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Co-phylogeography and morphological evolution of sika deer lice (*Damalinia sika*) with their hosts (*Cervus nippon*)

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SUMMARY

Lice are obligate parasites of mammals and birds and have become an important model for studies of host–parasite coevolution and cophylogenetics. Population genetic and phylogeographic studies represent an important bridge between microevolution and cophylogenetic patterns. We examine co-phylogeographic patterns in sika deer and their parasitic lice. Co-phylogeographic patterns in deer and lice were evaluated using homologous regions of mitochondrial COI sequences. The phylogeographic breaks recovered for deer populations matched those of previous studies. Comparisons of the phylogeographic tree topology for deer lice with that of their hosts revealed a significant level of congruence. However, comparisons of genetic distances between deer and lice suggested one of the estimated co-divergence events is more likely a recent host switch. Taking into account genetic divergence, there is not strong evidence for complete phylogeographic co-divergence between deer and their parasitic lice. However, mitochondrial phylogenies only track genetic structure of female lineages, and the incongruence between deer and louse phylogeography may be explained by louse migration mediated by male deer. Morphological analysis of head shape variation based on an elliptic Fourier descriptor showed that overall morphological variation contained phylogenetic signal, suggesting that in general morphology of these lice evolves congruent to population history.

Key words: co-phylogeography, population structure, microevolution, morphology, parasitic louse, mammal

INTRODUCTION

Parasitic lice (Insecta: Psocodea: "Phthiraptera") are obligate parasites of mammals and birds and spend their entire lifecycle on the hosts. Because of this permanent association with their hosts, lice have been used as a model system for studies of host–parasite codivergence (Page, 2002). Several studies comparing louse and host phylogenies have provided evidence for codivergence between lice and their hosts (e.g., Hafner *et al.* 1994; Page *et al.* 1998; Johnson *et al.* 2002*b*; Light and Hafner, 2008; Smith *et al.* 2008). In contrast, several cases in which substantial incongruence between host and parasite trees exists have been identified (Johnson *et al.* 2002*a,b*; Weckstein, 2004; Banks *et al.* 2006). All of these studies have focused on species level phylogenies, and there are only a few studies focusing on codivergence within species, among populations (Whiteman *et al.* 2007; ˇStefka *et al.* 2011). Thus, it is still generally unclear whether codivergence at the species level is driven by population level codivergence that precedes speciation. Population genetic and phylogeographic studies will be an important bridge between microevolution and cophylogenetic patterns (Johnson *et al.* 2003*a*).

To understand the causes of congruence and incongruence between host and parasite phylogenies, careful consideration of host and parasite behaviors, effects of other members of the parasite community (e.g. phoresis by hippoboscid flies), and geographical information are needed (Johnson *et al.* 2002*a,b*). As long as these factors are accounted for, parasite phylogeny could also provide insights regarding the host's phylogeny (Hopkins, 1942; Reed *et al.* 2004; Johnson *et al.* 2006; Whiteman *et al.* 2007). In particular, the molecular substitution rate for lice is generally much faster than that of their hosts (Hafner *et al.* 1994; Huelsenbeck *et al.* 1997; Page, 1996; Page *et al.* 1998; Johnson *et al.* 2003*b*), and substitutional differences may accumulate between diverging louse populations at shallow timescales even though their hosts have not yet accumulated any mutational differences. Therefore, population genetic and phylogeographic studies of parasitic lice also have the potential to uncover hidden population structure in their hosts.

In addition to studies of codivergence, lice have also been used as an important system in studying morphological evolution of coevolving systems. The evolution of morphological traits can be affected by many factors, such as phylogenetic constraints, gene flow, genetic drift, sexual selection, and, most importantly, selection pressure from the environment. Generally, it is very difficult to separate these factors clearly. Parasitic lice spend their entire life cycle on the body of the host and thus the environmental context for their morphological evolution can be more easily identified than that for free-living organisms. For this reason, lice are good models for studying the impact of the host on morphological evolution of these parasites. Previous studies have shown interesting coevolutionary patterns between lice and their hosts. For example, Reed *et al.* (2000) clearly showed that the head groove width of gopher chewing lice is strongly correlated with the hair diameter of the host. Mammal lice hang onto the host hair using the head groove, so that a mismatch between groove size and hair diameter likely increases the risk to the louse of falling off or being groomed off the host. Johnson *et al.* (2005) also found that the body width of wing lice from pigeons and doves is strongly correlated with host body size. Host body size dictates the width of the feather interbarb space, in which wing lice insert themselves to escape host preening defenses. As in the case of the gopher lice, a mismatch between the body width of the louse and the feather interbarb space increases the risk of being preened off the host (Clayton *et al.* 2003; Bush and Clayton, 2006).

Most previous studies of louse morphological evolution have focused only on functionally significant characters, such as louse body width vs feather interbarb space or louse head groove size vs hair diameter. However, the functional significance (if any) of many taxonomically and phylogenetically useful morphological characters in lice is not clear. In addition, recent progress on the higher level molecular phylogeny of parasitic lice has revealed that morphological convergence can be very frequent for characters used in louse taxonomy and phylogeny reconstruction (Johnson *et al.* 2004, 2012; Smith *et al.* 2004; Yoshizawa and Johnson, 2006, 2010). For lice, little is known about how these taxonomically and

phylogenetically useful morphological characters vary across populations and how these characters evolve over microevolutionary timescales. Such knowledge would be useful to understand the evolution of morphology over macroevolutionary time. Quantitative comparisons of morphometric data and phylogeny are an effective way to study these characters (Klingenberg and Gidaszewski, 2010), but no such study has been done for lice to date.

Here, we undertake a co-phylogeographic study of sika deer and their parasitic lice. The sika deer (*Cervus nippon*) is distributed in east Asia and throughout the Japanese Archipelago. Japanese populations are subdivided into six subspecies and are highly variable in body size (from 30 to 120 kg in male's body mass: Ohtaishi, 1986). Such a large variation in body size within a species of mammal is exceptionally rare (Ohdachi *et al.* 2009). However, morphology-based subspecific subdivisions within sika deer are not supported by mitochondrial phylogeny, and two distinct mitochondrial lineages have been identified for Japanese sika deer. Sika deer host a parthenogenetic chewing louse species, *Damalinia sika*, making this a potentially ideal system to evaluate patterns of co-phylogeography and morphological evolution. As mentioned above, morphological evolution can be affected by many factors, including gene flow via interbreeding. *D. sika* is an obligately parthenogenetic louse, and thus gene flow via interbreeding among populations can be excluded as a factor affecting morphological evolution, making this species useful for distinguishing other factors impacting morphological evolution of this species.

In this study, we estimated the mitochondrial population level phylogenies of sika deer and their lice, based on sequences of the COI gene. We investigated co-phylogeographic patterns by comparing tree topologies and genetic divergence between hosts and parasites. We also discuss novel insights regarding deer population structure based on phylogeographic patterns observed in their lice. To examine patterns of louse morphological evolution, we analyzed head shape using morphometric analyses. We compared the louse population tree from COI sequences with the results of cluster analysis of morphometric data.

MATERIALS AND METHODS

Samples

Deer and deer louse samples were collected throughout the Japanese archipelago (Fig. 1). Most louse samples were collected by the authors and fixed in 99% ethanol immediately. Hairs of deer were also sampled with lice and were stored in 99% ethanol. Some deer samples were collected by local hunters, and frozen skins were sent to the authors. Lice were collected from the skins and fixed with 99% ethanol. Numbers of deer and lice samples examined are listed in Table 1. In most cases, samples of lice came from the individual deer included in this study. However, the Taiwanese population of louse was represented from a host individual that was unavailable for sequencing. Sequences from the host in this case (*C. n. taiounus*) was represented by sequences obtained from GenBank. In addition, sequences from the Far East Russian population of sika deer were obtained from GenBank and included in the phylogeographic analysis of deer, even though louse samples were not available for analysis. This was done to stabilize the tree search by breaking the long branch leading to Taiwanese sika deer (Table 1). *Cervus elaphus* and *Damalinia forficula* were used as outgroups for the host and parasite trees, respectively. These species are not associated, but are simply used for rooting the ingroup topologies because they are among the closest relatives of *C. nippon* and *D. sika*.

