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SHORT COMMUNICATION

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Mitochondrial genome sequence comparisons indicate that the elephant louse *Haematomyzus elephantis* (Piaget, 1869) contains cryptic species

Sarah Kelly[1,2] | Yalun Dong[1,2] | Wei Wang[1,2] | Sonja Matthee[3] | Jeanette M. Wentzel[4,5,6] | Lance A. Durden[7] | Renfu Shao[1,2]

[1]Centre for Bioinnovation, University of the Sunshine Coast, Sippy Downs, Queensland, Australia

[2]School of Science, Technology and Engineering, University of the Sunshine Coast, Sippy Downs, Queensland, Australia

[3]Department of Conservation Ecology and Entomology, Stellenbosch University, Matieland, South Africa

[4]Hans Hoheisen Research Station, Faculty of Veterinary Science, University of Pretoria, Pretoria, South Africa

[5]Department of Veterinary Tropical Disease, Faculty of Veterinary Science, University of Pretoria, Pretoria, South Africa

[6]Centre for Veterinary Wildlife Research, University of Pretoria, Onderstepoort, South Africa

[7]Department of Biology, Georgia Southern University, Statesboro, Georgia, USA

Correspondence
Renfu Shao, Centre for Bioinnovation, University of the Sunshine Coast, 90 Sippy Downs Drive, Sippy Downs, Queensland 4556, Australia.
Email: rshao@usc.edu.au

Funding information
University of the Sunshine Coast

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Abstract

The parvorder Rhynchopthirina contains three currently recognised species of lice that parasitize elephants (both African savanna elephant *Loxodonta africana* and Asian elephant *Elephas maximus*), desert warthogs (*Phacochoerus aethiopicus*) and Red River hogs (*Potamochoerus porcus*), respectively. The Asian elephant lice and the African savanna elephant lice are currently treated as the same species, *Haematomyzus elephantis* (Piaget, 1869), based on morphology despite the fact that their hosts diverged 8.4 million years ago. In the current study, we sequenced 23 mitochondrial (mt) genes of African savanna elephant lice collected in South Africa and analysed the sequence divergence between African savanna elephant lice and previously sequenced Asian elephant lice. Sequence comparisons revealed >23% divergence for the 23 mt genes as a whole and ~17% divergence for *cox1* gene between African savanna and Asian elephant lice, which were far higher than the divergence expected within a species. Furthermore, the mt gene sequence divergences between these lice are 3.76–4.6 times higher than that between their hosts, the African savanna and Asian elephants, which are expected for the co-divergence and co-evolution between lice and their elephant hosts. We conclude that (1) *H. elephantis* (Piaget, 1869) contains cryptic species and (2) African savanna and Asian elephant lice are different species genetically that may have co-diverged and co-evolved with their hosts.

KEYWORDS
co-divergence and co-evolution, DNA barcode, ectoparasites, elephant lice, *Haematomyzus elephantis*, mitochondrial genome, Rhynchopthirina

INTRODUCTION

There are approximately 5000 known species of parasitic lice associated with birds and mammals that are classified into five parvorders: Amblycera, Anoplura, Ischnocera, Rhynchopthirina and Trichodectera (De Moya et al., 2021; Durden & Musser, 1994; Price et al., 2003). The parvorder Rhynchopthirina comprises three currently recognised species in the family Haematomyzidae: *Haematomyzus elephantis* (Piaget, 1869; elephant louse), *H. hopkinsi* (Clay, 1963; desert warthog louse) and *H. porci* (Emerson & Price, 1988; Red River hog louse) (Price et al., 2003). Rhynchopthirina lice have snout-like prolonged anterior mouthparts that differentiate them from lice in the other parvorders (Price et al., 2003).

