on the phylogenetic patterns of their lice, with the latter dictated instead by the biogeography of the hosts.

Over time, both processes may influence the louse populations, leading to a situation where a host species along one flyway have the same lice as all other potential host species along that flyway,

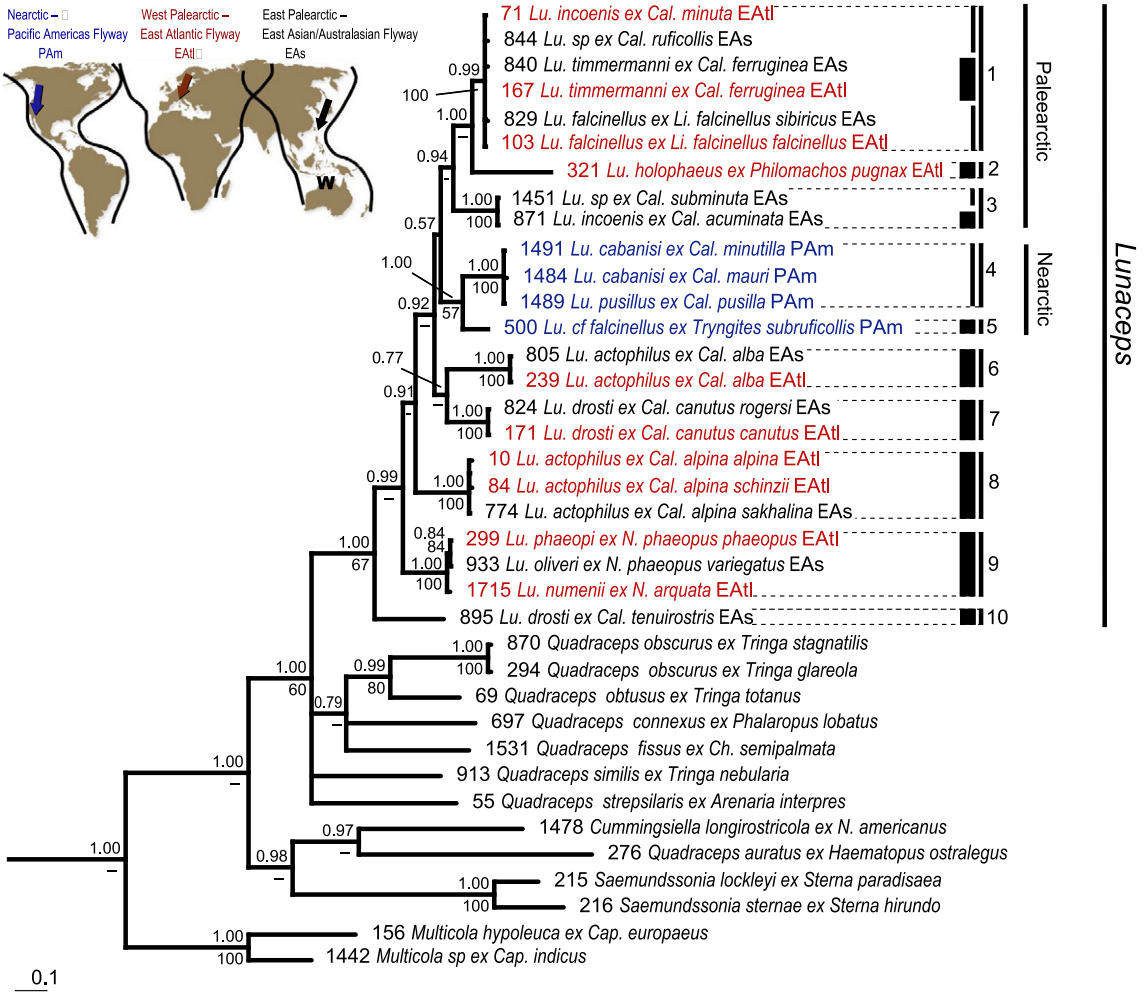

Fig. 1. Majority rule (50%) consensus tree of Quadraceptinae sensu Eichler, 1963. This clade was pruned from a larger phylogeny based on mitochondrial CO1, 12S and 16S sequences, inferred by Bayesian inference under the GTR+G+I model. Posterior probabilities (⩾50%) are indicated above the nodes and parsimony bootstrap values (⩾50%) below the nodes. Numbered bars delimit clades discussed in Sections 3 and 4. The specific identity of the host is given directly after the name of each individual louse sample. Numbers before names are sample identifiers (see Table 1). The approximate size of the host is denoted by the narrow (“small sandpipers” and stints, generally smaller than 180 mm) and broad (“large sandpipers”, generally larger than 180 mm) bars (measurements from Message and Taylor, 2005). Abbreviations after taxon names correspond to flyway affiliation (PAm = Pacific American flyway; EAtl = East Atlantic flyway; EAs = East Asian/Australasian flyway), as outlined in the inset, where arrows denote approximate collection localities for migrating birds, and “W” approximate collection localities for wintering birds.

but may be parasitised by a different louse species in a different flyway. In this paper, we use the louse genus *Lunaceps* as a model to test the relative importance of flyway differentiation compared with flyway homogenisation, and present a phylogeny based on three molecular markers (CO1, 12S, 16S) from lice collected from a wide variety of shorebirds from three different flyways.