Sequences of a portion of the mitochondrial COI gene were obtained for both deer (838 bp) and lice (776 bp). Though the length of these sequences were slightly different, the sequence for the deer completely overlapped that for the lice, making these regions homologous. Therefore, the sequence data can be utilized for the comparison of host–parasite substitution rates (see below). The mitochondrial control region is known to evolve rapidly and thus is commonly used for phylogeography of mammals (Nagata *et al.* 1999). However, because mitochondrial genome of deer lice is composed of minicircles (Cameron *et al.* 2011), homology of the control regions between deer and deer lice cannot be warranted. No nuclear

markers for phylogeographic studies are known for deer (Barbosa and Carranza, 2010). Therefore, to compare homologous gene regions between hosts and parasites, COI is the best option to date. In addition, because sika deer lice are parthenogenetic, mitochondrial DNA is one of the best markers for tracking the history of these parthenogenetic lines. Procedures for total DNA extraction, PCR, and sequencing followed Yoshizawa and Johnson (2010), but primers were newly designed here (supplementary data). Alignment was done by eye because of the lack of indels in this protein coding gene. The aligned matrix in nexus format and csv file for statistical analyses (see below) are available as Supplementary Data. Sequence data also are deposited at GenBank (Table 1). Uncorrected and likelihood-based genetic distances were calculated using PAUP* 4b10 (Swofford, 2002).

Phylogeographic Analyses

Phylogeographic analyses were performed with the parsimony (PAUP*), likelihood (PAUP* and PhyML 3.0: Guindon *et al.* 2010), and Bayesian (MrBayes 3.12: Ronquist and Huelsenbeck, 2003) criteria. Best fit models for the ML and Bayesian analyses were calculated by hLRT using Modeltest (Posada and Crandall, 1998) and MrModeltest (Nylander, 2004), respectively (Supplementary Data). For ML analyses, using PAUP* a heuristic search with TBR branch swapping was implemented for each data set, using a NJ starting tree. For MP analysis, 100 random addition replicates using TBR heuristic branch swapping were performed. PAUP* was also used for MP bootstrapping, with 1000 replicates. PhyML was used to conduct likelihood-based 1,000 bootstrap replicates, using NNI branch swapping, each with Bio-NJ as a starting tree. For Bayesian analyses, we performed 2 runs, each with 4 chains for 10,000,000 generations, and trees were sampled every 1,000 generations. For each run, the first 5,000 trees were excluded as a burnin and the 50% majority consensus tree of the remaining trees was calculated to estimate posterior probabilities.We also estimated ParsimonySplits networks as implemented in SplitsTree4 (Huson and Bryant, 2006) using default settings. The

outgroups were excluded from the splits analyses.

Codivergence Analyses

Codivergence analysis was performed using the Jungle algorithm (Charleston, 1998) in TreeMap 2.02b (Page and Charleston, 1998). Samples with identical haplotypes were trimmed to a single terminal taxon. All non-codivergence events were given equal costs of 1 and codivergence was given a cost of 0. As discussed below, a number of non-codivergence events were identified from the Jungle analysis. To test whether there was significant evidence for a lack of strict codivergence, some alternative genealogical topologies were tested using the approximately unbiased test (AU test: Shimodaira, 2002) using CONSEL (Shimodaira and Hasegaea, 2001). The partition homogeneity test (Farris *et al*, 1994, 1995) was also performed using PAUP* to evaluate whether host and parasite data sets represented significantly different topologies (Johnson *et al.* 2001). To perform the test, a data matrix in which hosts and parasites were matched in 1:1 associations (as described by Johnson *et al.* 2001) was prepared by excluding all the samples lacking a host-parasite association (i.e., outgroups and sika deer from Ussuri) and also eliminating the samples for which both the hosts and parasites from an identical locality show exactly identical haplotypes. Using this 1:1 data set, 1,000 replicates of the partition homogeneity tests were performed each with TBR branch swapping. This 1:1 association data set was also used to compare the genetic distance between deer and lice. PAUP* was used to estimate the genetic distances, and uncorrected pairwise distances of total data, uncorrected pairwise distances of four-fold degenerate sites, and likelihood-based distances using the models estimated above were calculated. MEGA5 (Tamura *et al.* 2011) was used to identify four-fold degenerate sites.

Analyses of Louse Morphology

For parasites, host imposed selection may be more important than the parasite population or phylogenetic history for shaping the patterns of

morphological evolution. To evaluate the correspondence between louse morphology and population structure of both deer and lice, we analyzed the shape of the louse head. We used elliptic Fourier analysis (Kuhl and Giardiana, 1982) as implemented in the software package SHAPE (Iwata and Ukai, 2002). Morphological terminology follows Lyal (1985).

For morphological analysis, we examined 138 louse specimens from 15 localities, and each locality was treated as a terminal. The morphological samples did not include those used for DNA extraction when Proteinase K during the extraction caused breakage of the head capsule. Specimens that were used only for morphological analyses were cleared using 10% KOH solution for one night at room temperature. All specimens were rinsed with 80% ethanol (3 times), dehydrated with 99.5% ethanol (2 times), and slide mounted with Euparol.

The head capsule was photographed through the microscope using a digital camera. Using the digital image, the head outline was traced and scanned. Because head shape of *D. sika* is close to a circle, the initial alignment of head shape had to be re-orientated. To do this, the right margin of the outline was oriented on the right margin of ventral carina. Following Smith (2000), 20 harmonics were calculated from the digitized outline, while excluding effects of size. The resulting 80 coefficients were subjected to principal component analysis using SHAPE. The 13 principal component scores (PC 1-13) that represented over 95% of the variation were used for canonical discriminant analysis. We then calculated Mahalanobis distances between each group of specimens from the same locality. Then, using the Mahalanobis distances among locality groups, we performed cluster analysis using the group average method. Discriminant and cluster analyses were performed using JMP 9.0 (SAS, 2011).

RESULTS

Phylogeography of Deer and Deer Lice

ML analysis of each data set recovered a single most likely tree (Figs 2-3). MP analyses recovered 18 (for deer) and 11 (for louse) trees of equal length, and

one of these was the same as the tree estimated from the ML analyses. ParsimonySplits networks are provided as Supplementary Figs 1–2.

Three major lineages (North, South, and Continental + Taiwanese Clades) were recovered for sika deer (Fig. 2, Suppl. Fig. 1). Support values for all clades were generally strong (>77% BS for all clades and 98% PP for South Clade), but only low Bayesian support values were obtained for North (98–100% BS vs 82% PP) and Continental + Taiwanese Clades (77–83 BS vs 81% PP). Two Japanese lineages (Northern and Southern lineages) composed a clade, and the Continental + Taiwanese samples formed the other clade. However, resolution of the basal relationships among these lineages was weakly supported (59% BS and 56% PP). Low resolution of the basal divergences was also evident from the splits network (Suppl. Fig. 1). Analysis of the smaller data set (1:1 data, see Materials and Methods) placed Taiwanese sika deer as the sister of the Southern clade, making Japanese sika deer paraphyletic (Supplementary Data). The Southern Clade included all samples from Kyushu and its adjacent islands. The Northern Clade consisted of the samples from Hokkaido and Honshu.

The analyses of the louse data set recovered two distinct lineages (Clades I and II: Fig. 3 and Suppl. Fig. 2). Support values for both clades were very strong. Clade I was subdivided into two clades (Ia and Ib). Clade Ia was well supported and included samples from Hokkaido, northern Honshu and Fukuoka. The basal branch of Clade Ib was extremely short and weakly supported. This clade was composed of samples from Nikko, southern Honshu, Tsushima and Kagoshima. In the split network, samples of Tsushima were placed closer to Clade Ia than the other samples of Clade Ib, although network connecting them was extremely short (Suppl. Fig. 2). Clade II was composed of the samples from southern Kyushu and adjacent islands, and the sample from Taiwan.

Codivergence Analysis

Initial Jungle analysis using TreeMap 2 recovered four equally optimal results with four codivergence events and 13 non-codivergence events (Figs 4–5).

Standard randomization test showed that the number of codivergence events was significantly higher than that expected by chance in 1000 randomizations of parasite tree (P=0.005). However, most cophylogenetic analyses do not accommodate parasites that occur on multiple host terminals, such as a single species of parasites on multiple host species or parasite populations found on multiple host populations (Johnson *et al.* 2002*a*). Analytically these are often *post hoc* considered to be recent host switching events. Because of these difficulties in dealing with widespread parasites, the results from the TreeMap analysis could not account for two shallow host switching events of lice from deer in Fukuoka to North1 and to Toya populations (asterisks in Fig. 5). There still was significant congruence between host and parasite trees (P=0.034) even if these two additional non-codivergence events were allowed.

Because louse phylogeography did not closely correspond to host deer divergence patterns, we also examined these topological incongruences statistically. When the distinction between the South and North populations in the deer was forced as a constraint on the louse tree (excluding the Taiwanese deer louse, which was kept within the Southern Clade, together with Kyushu deer louse, as in the original louse phylogeny), the resulting tree was significantly worse than the unconstrained tree using the AU test (P<0.001). In contrast, when only the basal divergence between the Taiwanese and Japanese populations as estimated from the deer phylogeny was constrained in the louse tree, this constraint tree was not significantly worse than the original using the AU test (P=0.217). Similarly for the deer, when the two major clades identified by the phylogenetic analyses of the louse was constrained in the tree for sika deer (excluding the Taiwanese sika deer which was kept separate from Japanese population as in the original deer phylogeny), the constrained tree was significantly worse using the AU test (P=0.027). Likewise, the partition homogeneity test recovered significant heterogeneity between the host-parasite COI sequences (P=0.001).