Although *H. elephantis* (Piaget, 1869) is currently recognised as the only louse species of both the African savanna elephant (*Loxodonta africana*) and the Asian elephant (*Elephas maximus*) (Price et al., 2003), the species status of elephant lice is not without contention. Five other species or subspecies of *Haematomyzus*, currently recognised as invalid, were described from African savanna and Asian elephants: *H. elephantis* (Walker, 1872, ex. Sri Lankan elephant, *E. maximus ceylanicus*), *H. elephantis sumatranus* (Fahrenholz, 1910, ex. Sumatran elephant, *E. maximus sumatranus*), *H. longirostris* (Piaget, 1869, ex. *L. africana*), *H. paradoxus* (Lahille, 1908, ex. *E. maximus*) and *H. prohoscideus* (Piaget, 1880, ex. *L. africana*). There is no published record to date of lice from the third living elephant species, the African forest elephant *Loxodonta cyclotis* (Price et al., 2003). African savanna and Asian elephant lice were recognised as different species in the five currently invalid species or subspecies. From a speciation and evolutionary perspective, if elephant lice co-diverged and co-evolved with their hosts, elephant lice would be expected to be different species or subspecies as African savanna and Asian elephants diverged 8.4 million years ago (median time; Kumar et al., 2017), are widely separated geographically and are classified into different genera in the family Elephantidae (Shoshani, 1998). In general, parasitic lice of mammals are highly host-specific and often co-diverge and co-evolve with their hosts, although host-switching also occurs (Barker, 1994). The current recognition and classification of elephant lice as a single species based on morphology (Ferris, 1931; Price et al., 2003) are difficult to account for given the long divergence and wide geographic separation between African savanna and Asian elephants.

In the current study we sequenced the partial mitochondrial (mt) genome of African savanna elephant lice. We compared the mt genome sequences between African savanna and Asian elephant lice. Asian elephant lice have a fragmented mt genome with 10 circular minichromosomes; each minichromosome has two to six genes (Shao et al., 2015). All other mammal lice in the parvorders Anoplura and Trichodectera studied to date also have fragmented mt genomes with 9–20 minichromosomes (Shao et al., 2009; Song et al., 2019). We also compared the mt genomes between elephant lice and their hosts. The sequence divergences revealed by our analyses indicate that African savanna and Asian elephant lice are different species that may have co-diverged and co-evolved with their hosts.

MATERIALS AND METHODS

Sample collection, DNA extraction and polymerase chain reaction amplification

Parasitic lice were collected in May 2019 from an African savanna elephant in Manyeleti Nature Reserve, Mpumalanga Province, South Africa. The specimens (RS460) consisted of one adult female louse and four eggs and were stored in 95% ethanol (Figure 1). The female louse specimen was mounted on a microscope slide for morphological examination. Genomic DNA was extracted from individual eggs (not pooled) using DNeasy Blood and Tissue kit (QIAGEN). Fragments of mt *cox1* (gene for cytochrome c oxidase subunit 1) and *rrnS* (gene for small subunit ribosomal RNA) were amplified by polymerase chain reaction (PCR) with broadly conserved primer pairs mtd6-mtd11 (for *cox1*) and 12SA-12SB (for *rrnS*); partial coding regions of multiple mt minichromosomes were amplified with primer pair ELF-ELR designed based on Asian elephant louse sequences (Shao et al., 2015, Supplementary Material S1). PrimeSTAR® MAX DNA Polymerase kit was used in PCR with the following optimised cycling conditions: 94°C for 1 min, 40 cycles of 98°C for 10 s, 53°C (for *cox1*) and 40°C (for *rrnS*) for 5 s, 72°C for 10 s and 72°C for 20 s. Specific primers for African savanna elephant lice were then designed from sequenced mt genes produced in the above steps. Primer pairs SKrrnSF-SKrrnSR, SKtrnYF-SKtrnYR and SKnad4LFO2-SKnad4LRO2 (Supplementary Material S1) were used to amplify full-length L_2-*rrnS* minichromsome, *Y-cox2-E* minichromsome and S_2-*R-nad4L-M-G-nad3* minichromsome, respectively (details of mt minichromsomes of the Asian elephant louse are reported in Shao et al., 2015) with the following cycling conditions: 94°C for 1 min, 50 cycles of 98°C for 10 s, 60°C for 5 s, 72°C for 30 s and 72°C for 30 s. PCR amplicons were visualised with agarose gel (1%) electrophoresis. PCR amplicon size was estimated by comparison with a 1-kb Ladder (Axygen Biosciences). Wizard® SV Gel and PCR Clean-Up System (Promega) were used to purify PCR amplicons for sequencing.