2. Materials and methods

2.1. Collection of lice

To test whether or not host biogeography has influenced the distribution and speciation patterns in *Lunaceps*, material was collected from birds following one West Palearctic flyway (the East Atlantic), one East Palearctic flyway (the East Asian/Australasian), and one Nearctic flyway (the Pacific American flyway), during migration or wintering (Table 1, Fig. 1). As the sandpipers are both the most species-rich and the easiest to catch of the hosts of *Lunaceps*, collection was focused on this group. Six host species were sampled from more than one flyway (in all cases both the East Atlantic and East Asian/Australasian flyways), nine *Lunaceps* hosts were collected from the East Atlantic flyway, nine from the East Asian/Australasian flyway, and three from the Pacific American flyway. Four host species (*Calidris alpina, Calidris canutus, Limicola falcinellus, Numenius phaeopus*) use more than one flyway, and each flyway population is considered diverse enough to be treated as a subspecies (Dickinson, 2003; Message and Taylor, 2005; see Table 1). To test the monophyly of *Lunaceps*, we included members of other genera placed by Eichler (1963) in Quadraceptinae, where *Lunaceps* was also placed, as well as some samples of *Carduiceps*, which is also specific to more or less the same host genera as *Lunaceps*.

Fresh material was collected in Sweden during 2007–2008, in Japan and Australia during 2008, and in Canada during 2009. Additional material was obtained from Finland, Belarus and Romania in 2009 and 2010. Birds were captured by mist-netting, Ottenby-style walk-in traps, wilster-nets or cannon nets, during normal banding activities. Captured birds were banded, fumigated with ethyl acetate for 15–20 min in glass jars and then released. This procedure is known to produce a lower yield than some other methods (Vas and Fuisz, 2010), but was a necessary compromise as louse collection was done in connection with bird banding and handling time had to be minimised. At all times the heads of the birds were outside the fumigation chambers and fumigation was stopped immediately if the birds showed signs of drowsiness. In Sweden and Japan, collections were made indoors or in sheltered spots and ruffling could be performed after fumigation, but this was not possible in Australia and Canada, where collection was made directly on the beach. Ruffling was not standardised, and only wings, stomach and back were ruffled. Lice were collected from the bottom of the fumigation chamber and stored in 95% ethanol at ambient temperature in the field, but -20° in the laboratory.

Lice were assigned to species initially based on the host they were collected from, but the material was later compared with specimens in the collections of the Natural History Museum (London, UK), the Museum of New Zealand Te Papa Tongarewa (Wellington, New Zealand), the Essig Museum of Entomology (Berkeley, USA), the Price Institute of Phthirapteran Research (Salt Lake City, USA) and the Oklahoma State University Museum (Stillwater, USA).

2.2. Extraction and sequencing

Prior to DNA extraction, the head and prothorax were cut off from the posterior part of the body, and both parts were extracted thoroughly using a DNeasy Blood and Tissue Kit (Qiagen, Sweden), following the manufacturer's instructions, with the following exceptions: extraction was allowed to continue in the water bath for 36 h and only one elution (with 100 ml elution fluid) was carried out. Exoskeletons were retrieved after extraction and mounted on slides in Canada balsam, forming vouchers. These vouchers for European, Australian and Canadian material were deposited at the Gothenburg Museum of Natural History (Gothenburg, Sweden) or the Swedish Museum of Natural History (Stockholm, Sweden), while the Japanese material was deposited at the Yamashina Institute for Ornithology (Chiba, Japan).

Sequencing of COI used the primers L6625 and H7005 (Hafner et al., 1994); 12S was sequenced using the primers 12SAI and 12SBI (Simon et al., 1994); and for 16S the primers 16SAR and 16SBR were used (Simon et al., 1994). PCRs were performed using GE Healthcare's Ready-To-Go beads. PCR protocols followed Yoshizawa and Johnson (2003) for 12S and 16S, and Hafner et al. (1994) for COI. A small sample from each PCR product was visualised on an ethidium bromide or GelRed (Biotium, Sweden) gel, and samples showing satisfactory bands were purified using EZNA Cycle Pure Kit (Omega, Sweden) or Exonuclease I + FastAP (Fermentas Life Sciences, Sweden) following the manufacturers' instructions. Sequencing of purified DNA, using the same primers as during PCR, was performed in both forward and reverse directions at Macrogen Inc., South Korea.

In addition to these mitochondrial markers, a total of four nuclear primer sets were examined: EF1-For3 and EF1-Cho10 (Danforth and Ji, 1998), LWRhF and LWRhR (Mardulyn and Cameron, 1999), F6999, F7081 and R7495 (Yoshizawa, 2004), and LepWG1 and LepWG2a (Brower and DeSalle, 1998). None of these primer sets produced any products visible on ethidium bromide gel. PCRs using nuclear primer sets were performed in standard, touch-down (Don et al., 1991) and touch-up (Meusnier et al., 2008) mode for all primer sets, with no results.

2.3. Analysis

Sequences were assembled in SeqMan II (DNAStar, Inc., USA), aligned in MegAlign (DNAStar, Inc.) individually for each locus, and manually inspected and adjusted in Se–Al (http://tree.bio.ed.ac.uk/software/seal/). For the combined data set, one louse individual from each host species was selected and aligned if possible, but for some individuals we did not obtain sequences for all loci. For all host species occurring along different flyways, we included one individual from each flyway, when available.

Uncorrected (p) distances were calculated in PAUP* (Swofford, 2002. PAUP*: Phylogenetic Analysis Using Parsimony (* and Other Methods). Version 4.0b10. Sinauer Associates, Sunderland, MA; (hereafter: Swofford, 2002)) for the COI data set separately in order to compare with previous studies.

Boostrap analysis was carried out separately for each locus and on the combined data set in PAUP* (Swofford, 2002) with default settings except that branches were collapsed if the minimum length was zero ("amb-"). A heuristic search under the Maximum Parsimony criterion continued for 1,000 replicates, with starting trees obtained by stepwise addition (random addition sequence, 10 replicates) and tree bisection and reconnection (TBR) branch swapping.