Comparisons of Genetic Distances

Fig. 6 shows plots of the uncorrected p-distances between pairs of lice and those from their deer hosts. These plots contain roughly five clusters of points (Groups A–E). The divergence events of deer and lice corresponding to the plot groups are indicated by shade in the figure, and the plots corresponding to the codivergence events are indicated by circled numbers (Figs 2–3, 5–6). The uncorrected p-distances of four-fold degenerate sites and likelihood-based distances also showed identical trends (not shown). When points in groups B and C were excluded (because they clearly represent some incongruence between louse and deer population histories) and correlation between deer and louse genetic distances were calculated, the substitution rate of the COI gene for lice was estimated to be about 4.8 times faster than that of deer (Fig. 6). This estimate also accords with the fact that the highest divergences among lice are around 12%, while the highest among deer are only 2.5%. Although plot group E corresponds to the co-divergence event between Kumamoto and Yakushima + Kuchinoerabu populations (Fig. 5), these points are also outliers of the estimated slope (Fig. 6).

Morphological Variation of Lice

Except for the populations from Kinkazan and Nara, all clusters identified by morphological analysis corresponded to the lineages recovered from the louse molecular phylogenetic analysis (Figs 7–8). Note that molecular phylogenetic analysis divided the Nikko population into two distinct lineages (Fig. 3), but the majority of these specimens were placed in the Clade Ia, which corresponded to the cluster identified by morphological analysis. In contrast, except for two populations collected from the same subspecies of deer (i.e., *C. n. yakushimae*: note that lice from these two populations are also genetically closely related: Fig. 3), the clusters identified from louse head morphology did not show any grouping corresponding to deer subspecies or geography.

PC1 explained variation in the shape of conus, depth of the antennal groove, head width, and width of the pulvinus (Fig. 7: proportion = 32.6%). PC2 also explained variation in the protrusion of the antennal groove margin, protrusion of

the temple, shape of the conus, and width of the pulvinus (proportion = 26.9%). PC3 explained variation in the antennal groove (proportion = 10.1%). PC4 explained clear variation in the depth of the pulvinus, and explained some variation in the depth of the antennal groove (proportion =9.1%). The proportion of morphological variation explained by PC5–13 was less than 5% (not shown). However, PC5 (proportion = 4.7%) still contained detectable variation in the depth of the antennal groove, and PC6 (proportion = 2.7%) explained clear variation in the length of the conus. PC7–13 explained very little of the overall variation.

DISCUSSION

Codivergence between Sika Deer and Lice

Comparing deer and louse trees using Jungle analyses recovered significant patterns of topological congruence. However, these analyses only account for the branching pattern and do not take into account the relative timing of branching events. Using information from the genetic divergence plots, three of four reconstructed codivergent events are also concordant with the relative timing of branching events (1, 3 and 4 in Fig. 6), providing reciprocal support for the results of both types of analyses. However, plot Group E, which would correspond to a codivergence between Kumamoto and Yakushima + Kuchinoerabu population (2 in Fig. 6), is apparently an outlier for genetic distance as compared to the other codivergence events. Therefore, even though the branching patterns of these populations might indicate codivergence, based on genetic distances this is more likely a recent host switch. When one of the codivergence events was excluded (i.e., number of codivergence events was set to three and one more host switching event was allowed), the tree randomization test showed that the number of co-divergence events was not significantly higher than that expected by chance (P=0.19). Significant mismatches between the deer and louse trees are also evident from the plots of the genetic distances, the AU test, and the partition homogeneity test.

In contrast, the AU test did not reject the possibility of basal diversification

of the Taiwanese deer louse from the Japanese population. Alternatively, when the smaller data set was analyzed, the Taiwanese sika deer was placed to the sister of the Southern Clade, agreeing with the louse tree as far as the placement of Taiwanese population is concerned. However, there is a significant discordance between pairwise genetic distance comparisons for these deer and louse populations (plot group C in Fig. 6). Therefore, by considering both topology and genetic distances, the close relationship between louse populations of Taiwan and southern Kyushu is unlikely to be a result of their codivergence and, as estimated from Jungle analyses, this should also be interpreted as a result of a recent host switch.

The louse populations in Kyushu are highly structured genetically, even within Clade I, whereas those of Hokkaido and Honshu show much less genetic variability (Fig. 3). Jungle analysis suggests that the louse populations in Hokkaido and Honshu originated via a recent host switch from deer in Kyushu. The deer populations in Honshu and Hokkaido show some genetic structure (Fig. 2), but the genetic variation of lice in Honshu and Hokkaido is apparently less structured and less variable (Fig. 3), even though the substitution rates of lice are much faster than those for deer (Fig. 6). The lack of genetic variability and structure in these louse populations also supports the hypothesis, from Jungles analysis, that they originated from a recent host switch, which may have been associated with a genetic bottleneck.

The evidence for multiple host switching events suggests that deer lice migrate among deer populations quite readily and independent of deer population structure. Mismatches between host and louse phylogenies at species level are quite frequent for bird lice (Johnson *et al.* 2002*a,b*; Weckstein, 2004; Banks *et al.* 2006). In contrast, all previous studies of mammal lice have identified highly significant codivergence patterns between mammal lice and their hosts at the species (Hafner *et al.* 1994; Light and Hafner, 2007; Light *et al.* 2008; Smith *et al.* 2008) or population levels (Demastes *et al.* 2012). Although trends similar to other mammal lice were expected in these deer lice, it appears that different dynamics may operate for the deer–lice system, and these are explored below.

Phylogeography of Deer Lice

The most basal divergence within deer lice was estimated to correspond to the divergence between continental and Japanese deer lineages (Figs 2–3). Clade II appears to have its ancestor in Taiwanese populations, and Japanese populations seem to have originated by a recent host switch from the Taiwanese population to the South Clade (Fig. 5). The common ancestor of Clade I was estimated to parasitize the South deer clade, and the louse populations on the North deer clade seem to have originated by a very recent host switch from louse populations parasitizing the South deer clade (Fig. 5).

This scenario is reasonable based on the additional evidence provided by genetic distances (Figs 3, 6, Suppl. Fig. 2). Deer lice collected from Kyushu contain haplotypes from all major louse clades (Clade Ia, Ib and II) and, even within Clade I, they are quite variable genetically (shown by dotted circles: Fig. 3). In contrast, although lice from Hokkaido and Honshu are also divided into two subclades (Clade Ia and Ib), their haplotypes are either identical with (Clade Ia) or only slightly different from (Clade Ib) those of Kyushu (Fig. 3). Therefore, recent host switches of deer lice from Kyushu to Hokkaido + Honshu populations are highly plausible.

This scenario implies two possibilities. The first possibility is that the common ancestor of the Northern deer clade might not have hosted any louse. The Northern deer clade is thought to have colonized the Japanese Archipelago during the last ice age via Sakhalin (see below). If deer lice are not tolerant of cold temperatures, this scenario might be plausible. Although no quantitative data are available, we only collected two lice from two deer at Nishiokoppe, the northern most locality of the present sampling area (Fig. 1), and the field work was conducted in mid-winter (Table 1). Based on other collections we have made, this was an exceptionally small number of lice infecting these deer. A second possibility is that the louse populations originally parasitizing deer in Hokkaido and Honshu were completely replaced by those from Kyushu following the recent host switch

437 events. Recently, *Damalinia sika* was introduced to the USA has since become
438 widespread and is replacing native lice very rapidly in some native deer populations
439 (Mertins *et al.* 2011, unpub. data). This may indicate that certain lineages of deer
440 lice, particularly parthenogenetic species such as *Damalinia sika*, may have higher
441 population growth rates, giving them the potential to replace other populations.
442
443 *Implications of Louse Population Structure for Deer Phylogeography*
444 Previous studies from the mitochondrial D-loop and CytB sequences show
445 that Japanese sika deer possess two genetically distinct lineages (Tamate *et al.*
446 1998; Nagata *et al.* 1999). Using sequences of the COI gene, the present analysis
447 also supported this previous result (Fig. 2). However, as discussed above, louse
448 phylogeography is highly incongruent with deer population structure (Fig. 3), even
449 though one would expect that dispersal of these permanent parasites is highly tied
450 to that of their hosts.
451 One possible explanation for the topological incongruence may be phoresis of
452 these lice on hippoboscid flies as has been convincingly demonstrated for pigeon
453 lice (Harbison *et al.* 2009; Harbison and Clayton, 2011). Unlike lice, hippoboscid
454 flies have wings and can disperse between host individuals relatively freely. Sika
455 deer host three hippobiscid flies, *Lipoptena fortisetosa*, *L. sikae* and *L. japonica*
456 (Mogi, 1975; Mogi *et al.* 2002). The final instar larvae of these species leave the
457 deer for pupation and, after emergence, louse-free adult flies fly to new hosts. Soon
458 after the arrival to new host deer, the flies cut off their wings and thus are not
459 likely to disperse to a new host (Yamauchi and Nakayama, 2006; Yamauchi,
460 personal communication). In addition, there are no records of *Damalinia sika*
461 attached to hippoboscid flies. Therefore, although the possibility cannot be
462 excluded completely, phoresis does not seem a likely explanation for the topological
463 incongruence between deer and louse trees.
464 The more likely explanation for incongruence between deer and louse trees is
465 that lice migrate via male deer. The two geographically distinct lineages of Japanese
466 sika deer have only been recovered using maternally inherited mitochondrial

markers, which only represents the genetic structure of female deer (Nagata *et al.* 1999). In contrast, two such geographically distinct groups are not recovered in analyses of microsatellite markers (Goodman *et al.* 2001). In fact, the genetic structure of Japanese sika deer recovered using microsatellite markers is more congruent with the louse phylogeny, because the deer populations from Kyushu are divided into two clusters based on microsatellites. Furthermore, microsatellite data clusters a deer population from northern Kyushu together with populations from northern Honshu and Hokkaido (Goodman *et al.* 2001), which is also concordant with the louse phylogeny (Figs 1 and 3). Sika deer are polygamous, and females compose maternal groups that do not migrate and tend to stay within a male's territory. In contrast, young male deer (3–7 year old) migrate widely before obtaining a harem (Miura, 1984; Takatsuki, 2006). Therefore, deer lice could be dispersed widely by male deer, and the dispersal of males is likely to be the main source for the incongruence between deer and lice mitochondrial population histories. Observations from deer nuclear markers suggest that the louse phylogeny does at least partially reflect the deer's population structure, with respect to the fact that male gene flow is quite frequent between two mitochondrial lineages of sika deer. The louse population structure also provides further support for the idea that subspecific division in sika deer does not reliably reflect population subdivision.

The two Japanese mitochondrial lineages of sika deer have been estimated to have already diverged on the continent before their invasion of the Japanese Archipelago (around 0.3–0.5 Mya), based on mitochondrial genetic distances and geographic history of the region (Nagata *et al.* 1999; Okada, 2008). The Southern Clade is hypothesized to have invaded via the Korean Peninsular in the mid Pleistocene (~0.17 Mya), and the Northern Clade is estimated to have invaded via Sakhalin in late Pleistocene (60,000–10,000 ya). However, as mentioned above, these estimates are based on only female line genetic structure. In the present analysis, the Taiwanese deer louse is placed very close to the deer louse from southern Kyushu (Fig. 3), which implies that southern Japanese populations of sika

497 deer may have had contact with continental deer more recently than estimated from
498 the mitochondrial phylogeny of the deer alone.
499 As pointed out in previous studies (Johnson *et al.* 2002*a*; Clayton and
500 Johnson, 2003; Weckstein, 2004; Poulin *et al.* 2011), detailed information about
501 louse and host behaviors is important for understanding the causes of similarities
502 and discordance between the host and louse phylogenies. The behavior of the sika
503 deer is well studied (reviewed in Takatsuki, 2006), which greatly aided in
504 interpreting the discordance between the deer and louse phylogenies. Alternatively,
505 studies of louse phylogeography could also prove important for understanding the
506 host's population structure. The substitution rate of louse mitochondrial genes is
507 much faster than that of their vertebrate hosts (see below), and these insects can
508 be transmitted both paternally as well as maternally. In the case of sika deer, good
509 nuclear markers for population genetic studies, including those on Y-chromosome,
510 have not been discovered to date (Barbosa and Carranza, 2010), and long distance
511 dispersal of deer lice apart from their hosts is quite unlikely. In such cases, louse
512 population genetic trees can be good indicators of hidden population structure in
513 their hosts (Reed *et al.* 2004; Whiteman *et al.* 2007; Zohdy *et al.* 2012). This
514 notion seems to be supported in our study by the fact that the louse tree is more
515 similar to the deer population structure estimated from nuclear microsatellite
516 markers (Goodman *et al.* 2001) than to that estimated from mitochondrial genes
517 (Nagata *et al.* 1999).
518
519 *Comparisons of Substitution Rates*
520 By plotting pairwise uncorrected genetic distance comparisons for lice against
521 those for their deer, roughly five groups of points are evident (Fig. 6). Group A
522 represents the shallowest divergences within both deer and lice. The phylogenetic
523 trees do not show significant evidence for unevenness of substitution rates among
524 populations for either deer or lice. Therefore, one of the slopes connecting A-B,
525 A-E-C or A-D likely estimates the relative rate of substitution for lice vs. deer of
526 the COI gene. As discussed above, plot groups B and C clearly represent some

incongruence between louse and deer population history (Figs 4–6). Given these considerations, the slope of the line connecting groups of comparisons A and D seems to be the best estimate for the relative rate of substitution between lice and deer (Fig. 6). Most of the comparisons between lineages involved in codivergence (see below and Results) are also in Groups A and D.

This slope estimates the relative substitution rate to be around 4.8 times faster in lice than in deer when uncorrected pairwise distances are used. Comparisons of four-fold degenerate sites (i.e. neutral sites: 137 bp for deer and 131 bp for lice) also provided a comparable value (4.9 times). This relative rate is greater than those estimated previously (two to four times faster) for lice and their hosts (Hafner *et al.* 1994; Page, 1996; Huelsenbeck *et al.* 1997; Page *et al.* 1998; Reed *et al.* 2004; Light & Hafner, 2007; Demastes *et al.* 2012). Phylogenetic studies show that mitochondrial substitution rate of deer lice is faster than that of other lice (Yoshizawa and Johnson, 2003, 2010: note that the species is mislabeled as *Bovicola tibialis* in Yoshizawa and Johnson, 2003: see Mertins *et al.* 2011 for further information). Therefore, the presently estimated relative rate (4.8 times) can be regarded as reasonable. Although a far greater relative rate (11 times) was estimated by comparing the branch lengths of likelihood trees that were congruent between doves and their wing lice (Johnson *et al.* 2003*b*), a smaller estimate of relative rate of 1.6 times was obtained based on parsimony. Although not shown, comparisons of likelihood-based pairwise distances for deer and louse COI sequences resulted in a greater relative substitution rate (7.1 times). This value does not account for topology because of the many non-codivergence events identified in the present analyses. Estimates based on branch lengths in a tree topology probably provide higher relative rates because more multiple substitutions can be accounted for using a tree based approach, as was done for doves and their wing lice (Johnson *et al.* 2003*b*). As mentioned above, the substitution rates of deer louse mitochondrial genes are faster than other lice sequenced to date (Yoshizawa and Johnson, 2003, 2010), possibly related to its parthenogenesis (Bengtsson, 2003; Henry *et al.* 2011; Shreve *et al.* 2011).

557

558 *Microevolution of Louse Head Morphology*

559 The dendrogram obtained by cluster analysis of louse head morphology was
560 highly concordant with the louse phylogenetic tree (Figs 3, 8). The only obvious
561 discordance was a difference in the placement of populations from Nara and
562 Kinkazan, otherwise all the major clusters were concordant with the louse
563 molecular tree. Thus, the shape of the louse head capsule appears to contain
564 significant phylogenetic signal.

565 The morphology of the conus and neighboring structures shows the greatest
566 variation and these variables are the major factors in PCs 1–3 and major or minor
567 factor in PCs 4–6 (Fig. 7). The function of the conus is poorly understood.
568 However, this structure often shows sexual dimorphism and is frequently absent in
569 male Trichodectidae (Lyal, 1985) so that the conus may not be a highly functional
570 structure and thus selective pressure on this character may be relatively weak. The
571 second structure showing large variation is the anterior margin of the pulvinus (Fig.
572 7). This character is one of the factors in PCs 1, 2 and the major factor in PC 4.
573 This structure is involved in grasping the host hair and thus is functionally
574 important. Previous study has shown that this character is strongly correlated with
575 host size and thus is likely to be under strong selection pressure (Reed *et al.*
576 2000).