The choice of model for the partitions in Bayesian Inference (BI) was determined based on the Akaike Information Criterion (Akaike, 1973) calculated in MrModeltest 2 (Nylander, 2004. MrModeltest v2. Evolutionary Biology Centre, Uppsala University.). In COI first, second and third position were modelled separately. For all loci posterior probabilities (PP) were calculated under the general time-reversible (GTR) model (Lanave et al., 1984; Tavaré, 1986; Rodríguez et al., 1990), assuming rate variation across sites

Table 1
Taxa used in this study.

Taxon information					Genbank Accession Nos.		
Species	Host	Flyway (location)	Sample	Voucher	COI	12S	16S
Ingroup							
Lu. actophilus	*Cal. alba*	EAtl (S)	239	NRM	JN900111	–	JN900184
		EAs (A)	805	NRM	JN900130	–	JN900192
			807a-1	GNM	JN900129	–	–
	Cal. a. alpina	EAtl (S)	10a-1	NRM	JN900083	JN900198	JN900160
			10a-2	GNM	JN900084	JN900199	JN900161
	Cal. a. sakhalina	EAs (J)	774	YIO	JN900133	–	JN900194
			782	YIO	JN900132	–	JN900193
	Cal. a. schinzii	EAtl (S)	84-1	NRM	JN900093	JN900208	JN900169
			84-2	GNM	JN900092	JN900207	JN900168
		EAtl (F)	1640-1	NRM	JN900146	–	–
			1640-2	GNM	JN900147	–	–
Lu. cabanisi	*Cal. mauri*	PAm (C)	1484	NRM	JN900148	–	–
			1487	NRM	JN900149	–	–
			1586	GNM	JN900150	–	–
	Cal. minutilla	PAm (C)	1491	NRM	JN900151	–	–
			1494	NRM	JN900152	–	–
			1545	GNM	JN900154	–	–
			1548	GNM	JN900153	–	–
Lu. drosti	*Cal. c. canutus*	EAtl (S)	171-1	NRM	JN900107	JN900221	JN900180
			177-1	NRM	JN900106	JN900220	JN900179
			220	GNM	JN900112	JN900225	–
	Cal. c. rogersi	EAs (A)	823	NRM	JN900128	–	–
			824b-1	GNM	JN900127	JN900231	–
	Cal. tenuirostris	EAs (A)	895	NRM	JN900142	JN900235	–
			900	GNM	JN900141	JN900234	–
Lu. falcinellus	*Li. f. falcinellus*	EAtl (S)	103-1	NRM	JN900091	JN900206	JN900167
			103-2	GNM	JN900090	JN900205	JN900166
			106-1	NRM	JN900102	JN900216	JN900176
			106-2	GNM	JN900101	JN900215	JN900175
			134-1	NRM	JN900100	JN900214	JN900174
			134-2	GNM	JN900099	–	JN900173
	Li. f. sibirica	EAs (A)	829	NRM	JN900126	–	–
			830	GNM	JN900125	–	–
	Tryngites subruficollis	PAm (S)	500	NRM	JN900115	–	JN900187
Lu. holophaeus	*Philomachus pugnax*	EAtl (S)	321c-1	NRM	JN900118	–	–
Lu. incoenis	*Cal. minuta*	EAtl (S)	71-1	NRM	JN900086	JN900201	JN900162
			71-2	GNM	JN900085	JN900200	–
			77-1	NRM	JN900095	JN900210	JN900170
			77-2	GNM	JN900094	JN900209	–
	Cal. acuminata	EAs (J)	784a-1	YIO	JN900131	–	–
		EAs (A)	871	NRM	JN900143	JN900233	–
Lu. numenii	*N. arquata*	EAtl (R)	1715	NRM	JN900145	–	–
Lu. oliveri	*N. ph. variegatus*	EAs (A)	933a-1	NRM	JN900130	–	–
Lu. phaeopi	*N. ph. phaeopus*	EAtl (S)	299	NRM	JN900119	–	JN900190
Lu. pusillus	Cal. pusilla	PAm (C)	1489	NRM	JN900155	–	–
			1499	GNM	JN900156	–	–
			1544	GNM	JN900157	–	–
Lu. timmermanni	*Cal. ferruginea*	EAtl (S)	167	NRM	JN900097	JN900212	JN900172
			168a-2	GNM	JN900096	JN900211	JN900171
			196-1	NRM	JN900105	JN900219	JN900178
			196-2	GNM	JN900104	JN900218	–
			208-1	NRM	JN900103	JN900217	JN900177
			250a-1	GNM	JN900110	JN900224	JN900183
		EAs (A)	840	NRM	JN900124	–	–
			842	GNM	JN900123	–	–
Lunaceps sp. 1	*Cal. subminuta*	EAs (A)	1451a-1	NRM	JN900137	–	–
			1451a-2	GNM	JN900136	–	JN900196
Lunaceps sp. 2	*Cal. ruficollis*	EAs (A)	844	NRM	JN900122	–	
Other Rallicolidae sensu Eichler (1963)							
Cu. longirostricola	*N. americanusu*	– (U)	1478	NRM	JN900159	–	–
M. hypoleucus	*Cap. europaeus*	– (S)	156	NRM	JN900098	JN900213	–
M. sp.	*Cap. indicus*	– (J)	1442	YIO	JN900138	JN900232	–
Q. auratus	*H. ostralegus*	– (S)	276	NRM	JN900109	JN900223	JN900182
Q. connexus	*Phalaropus lobatus*	– (J)	697	YIO	JN900134	–	–
Q. fissus	*Ch. semipalmata*	– (C)	1531	NRM	JN900158	JN900236	JN900197
Q. obscurus	*Tringa glareola*	– (S)	294	NRM	JN900120	JN900230	–
	Tringa stagnatilis	– (A)	870	NRM	JN900144	–	–
Q. obtusus	*Tringa totanus*	– (S)	69-1	NRM	JN900087	JN900202	JN900163
Q. similis	*Tringa nebularia*	– (A)	913	NRM	JN900140	–	–
Q. strepsilaris	*Arenaria interpres*	– (S)	55a-1	NRM	JN900088	JN900203	JN900164
S. lockleyi	*Sterna paradisaea*	– (S)	215a-1	NRM	JN900114	JN900227	JN900186