577 The factors explaining the largest variation in louse head morphology appear
578 to be both relatively neutral as well as characters expected to be under stronger
579 selection. Variation in these characters clearly contains signal concordant with the
580 louse phylogeographic history (Figs 2, 8). In this case, the neutral characters
581 appeared to comprise a larger fraction of the variation compared to those expected
582 to be more functionally significant. This suggests that random drift of more neutral
583 characters plays a major role in the microevolution of morphology for these lice.
584 The evolution of functionally significant characters is slower compared to that of
585 less functional characters, perhaps because of stabilizing selection. Interestingly,
586 the shape of conus is frequently used in taxonomic and phylogenetic studies, but

the shape of pulvinus is rarely used (e.g., Lyal, 1985; Smith, 2001). A high level of variation and correlation with phylogeny of taxonomically important characters, such as dorsal head pattern and abdominal pigmentation, have also been identified in the louse genus *Brueelia* (Johnson *et al.* 2002*a*), though functional significance of these characters is less clear. Many important diagnostic characters used in louse taxonomy possibly originated by the accumulation of more neutral variation along independent phylogenetic lineages.

The present analysis shows that microevolutionary changes in deer louse morphology accumulate along phylogenetic lines (Figs 3, 8). In contrast, Smith *et al.* (2004) and Johnson *et al.* (in press) showed that, across bird feather lice, convergence is frequent in functionally significant characters, such as body width (i.e., body vs wing lice) or the shape of the anterior head margin. Such convergence is evidence that natural selection produced the variation seen in louse morphology across bird lice. The morphology of deer lice also provides a potential example of convergence caused by similar selection pressures. Although most morphometric clusters are concordant with the louse phylogenetic tree, populations from Kinkazan and Nara were placed at discordant positions (Fig. 8). Interestingly, sika deer of these populations have unusual histories. Kinkazan is a small island (9.6 km2), and the population size of sika deer on this island has been highly variable. Given this population fluctuation in their hosts, it is also likely that the lice would have experience population bottlenecks. Dwarfishness of deer on Kinkazan also occurs because of the occasional high deer density on the island. Similarly, sika deer in Nara inhabit an urbanized environment (Nara Park), and their population is controlled by humans. Large decreases in population size have occurred in the past. In addition, these deer exhibit dwarfishness in body size and delay of sexual maturity (Torii and Tatsuzawa, 2009). These phenomenon are thought to be a result of selection when the deer in Nara Park are at high density. These unusual environmental conditions and/or population bottlenecks probably resulted in accelerated morphological evolution in deer lice that is not concordant with their phylogenetic history.

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842
843

844 Figure legends

845 Fig. 1. Map of collecting sites for sika deer and lice. Circles indicate the collection
846 sites, with different colors showing different louse clades. For numbers of deer
847 and lice samples from each locality, see Table 1.

848 Fig. 2. Phylogenetic tree of sika deer individuals estimated using maximum
849 likelihood. Branch lengths are proportional to substitutions per site. Numbers
850 associated with each branch indicate MP bootstrap/ ML bootstrap/ Bayesian
851 posterior probabilities. Shades indicate haplotype group names used for Figs
852 4–5. Numbers in circles indicate codivergence points corresponding to those
853 in Fig. 3. See also Figs 4–6.

854 Fig. 3. Phylogenetic tree of deer lice estimated using maximum likelihood. Branch
855 lengths are proportional to substitutions per site. Numbers associated with
856 each branch indicate MP bootstrap/ ML bootstrap/ Bayesian posterior
857 probabilities. Shades indicate haplotype group names used for Figs 4–5.
858 Numbers in circles indicate codivergence points corresponding to those in
859 Fig. 2. Broken-line circles indicate samples from Kyushu and adjunct islands.
860 See also Figs 4–6.

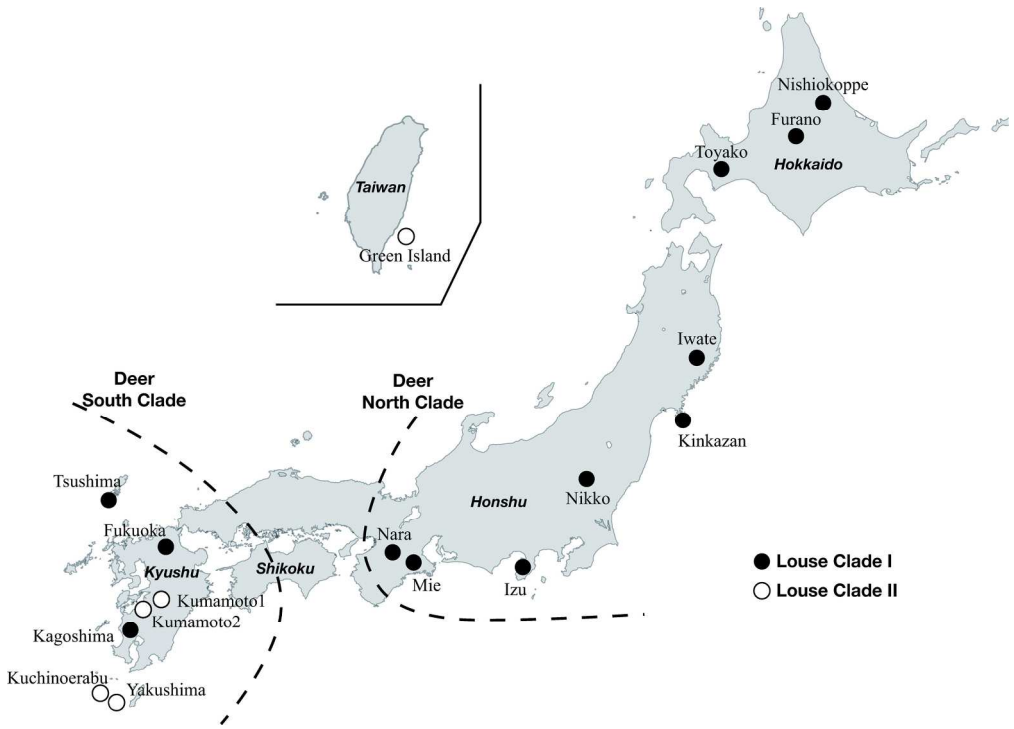
861 Fig. 4. Tanglegram of deer and louse trees with lines connection host–parasite
862 associations.

863 Fig. 5. Optimal Jungle reconstruction using TreeMap2. Note that two host switch
864 events indicated by asterisks were added by hand, because of the inability of
865 Jungles analysis to deal with parasite lineages occurring on multiple host
866 lineages. Labels for louse are distinguished from deer by a "p" heading. See
867 text for detailed explanation.

868 Fig. 6. Plots of uncorrected pairwise distances estimated from deer and louse COI
869 sequences. Shadings indicate deer and lice divergence events as noted on left
870 and lower margins. Dashed outlines and associated letters indicate plot
871 groups as discussed in the text. Numbers in circles indicate codivergence
872 points corresponding to those in Figs 2–3 and 5.

873 Fig. 7. Illustration of shape variation in louse head morphology, with variation

874 contained within each of the first four principal components indicated.
875 Fig. 8. Dendrogram resulting from cluster analysis of the louse head shape principal
876 components. Terminal labels indicate louse collection site (left), their host
877 subspecies (represented by first three letters of subspecific epithet: middle),
878 and the mitochondrial lineages for each louse population (right).
879
880 **Table 1.** List of the samples used in this study. Abbreviated labels corresponding to
881 those indicated in figures.
882
883 **Supplementary Figure 1.** ParsimonySplits network of sika deer.
884
885 **Supplementary Figure 2.** ParsimonySplits network of sika deer lice.
886
887 **Supplementary data.** Primer list and selected evolutionary models for likelihood and
888 Bayesian analyses (data.txt), raw data from Fourier analyses (sape.data.csv),
889 aligned sequences (Lice.coi.full.nex: full data for lice; Deer.coi.full.nex: full
890 data for deer; Louse-deer.coi.nex: combined 1:1 data set), and deer ML tree
891 estimated without a sample from Ussuri (exUssuri.tre).