Table 1 (*continued*)

Taxon information					Genbank Accession Nos.		
Species	Host	Flyway (location)	Sample	Voucher	COI	12S	16S
S. sternae	*Sterna hirundo*	– (S)	216b-1	NRM	JN900113	JN900226	JN900185
Outgroup							
Ai. rheinwaldi	*Branta bernicla*	– (S)	464	NRM	JN900116	JN900228	JN900188
Ae. sp.	*Branta bernicla*	– (S)	462	NRM	JN900117	JN900229	JN900189
Car. meinertzhageni	*Cal. a. alpina*	EAtl (S)	19b-1	NRM	JN900089	JN900204	JN900165
Car. scalaris	*Philomachus pugnax*	EAtl (S)	515-1	NRM	JN900135	–	JN900195
Car. zonarius	*Cal. canutus*	EAtl (S)	287a-1	NRM	JN900121	–	JN900191
	Cal. ferruginea	EAtl (S)	170	NRM	JN900108	JN900222	JN900181

Flyway abbreviations: EAs = East Asian/Australasian; EAtl = East Atlantic; PAm = Pacific Americas. Species not following these flyways have been denoted with a "–". Genus abbreviations used in louse names are: *Ae.* = *Anatoecus*; *Ai.* = *Anaticola*; *Car.* = *Carduiceps*; *Cu.* = *Cummingsiella*; *Lu.* = *Lunaceps*; *M* = *Mulcticola*; *Q.* = *Quadraceps*; *S.* = *Saemundssonia*. Genus abbreviations used in host names are: *Cal.* = *Calidris*; *Cap.* = *Caprimulgus*; *Ch.* = *Charadrius*; *H.* = *Haematopus*; *Li.* = *Limicola*; *N.* = *Numenius*.
Locality abbreviations: A = Australia; C = Canada; F = Finland; J = Japan; R = Romania; S = Sweden; U = United States.
Vouchers for European, Australian and Canadian material were deposited at the Gothenburg Museum of Natural History (GNM; Gothenburg, Sweden) or the Swedish Natural History Museum (NRM; Stockholm, Sweden), while the Japanese material was deposited at the Yamashina Institute for Ornithology (YIO; Chiba, Japan). Voucher numbers for slides are the same as sample numbers.
Missing data is denoted with a "–". Sample identifiers correspond to the same numbers in the figures.

according to an inverse gamma distribution with six rate categories for all models except COI third positions, in which a discrete gamma distribution with six rate categories was assumed (Γ; Yang, 1994).

Gene trees were estimated by BI using MrBayes 3.1.2 (Huelsenbeck and Ronquist, 2001, 2005) according to the following: (i) all loci were analysed separately (single-locus analyses, SLAs); (ii) sequences were concatenated – all loci together (multi-locus analysis). In the multilocus analysis, the data were partitioned by locus, using rate multipliers to allow different rates for the different partitions (Ronquist and Huelsenbeck, 2003; Nylander, 2004). Four Metropolis-coupled Markov chain Monte Carlo (MCMC) chains were run with incremental heating temperature 0.1 for 10×10^6 generations and sampled every 1,000 generations. The first 10% of the generations were discarded as "burn-in", well after stationarity of chain likelihood values had been established, and the posterior probability was estimated for the remaining generations.

To test whether flyway biogeography of the lice contains significant phylogenetic signal, *s* of Maddison and Slatkin (1991) was calculated from three different population tree models in Mesquite (Maddison and Maddison, 2010. Mesqite: a modular system for evolutionary analysis. Version 2.74. http://mesquiteproject.org). The three population tree models were: (1) lice were ordered entirely according to flyways but these flyways were unordered; (2) lice were ordered entirely according to flyways but the two Palaearctic flyways (East Atlantic and East Asian/Australasian) were placed as more closely related to each other than to the Pacific American flyway; (3) lice were ordered according to host phylogeny, which was obtained by pruning the tree of Gibson (2010) to remove all host species not sampled here. In models 1 and 2, flyway association was determined by where the individual louse in question was collected, even if the host species migrates along multiple flyways. In each case 1,000 gene trees were simulated, *s* of Slatkin and Maddison was calculated for each gene tree and compared with the observed value calculated by Mesquite (Maddison and Maddison, 2010). Effective population size was varied between 100, 10,000, and 1,000,000. Flyway biogeography and host affinities were assigned as in Table 1 and only the combined data set was used. Outgroup species were not included in the analysis.

We also calculated the genealogical sorting index (GSI_T; Cummings et al., 2008) of a pruned version of the combined tree, where all non-*Lunaceps* taxa were excised. Permutations (10,000) were performed on http://www.genealogicalsorting.org/ (Cummings et al., 2008) for the 52 most parsimonious trees obtained from PAUP* (Swofford, 2002) under the maximum parsimony criterion after a heuristic search of 1,000 random replicates. Each terminal taxon was assigned to a group corresponding to the three flyways (East Atlantic, East Asian/Australasian and Pacific American).

3. Results

Including the outgroup taxa, the aligned COI data set comprised 379 characters, of which 200 (52.8%) were parsimony informative; 12S comprised 393 characters, of which 295 (75.1%) were parsimony informative; and 16S comprised 526 characters, of which 276 (52.5%) were parsimony informative. Including outgroups, the combined data set comprised 1,304 characters of which 789 (60.5%) were parsimony informative. Models for all data partitions were GTR+I+G, except for third codon positions of COI, which was GTR+G.

Overall, all three single-locus datasets, as well as the combined set of concatenated sequences, gave good support for the terminal *Lunaceps* clades, although support for internal nodes was typically low (Figs. 1 and 2 and Supplementary Figs. S1 and S2). There were no strongly supported conflicts between the different single-locus alignments regarding the phylogeny of the genus *Lunaceps*. *Lunaceps* itself is monophyletic with good support (PP = 1.00) in all data sets except the COI dataset (PP = 0.77, bootstrap support (BS) <50; Fig. 2). Monophyly of Quadraceptinae (sensu Eichler, 1963) is supported in all analyses except the parsimony bootstrap of the combined data set (PP = 1.00, bootstrap support <50; Fig. 1).

Clade 1 (Figs. 1 and 2) contained material collected from four species of shorebirds of different size preferring marine habitats. There was very little genetic differentiation among these lice, even in the two cases where hosts were collected along different flyways (*Calidris ferruginea* and *Li. falcinellus* along both the East Atlantic and the East Asian/Australasian flyways). According to current taxonomy, at least three species of *Lunaceps* are part of this clade, challenging the validity of these species. Clade 2 is represented by material from a single host species collected along the East Atlantic flyway. Apart from curlews and godwits, this host species is the largest host of any *Lunaceps* sp. (Message and Taylor, 2005). *Philomachus pugnax* also occurs along the East Asian/Australasian flyway but we have no material from there. Clade 3 contained lice collected from *Calidris acuminata* and *Calidris subminuta*, from the East Asian/Australasian flyways. There was very little differentiation among these lice and all but one shared the same CO1 haplotype (Fig. 2, Table 2), but the hosts are of different size and, as far as known, not closely related (Gibson, 2010). Both of these hosts are restricted to the East Asian/Australasian flyway and tend to occur together in freshwater habitats on migration. Clade 4 contained

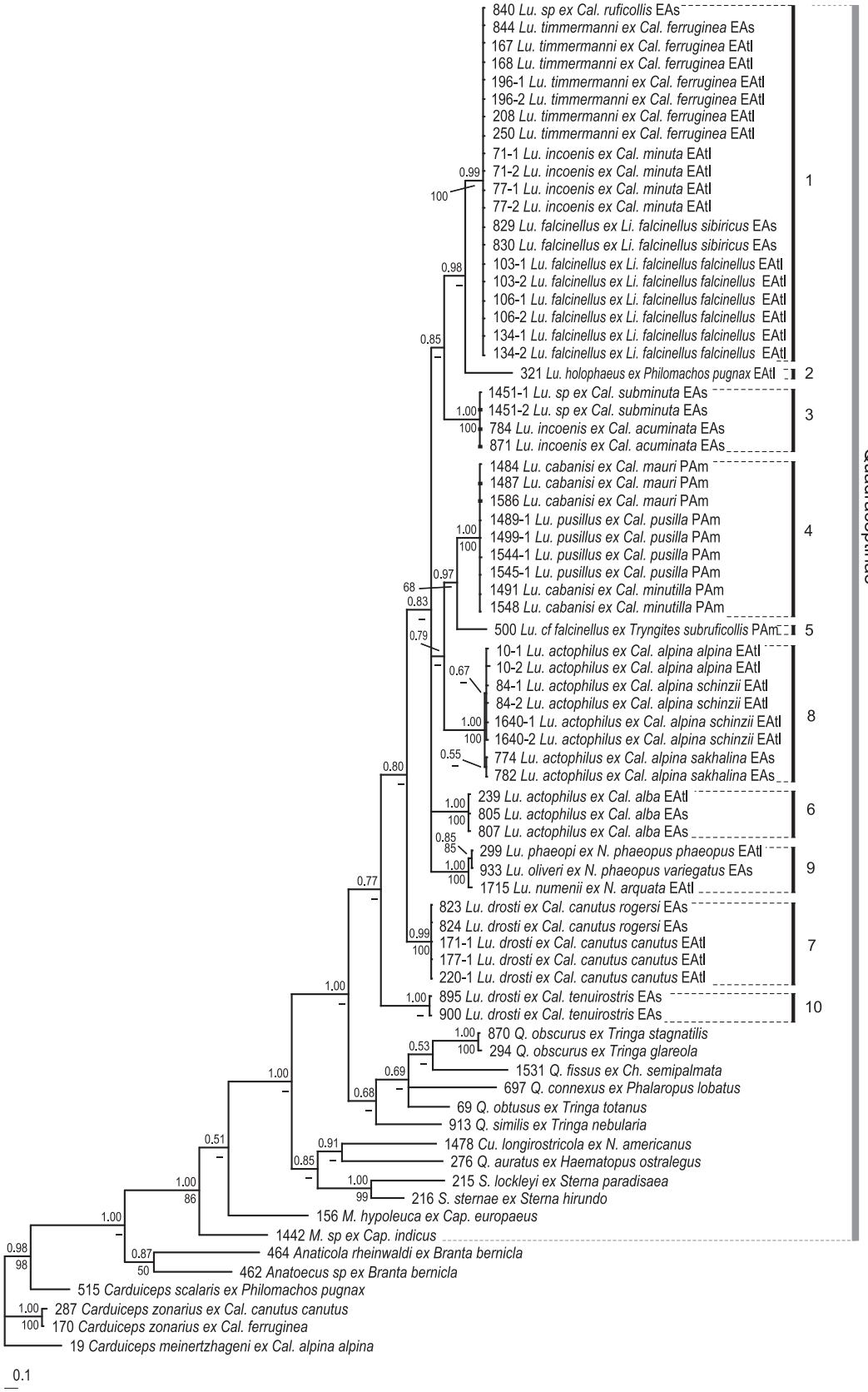

Fig. 2. Majority rule (50%) consensus tree of *Lunaceps* based on mitochondrial COI sequences, inferred by Bayesian inference under the GTR+G+I model. Posterior probabilities (⩾50%) are indicated above the nodes and parsimony bootstrap values (⩾50%) below the nodes. Bars delimit the same clades as in Fig. 1. Abbreviations used for louse genera: *Cu.* = *Cummingsiella*; *Lu.* = *Lunaceps*; *M.* = *Mulcticola*; *Q.* = *Quadraceps*; *S.* = *Saemundssonia*. Abbreviations used in host genera: *Cal.* = *Calidris*; *Cap.* = *Caprimulgus*; *Ch.* = *Charadrius*; *Li.* = *Limicola*; *N.* = *Numenius*. Numbers before names are sample identifiers (see Table 1). Flyway abbreviations at the end of terminals are: PAm = Pacific American; EAtl = East Atlantic; EAs = East Asian/Australasian.