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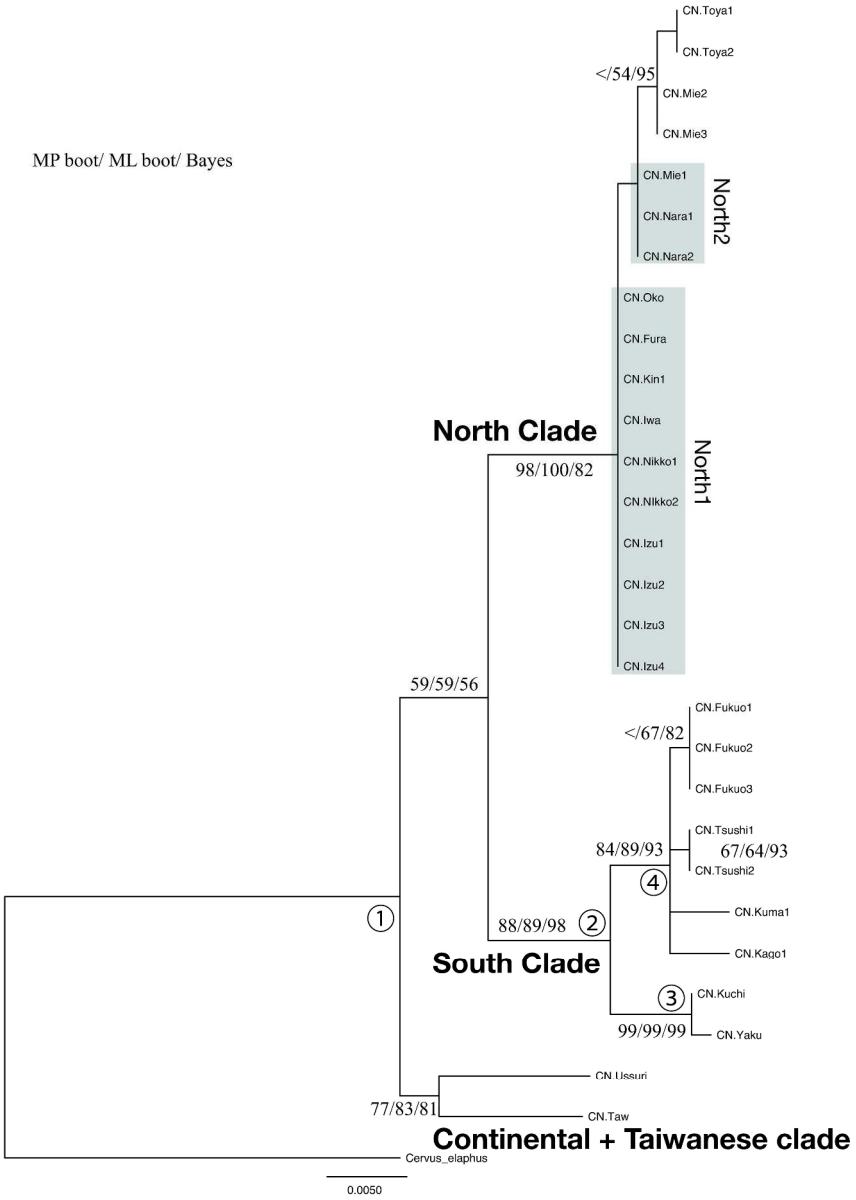

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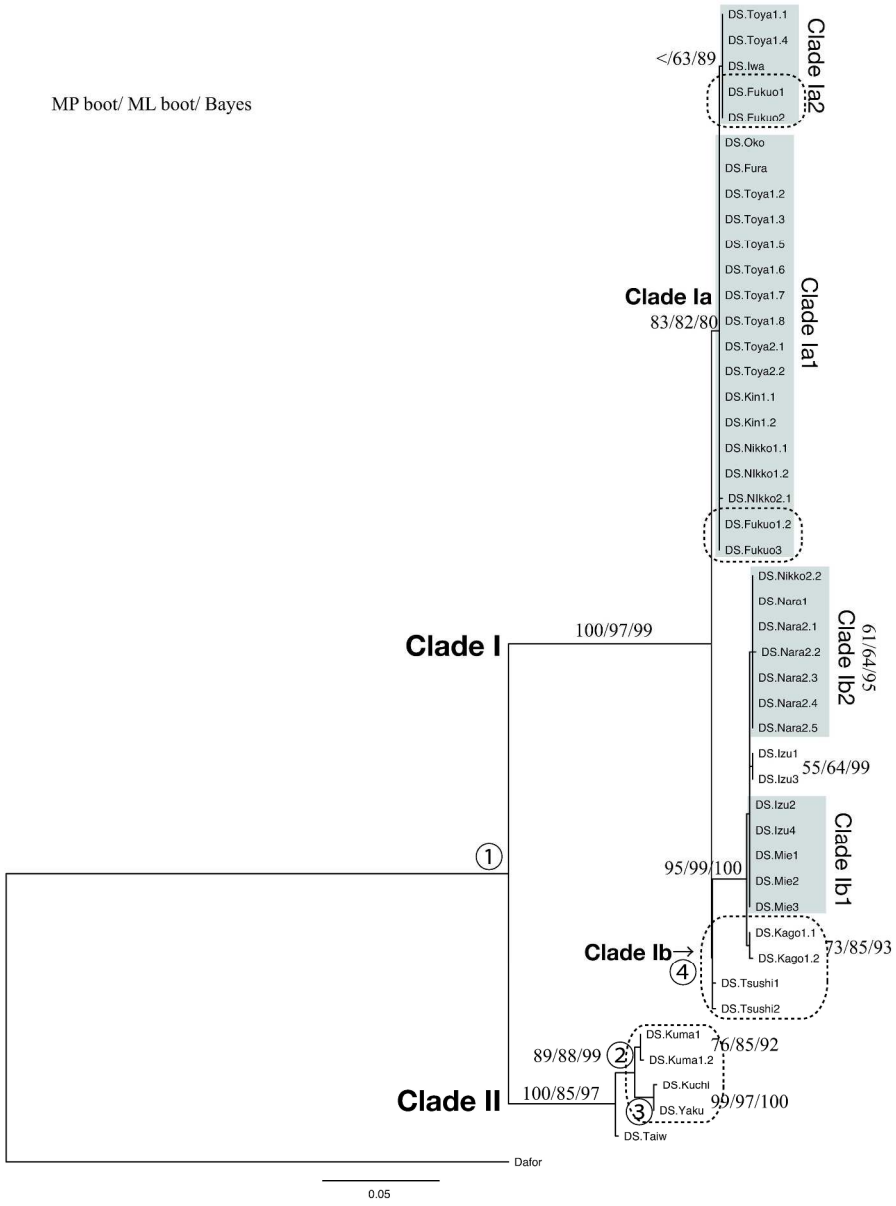

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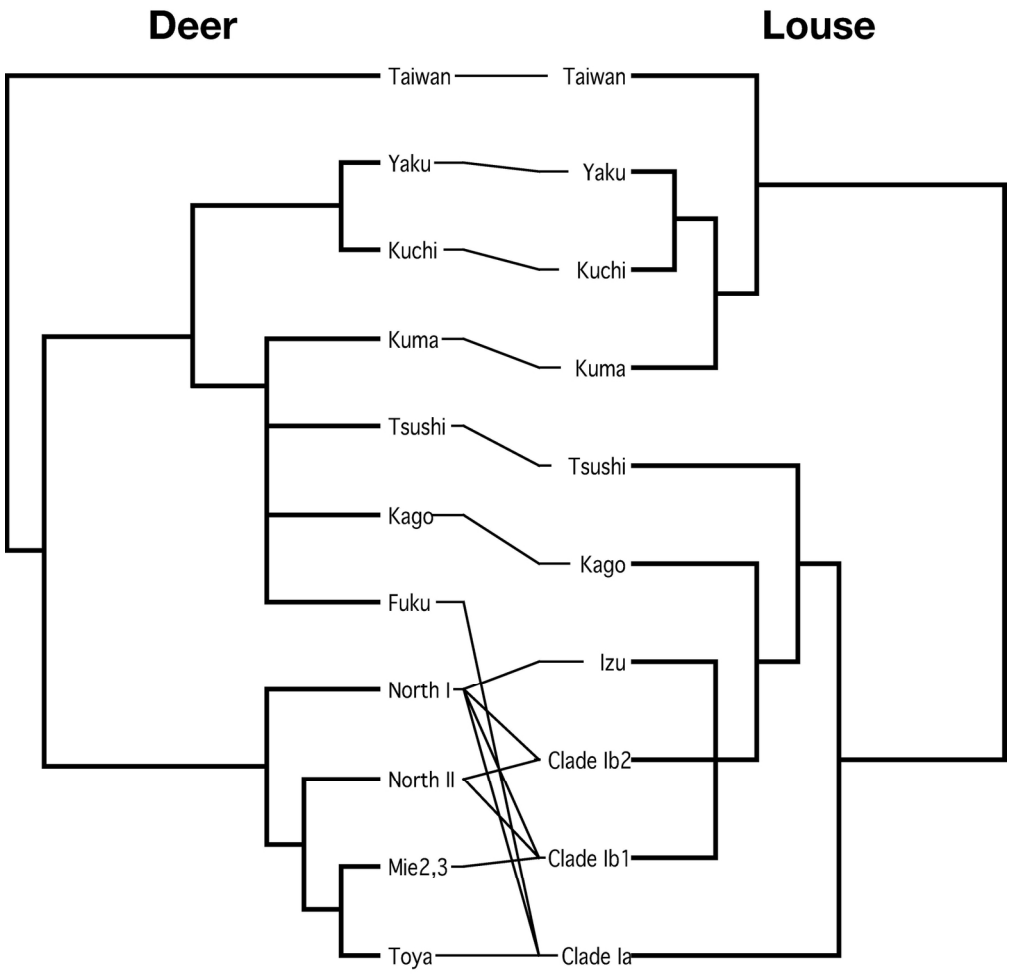