Table 2
Uncorrected (p) distances for the COI data set. Clade numbers as in Fig. 1. All numbers expressed in percentages, with dashes representing one-taxon clades within which no distances can be calculated. Only distances between *Lunaceps* spp. are included.

Clade number	1	2	3	4	5	6	7	8	9	10
1	0.0									
2	12.9	–								
3	14.3	16.7	0.1							
4	15.6	16.4	17.2	0.1						
5	14.8	15.8	16.9	11.1	–					
6	15.9	17.2	16.3	14.0	15.6	0.0				
7	18.1	19.4	16.2	16.0	17.3	15.9	0.2			
8	16.7	19.9	17.7	14.6	16.4	15.0	16.6	0.6		
9	15.1	15.1	17.0	17.5	17.4	17.1	17.5	15.6	0.5	
10	18.5	20.1	19.1	20.4	20.6	18.3	19.2	20.3	19.8	0.3

Lunaceps from three sandpipers of similar size (Message and Taylor, 2005) following the Pacific American flyway and was sister to clade 5. Together, these two latter clades formed an exclusively Nearctic clade of *Lunaceps* in the BI analysis but not in the bootstrap parsimony analysis (PP = 1.00, BS = 57%; Fig. 1).

Clades 6, 7, 8 and 10 each consisted of material collected from a single host species represented from more than one flyway, with the exception of the host of clade 10, which is restricted to the East Asian/Australasian flyway. The host species of clades 7 and 8 are taxonomically divided at the subspecies level. In none of these cases was there any apparent genetic divergence between the louse material collected from hosts with populations separated into different flyways. However, both *Lunaceps actophilus* and *Lunaceps drosti* are shown to be non-monophyletic. Clade 9 contained all of the material collected from curlews (Figs. 1 and 2). There was a slight but statistically unsupported tendency to differentiation between the lice from different species, but none between the two allegedly different species of lice from the two subspecies of *N. phaeopus* from different flyways. Clade 10 was the sister group of all other *Lunaceps* in the combined and COI data sets, but this position is supported only in the combined data set and even then only by BI (PP = 0.99; BS <50; Fig. 1). Supplementary Figs. S1 and S2 are congruent with Figs. 1 and 2 but are less complete, as amplification failed for the 12S and 16S loci in some cases.

In the ingroup of the COI data set, within-clade uncorrected (p) distances are between 0.0% and 0.6%, and between-clade uncorrected (p) distances between 11.1% and 20.6% (Table 2). Uncorrected (p) distances between clades 6 and 8 (Figs. 1 and 2), both nominally *Lu. actophilus*, were 15.0% and uncorrected (p) distances between the two nominal *Lu. drosti* clades (7 and 10, Figs. 1 and 2) were 19.2% (Table 2).

The observed value of Slatkin and Maddison's *s* was 12 steps, which is inside the 95% confidence interval of both model 1 (unordered flyways; 8–13 steps) and model 2 (ordered flyways; 8–13 steps), meaning neither model can be rejected. It was well outside the 95% confidence interval of model 3 (host phylogeny; 23–24 steps), and this model was therefore rejected. Varying effective population size had no effect on these results.

GSI_T for the Pacific American material (clades 4–5; Figs. 1 and 2) was 1, rejecting the null hypothesis of random distribution of group assignments ($P = 0.0003$). For both of the other flyway groups, the null hypothesis could not be rejected ($GSI_T = 0.1619433$; $P = 0.2585$ for the East Atlantic flyway; $GSI_T = 0.1503087$; $P = 0.2686$ for the East Asian/Australasian flyway).

4. Discussion

As detailed below, our results indicate several cases where phylogenetic information is at odds with current taxonomy. All of these groupings and splits received high support (Figs. 1 and 2), and in all clades, internal genetic variation was small (Table 2), whereas between-clade variation was high (Table 2). This pattern agrees with the proposed "barcoding gap" (Hebert et al., 2004), is similar to that of previous studies (Table 3) and suggests that the clades of *Lunaceps* included in our analyses are well separated. Together, the tree topologies and the distances between clades imply that current taxonomy of *Lunaceps* needs to be changed. Given that many of the species of *Lunaceps* were based mainly or only on a handful of measurements, host relationships or both (e.g., Timmermann, 1954), with no regard for morphology, the blurring of nominal species names (as in clade 1, Fig. 1) is expected, and an upcoming revision (Gustafsson and Olsson, unpublished data) will clarify the taxonomy of *Lunaceps*.

Flyway differentiation would occur if, in two populations of the same host species following different flyways during migration, the louse populations were different, resulting from isolation between the two louse populations for sufficient time to allow speciation to occur. In our data, there was no evidence of flyway differentiation in any *Lunaceps* species, except possibly for the louse collected from *Calidris alpina alpina, Caidris alpina schinzii* and *Calidris alpina sakhalina*, for which the CO1 haplotypes appear to cluster into different groups corresponding to a separation between the East and West Palearctic louse populations. However, as the clades formed by these haplotype groups are unsupported in our analyses (Fig. 2), no conclusions may be drawn from it except that they may be worthwhile to study further by different methods, e.g., microsatellites. This would not in any way be an example of flyway differentiation as the hosts are differentiated into different subspecies.

The Maddison and Slatkin (1991) test could not reject the hypothesis that *Lunaceps* are ordered along host flyways, but the genealogical sorting test (Cummings et al., 2008) could not reject the null hypothesis that flyway assignment for the East Atlantic and the East Asian/Australasian flyways were randomly distributed, with only the Pacific American flyway material being significantly ordered.

Several shorebird species are hypothesised to have gained their present large geographic distribution only between or after the last ice ages (Wenink et al., 1996; Kraaijeveld and Nieboer, 2000; Buehler et al., 2006; Rönkä et al., 2008), implying that the division of migratory behaviour among some species of Calidrinae into different flyways is too recent for speciation to have occurred in the lice of the respective flyways.

Flyway homogenisation would occur if several or all host populations following a flyway had the same flyway-specific species of louse, with no limits to gene flow between the lice of individual host species. In our data, *Lunaceps* from larger sandpipers (Clades 2, 5–8 and 10; Fig. 1, broad bars; larger sandpipers here arbitrarily defined as species generally larger than 180 mm, data from Message and Taylor, 2005) showed no evidence of flyway homogenisation and appear to be host-specific. In contrast, clades 1, 3 and 4, which include the lice of the smaller sandpipers and stints (Fig. 1, narrow bars; smaller sandpipers here arbitrarily defined as species generally smaller than 180 mm, data from Message and Taylor, 2005), all contain *Lunaceps* from more than one host species. According to current taxonomy, the lice of these clades belong to different species but in our view, clades 1, 3 and 4 are each made up of a single species of louse, as evidenced by the lack of genetic differentiation. In clade 3 (Figs. 1 and 2) two host species from the same flyway are parasitised, and in clade 4 (Figs. 1 and 2) three host species from the same flyway are parasitised. This may be interpreted as an indication of flyway homogenisation but in clade 1 (Figs. 1 and 2) in which four host species are parasitised, this homogenisation also extends to other flyways for two host species. One explanation for this may be that for some species of

Table 3
Selection of published genetic distances for other groups of lice. Also included are data from the present study. Only mitochondrial data has been included.

Louse group	Gene	Within-group	Between-group	References
Goniodidae	COI	<1%	8.8–17.2%	Johnson et al. (2001a)
Penenirmus	COI	"Identical"	7.6–28.7%	Johnson et al. (2001b)
Brueelia	COI	<1%	9.7–23.1%	Johnson et al. (2002a)
Columbicola	COI	–	3.1–29.8%	Johnson et al. (2003b)
Austrophilopterus	COI	0.0–3.9%	9.5–18.7%	Weckstein (2004)
Columbicola	COI	<1%	Up to 27%	Johnson et al. (2007)
Columbicola	12S	–	Up to 26%	Johnson et al. (2007)
Lunaceps	COI	0.0–0.6%	11.1–20.6%	This study

lice dispersal is easy both between species and between flyways, or that it is a case of ancient flyway homogenisation having its origin before the current flyways took shape. Clade 1 (Fig. 1) is most widely spread, occurring on four sampled host species along two flyways and with no internal genetic variation (Tables 2 and 3). The breeding and wintering distribution of the hosts of this clade are more or less the whole Palaearctic and the Palaeotropic, respectively. This distribution thus partially overlaps with that of clade 3 (Fig. 1), the hosts of which breed in the Arctic and Central Siberia, and winter in Australasia. Both clade 3 hosts were caught in freshwater locations and tend to favour these locations over estuaries, ocean shores and other saltwater stopover and wintering grounds (Paulson, 1993; Piersma, 2003; Message and Taylor, 2005). All other smaller sandpipers and stints were caught in saltwater locations, or are known to favour these during migration (Piersma, 2003). Louse distribution may be influenced by this ecological difference between the hosts. Both clades 1 and 3 are internally genetically homogenous regarding genetic divergence among the hosts and differ by 14.3% (uncorrected (p); Table 2). Lastly, clade 4 (Fig. 1) contains all material collected along the Pacific American flyway, from three different host species.

The six louse species of clades 2, 5–8 and 10, which contain the material from the larger host species, are each strictly host-specific in spite of hosts being relatively similar in size. On the other hand, both clades 1 and 3 (Figs. 1 and 2) contain material from host species of very different sizes, with *C. ferruginea* and *C. acuminata* being similar in size to *C. alpina* and *C. alba* (Message and Taylor, 2005), which occur along the same flyways. Thus, some species of lice (clades 1, 3 and 4; Figs. 1 and 2) appear to be capable of successful colonisation of new host species; in some cases also of hosts of different sizes; while others (clades 6–8; Figs. 1 and 2) appear not to be. It is possible that certain morphological traits facilitate colonisation of new host species. For instance, the *Lunaceps* spp. of clades 1–5 (Figs. 1 and 2) are typically more narrow in both head, pterothorax and abdomen but have longer heads than those of clades 6–8 and 10 (Figs. 1 and 2; D. Gustafsson, unpublished data). Harrison's rule (Harrison, 1915; Johnson et al., 2005) predicts that the size of a parasite will be approximately proportional to that of the host, which may have consequences for lateral spread. The size of a wing louse species is positively correlated to host body mass and interbarb space on flight feathers (Johnson et al., 2005), and barb diameter may also be a factor in the survivability of a louse on a novel host (Tompkins et al., 1999). Bush and Clayton (2006) demonstrated that an artificial transfer of lice to birds of much smaller or much larger body size has a clear impact on their fitness but that transfer to hosts of similar size had little or no effect. Size differences may also be prohibitive to the lateral spread of *Lunaceps* spp. to novel hosts and could be tested with a larger sample of *Lunaceps* from hosts with known feather characteristics. In the present study, data on interbarb space, barb diameter and other features of the feathers were not collected. However, if *Lunaceps* lice were prevented from lateral spread by size-dependent host characteristics alone, they would be expected to be able to spread to hosts of similar size, as is possible in doves (Bush and Clayton, 2006). That this appears not to be the case may imply that host size may not be the only factor that structure host-relationships in *Lunaceps*. Instead, other differences such as behaviour or ecology may be important.