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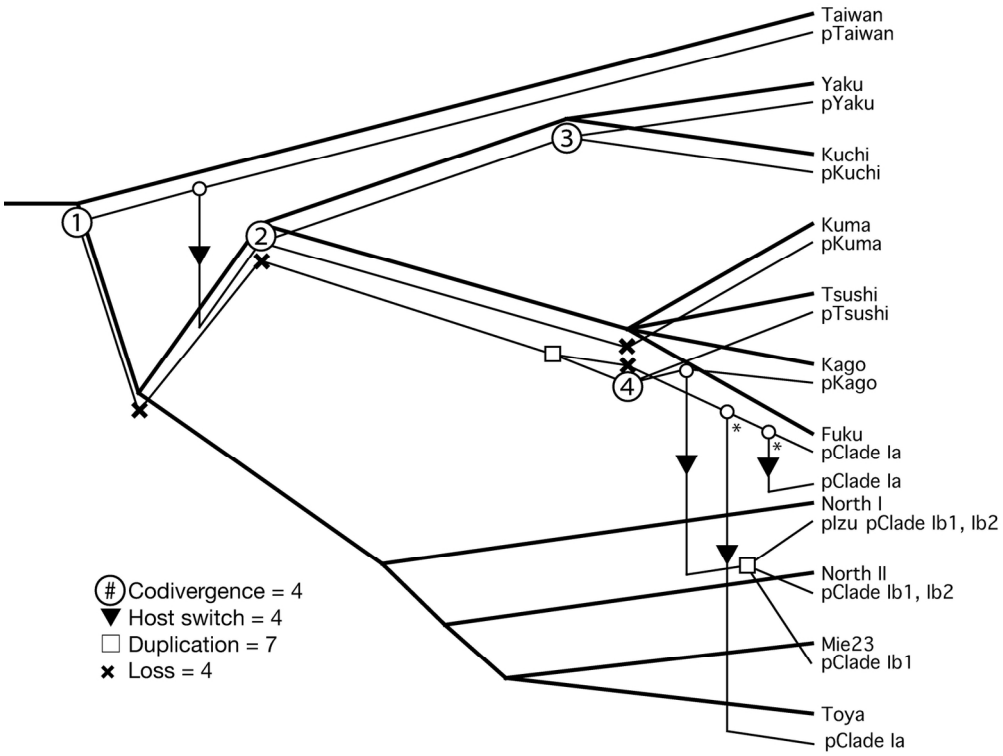
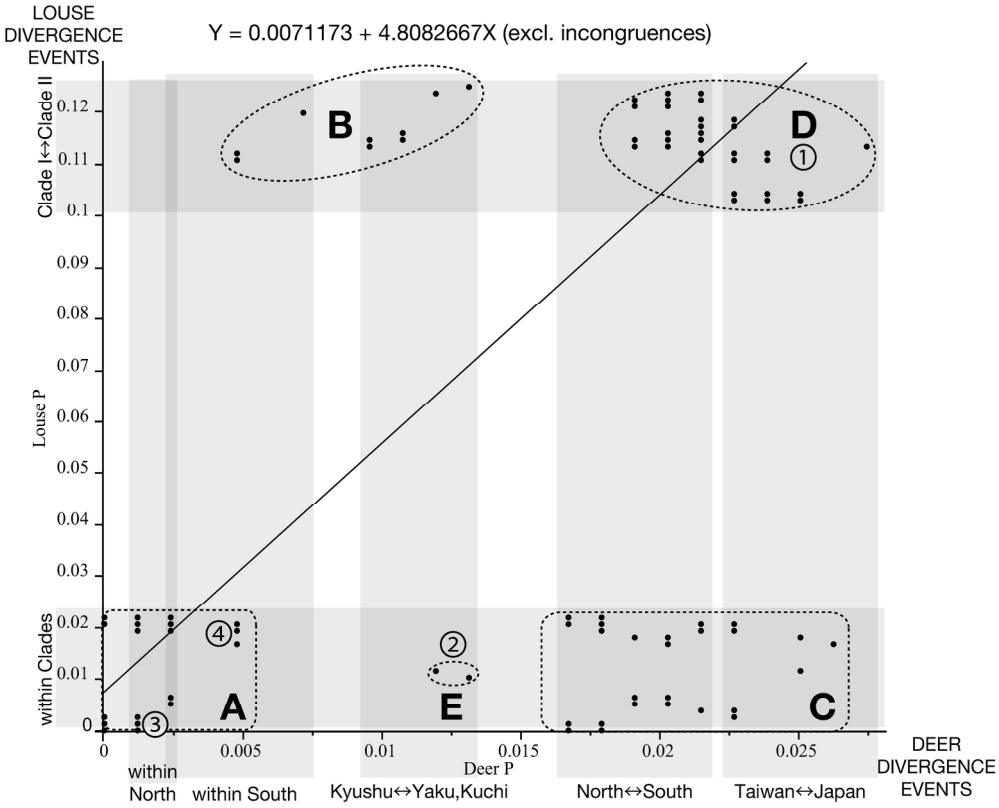
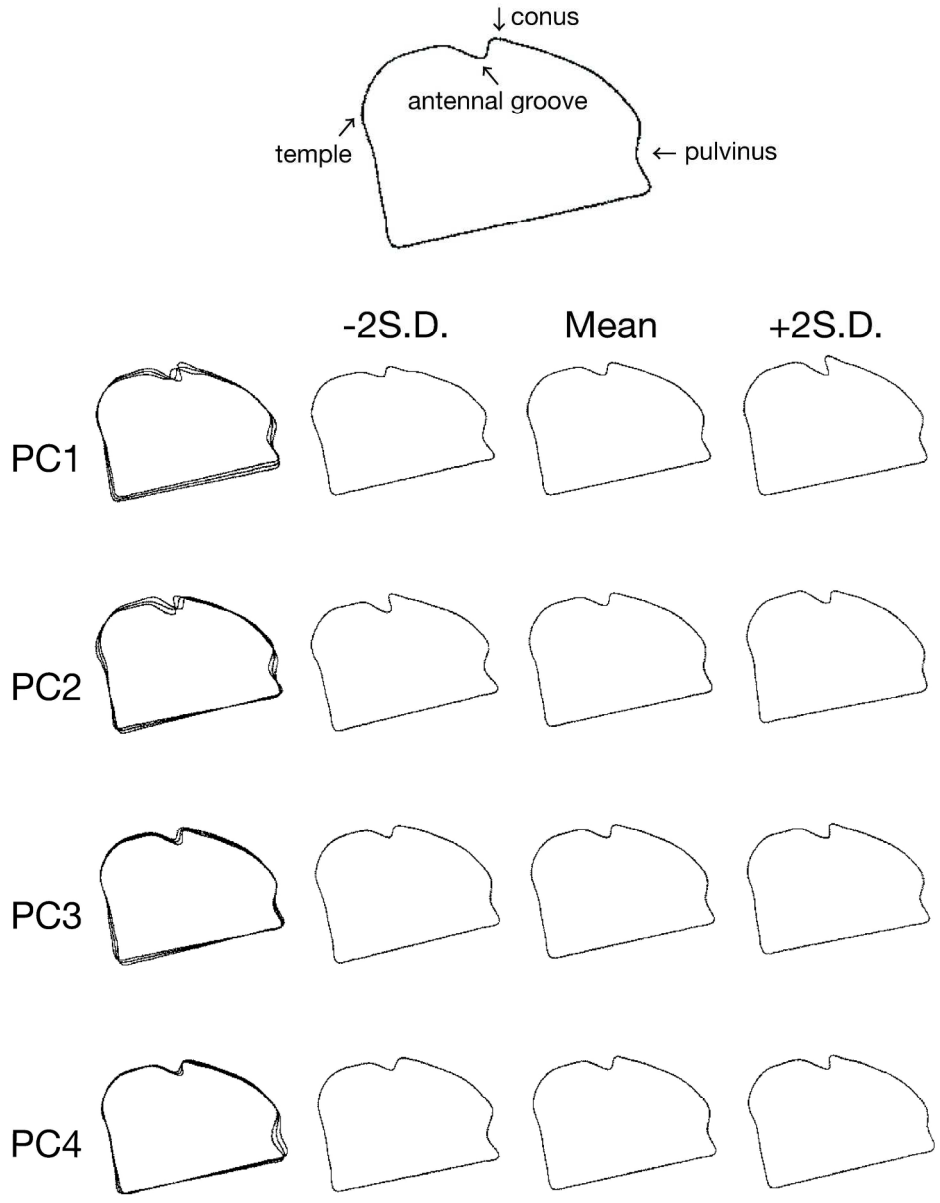


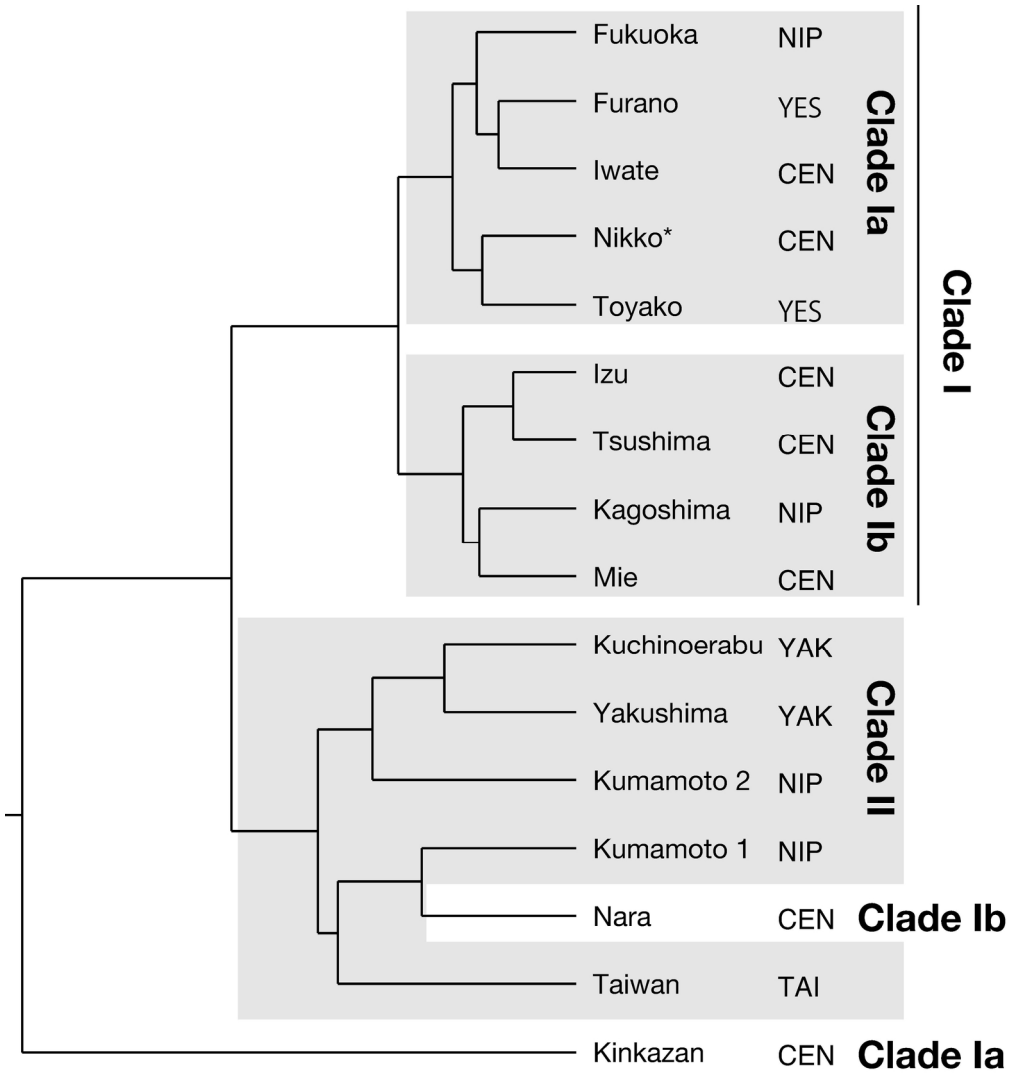
152x121mm (300 x 300 DPI)



179x143mm (300 x 300 DPI)



202x261mm (300 x 300 DPI)



179x192mm (300 x 300 DPI)

				DNA (n)		Morph (n)	GenBank	
Label	**Deer subsp.**	**Locality**	**Date**	**Deer**	**Louse**	**Louse**	**Deer**	**Louse**
Oko	*yesoensis*	Hokkaido: Nishiokoppe Villa.	20. ii. 2007	1	1	n.a.	AB713061	AB713088
Fura	*yesoensis*	Hokkaido: Furano City, Experimental Forest of the Univ. Tokyo	23. xii. 2007	1	1	10	AB713062	AB713089
Toya	*yesoensis*	Hokkaido: Lake Toyako, Nakajima	7. iii. 2002	2	10	10	AB713063-4	AB713090-9
Iwa	*centralis*	Iwate: Sumita Town	18. i. 2004	1	1	4	AB713065	AB713100
Kin	*centralis*	Miyagi: Kinkazan Is.	4. vi. 2001	1	2	2	AB713066	AB713101-2
Nikko	*centralis*	Tochigi: Nikko City	30. i and 26. ii. 2009	2	3	10	AB713067-8	AB713103-6
Izu	*centralis*	Shizuoka: Izu City, Mt. Tanabayama	4. iii. 2009	4	4	10	AB713069-72	AB713107-10
Mie	*centralis*	Mie: Odai Town	ii. 2009	3	3	10	AB713073-5	AB713111-3
Nara	*centralis*	Nara: Nara Park	1. v. 2008	2	6	10	AB713076-7	AB713114-9
Tsushi	*centralis*	Nagasaki: Tsushima Is., Izuhara Town	28. i and 8. ii. 2009	2	2	10	AB713078-9	AB713120-1
Fuk	*nippon*	Fukuoka: Asakura C.	17 and 24. i. 2009	3	4	10	AB713080-2	AB713122-5
Kuma1	*nippon*	Kumamoto: Izumi Villa., Shiibagoe	24. v. 1997	1	1	10	AB713083	AB713126
Kuma2	*nippon*	Kumamoto: Kuma Villa.	7. iv. 2010	n.a.	1	8	n.a.	AB713127

Kago	*nippon*	Kagoshima: Satsuma Town, Mt. Shibaoyama	10 and 14, iii. 2008	1	2	10	AB713084	AB713128-9
Kuchi	*yakushimae*	Kagoshima: Is. Kuchinoerabu	25. ii. 2008	1	1	5	AB713085	AB713130
Yaku	*yakushimae*	Kagoshima: Is. Yakushima, Miyanoura	3. iii. 2008	1	1	10	AB713086	AB713131
Taiw	*taiouanus*	Green Island, Taiwan	19. ix. 2006	1	1	2	EF058308	AB713132
Ussuri	*hortulorum*	GenBank	-	1	n.a.	n.a.	NC013834	n.a.
Dafor (louse outgroup)	-	Marin County, California, USA ex. Axis axis	22. vi. 2005	n.a.	1	n.a.	n.a.	AB713087
Cervul elaphus (deer outgroup)	-	GenBank	-	1	n.a.	n.a.	AB245427	n.a.

[supplementary data file for co-phylogeography of sika deer and deer lice: Mizukoshi, Johnson and Yoshizawa]

PCR and Sequencing primers for deer

COI.CN.F (GCA GCC GGA ATT ACA ATA CT) + COI.CN.R2 (GCA ACT ACA TAA TAT GTG TCA)
COI.CN.F2 (CTA GCA ACA CTC CAC GGA GG) + COI.CN.R (GCA TCC ATT TAG YCA CTC TA)

PCR and Sequencing primers for lice
COI.DS.F (ATY ACA ATG CTT CTT CTA GAT CG) + COI.DS.R2 (TGG AAR TGY GCT ACC ACR TA)
COI.DS.F2 (CAC CAT TGG RGG TCT CAC TG) + COI.DS.R (AAT TCG ATA AGA GAA CAA GGA GC)

Likelihood model selected for lice data
[!Likelihood settings from best-fit model (HKY+G) selected by hLRT in Modeltest 3.7 on Fri Oct 7 10:25:20 2011]
BEGIN PAUP;
Lset Base=(0.2101 0.1952 0.2412) Nst=2 TRatio=4.2614 Rates=gamma Shape=0.3251 Pinvar=0;
END;

Likelihood model selected for deer data
[!Likelihood settings from best-fit model (HKY+G) selected by hLRT in Modeltest 3.7 on Fri Oct 7 10:23:26 2011]
BEGIN PAUP;
Lset Base=(0.2935 0.2196 0.1632) Nst=2 TRatio=22.5927 Rates=gamma Shape=0.0106 Pinvar=0;
END;

Bayesian model selected for lice data
[!MrBayes settings for the best-fit model (HKY+G) selected by hLRT in MrModeltest 2.3]
BEGIN MRBAYES;
Lset nst=2 rates=gamma;
Prset statefreqpr=dirichlet(1,1,1,1);
END;

Bayesian model selected for deer data
[!MrBayes settings for the best-fit model (HKY+G) selected by hLRT in MrModeltest 2.3]
BEGIN MRBAYES;
Lset nst=2 rates=gamma;
Prset statefreqpr=dirichlet(1,1,1,1);
END;