None of the host groups of clades 1, 3 and 4 (Fig. 1) formed monophyletic clades to the exclusion of other hosts (Borowik and McLennan, 1999; Thomas et al., 2004a; Gibson, 2010). While *Calidris pusilla* and *Calidris mauri* (hosts of clade 4) are sister taxa, *Calidris minutilla* is more closely related to *Calidris minuta*, which is host to clade 1 *Lunaceps*. The other hosts of clade 1 are spread over almost all of the Calidrinae tree (Gibson, 2010), with *Li. falcinellus* (clade 1) being closely related to *C. acuminata* (clade 3), *Calidris ruficollis* (clade 1) being more closely related to *Cal. subminuta* (clade 3) and some taxa not surveyed here, and *C. ferruginea* being without close relatives, but perhaps being distantly allied to *Calidris himantopus*, which is host to highly aberrant *Lunaceps* (D. Gustafsson and U. Olsson, unpublished data).

Therefore, cospeciation is unlikely to have occurred, however as no complete set of host sequences were available to us, this could not be formally tested and cannot be ruled out as flyway homogenisation could be virtually impossible to separate from lack of speciation on closely related host species. In addition, it is possible that more basal co-speciation events have been blurred by a large number of host switches. Further, distribution and migration patterns among the hosts during the interglacial periods (Kraaijeveld and Nieboer, 2000; Buehler et al., 2006), different from current patterns, may also have obscured relationships. In any case, the lack of supported internal nodes for most of *Lunaceps* makes any comparison with the host phylogeny difficult. The Maddison and Slatkin (1991) test rejected the hypothesis that the louse phylogeny followed the host phylogeny but neither all host species, nor all *Lunaceps* species, were included.

Although the common ancestor of the Calidrinae may be as old as late Oligocene (Baker et al., 2007), the Pacific American host species are quite closely related to each other, and seem to have radiated both rather rapidly and comparatively recently (Gibson, 2010), suggesting that the identity of the *Lunaceps* on these hosts (Fig. 1, clade 4) may actually be a case of "failure to speciate". In parasite systematics, this concept implies a lack of divergence between the lice inhabiting a group of hosts undergoing cladogenesis due to continued gene flow between the lice (Johnson et al., 2003a). An argument against "failure to speciate" is the fact that *C. minutilla* and *C. minuta* are inferred to be sisters by Gibson (2010), in spite of being restricted to different flyways. Both of these hosts are parasitised by flyway-specific lice that are not closely related and occur on mutually exclusive sets of several other, more distantly related, host species. Such a pattern makes flyway homogenisation a more likely explanation than "failure to speciate". A scenario that may explain the composition of clade 4 could be that cospeciation followed by "failure to speciate" may have happened in the case of *C. pusilla* and *C. mauri*, with subsequent "flyway homogenisation dispersal" to *C. minutilla*. Alternatively,

the three host species may have been colonised by "flyway homogenisation dispersal" from one of the other calidrine species occurring along the Pacific American flyway, but which were not available for this study.

The one case where co-speciation seems likely is in the *Lunaceps* from curlews (clade 9; Figs. 1 and 2). There was a tendency for the lice of *N. phaeopus* subspecies to group together to the exclusion of that from *N. arquata* (PP = 0.84; BS = 84%; Fig. 1), and while the haplotypes of the two former are identical, they both differ from the latter by 0.7% (data not shown). The two hosts are not closely related (Gibson, 2010), but as no other *Lunaceps* from curlews were available, relationships of *Lunaceps* on non-calidrinae hosts could not be thoroughly investigated.

Notably, the three host groups of *Lunaceps* – calidrine sandpipers, curlews and whimbrels, and godwits – also do not form a monophyletic group (Chen et al., 2003; Paton et al., 2003; Thomas et al., 2004a,b; Baker et al., 2007; Gibson, 2010), but all three groups can often be found together in similar habitats during migrations and wintering. This suggests that, at least historically, transmission of *Lunaceps* between different taxa may have been commonplace. Why *Lunaceps* lice have not colonised other potential hosts that use the same stop-over and wintering grounds – plovers, dowitchers, shanks and others – is unknown, but *Lunaceps* have occasionally been recorded in small numbers from these hosts (e.g., Kellogg and Chapman, 1899; Blagoveshchensky, 1948; Rékási, 1993), suggesting that successful colonisation may be prevented by preoccupancy of the same niches by *Quadraceps* lice.

No evidence was found for flyway differentiation in *Lunaceps* but there is some evidence that flyway homogenisation has occurred in *Lunaceps* on smaller sandpipers and stints. In three cases, representatives of the same clade of lice were found on more than one host species following the same flyway (Figs. 1 and 2; clades 1, 3 and 4), although in one case (Figs. 1 and 2; clade 1) lice were identical across two Palaearctic flyways. In no case is more than one large sandpiper parasitised by the same species of *Lunaceps*, however both clades 1 and 3 (Figs. 1 and 2) parasitise one or several smaller sandpipers in addition to the large host. While genetic divergences are small within clades, they are comparable with between-species differences reported from other groups of lice, suggesting that all clades obtained in this study could be treated as species. A separate paper will review the genus *Lunaceps* on the basis of this phylogeny. The host associations are in no case monophyletic, rejecting strict cospeciation, except perhaps in the case of clade 4 *Lunaceps* on *C. pusilla* and *C. mauri*, and that of the *Lunaceps* on curlews.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ijpara.2011.11.003.

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