Illumina sequencing, data retrieval from sequence read archive and sequence analyses

Purified PCR amplicons from individual louse eggs produced above were sequenced with Illumina NovoSeq 6000 at Novogene (HK). Paired-end sequence reads produced were 250 bp each for mtd6-mtd11, 12SA-12SB and ELF-ELR amplicons, and were 150 bp each for SKrrnSF-SKrrnSR, SKtrnYF-SKtrnYR and SKnad4LFO2-SKnad4LRO2 amplicons. Illumina sequence reads from mtd6-mtd11, 12SA-12SB and ELF-ELR amplicons were assembled with Geneious Prime (version 2021.0.1, https://www.geneious.com/features/) using the published mt genome sequences of the Asian elephant louse (Shao et al., 2015; specimen number B1567; GenBank accession numbers KF933032-41) as the initial references to identify the mt genes of the African savanna elephant louse. The assembly parameters used were as follows: (1) minimum overlap 150 bp, (2) minimum overlap identity 80% or above, (3) maximum gaps per read 5% and (4) maximum gap size 5 bp. The *cox2*, *rrnS* and *nad4L* sequences of the African savanna elephant louse obtained in the steps above were used as the initial references to assemble the sequence reads from SKtrnYF-SKtrnYR amplicon, SKrrnSF-SKrrnSR amplicon and SKnad4LFO2-SKnad4LRO2 amplicon, respectively. The assembly parameters used were as follows: (1) minimum overlap 100 bp, (2) minimum overlap identity 95%, (3) maximum gaps per read 3% and (4) maximum gap size 3 bp. Consensus sequences were produced from assemblies with 50% threshold. Mitochondrial protein-coding and rRNA genes were identified with BLAST searches of the NCBI database (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Mitochondrial tRNA genes were identified with tRNAscan-SE and ARWEN based on their secondary structure (Laslett & Canback, 2008; Lowe & Eddy, 1997).

FIGURE 1 A female louse (left) and four louse eggs were collected from an African savanna elephant in Manyeleti Nature Reserve, Mpumalanga Province, South Africa. The louse and eggs were assigned the number RS460 in Renfu Shao's collection.

Genomic sequence reads of an Asian elephant louse (SRR5308122) and transcriptomic sequence reads of an Asian elephant louse (SRR2051491) were retrieved from the NCBI Sequence Read Archive (SRA). The SRA sequence reads were assembled with Geneious Prime using the published mt genome sequences of the Asian elephant louse (B1567, Shao et al., 2015) as the references. The assembly parameters were as follows: (1) minimum overlap of 100 bp for genomic reads (SRR5308122) and 60 bp for transcriptomic reads (SRR2051491), (2) minimum overlap identity of 90%, (3) maximum gaps per read of 3% and (4) maximum gap size 3 bp. Consensus sequences were produced with a 50% threshold. Mitochondrial gene identification was the same as described above.

Pairwise alignments of mt gene and genome sequences were generated using the NCBI BLAST tool *Global Align* (https://blast.ncbi.nlm.nih.gov/Blast.cgi) with the scoring parameters *Match/Mismatch 1/-2* and *Gap Costs Existence 5/Extension 2*. Sequence divergences (uncorrected p-distances) were calculated from pairwise alignments: (1) among the four elephant lice (i.e., RS460, B1567, SRR5308122 and SRR2051491), (2) between the African savanna elephant *L. africana* (GenBank accession number NC_000934) and the Asian elephant *E. maximus* (NC_005129) and (3) between eight pairs of closely related congeneric species of parasitic lice from different parvorders (Supplementary Material S2).

RESULTS

Twenty-three mitochondrial genes in nine minichromosomes were identified by analysis of Illumina sequence reads from African savanna elephant lice

We obtained 2,431,128 paired-end sequence reads, 250 bp each, from the mtd6-mtd11, 12SA-12SB and ELF-ELR amplicons, and 51,666,203 paired-end sequence reads, 150 bp each, from the *Y-cox2-E*, L_2-*rrnS* and S_2-*R-nad4L-M-G-nad3* amplicons produced from the African elephant louse eggs. Assembly of these Illumina sequence reads produced nine contigs, corresponding to nine mt minichromosomes reported in the Asian elephant louse (Table 1; Shao et al., 2015). Further analysis of these contigs identified 23 mt genes: eight protein-coding genes (*cob*, *cox1-3*, *nad1*, *nad3*, *nad4* and *nad4L*), two rRNA genes (*rrnL* and *rrnS*) and 13 tRNA genes (*A*, *C*, *E*, *F*, *G*, L_2, *M*, *R*, S_2, *T*, *V*, *W* and *Y*). Six of these genes were partially sequenced, and the other 17 genes were sequenced in full length (Table 1). No sequence variation was observed between different louse eggs. Two pseudogenes, ^{P}*nad5* and ^{P}*nad6*, were also identified. These pseudogenes were much shorter than the full-length *nad5* and *nad6* but shared high sequence identity with sections of *nad5* and *nad6* of the

TABLE 1 Mitochondrial genes and minichromosomes of the African savanna elephant lice identified in the current study.

Minichromosome	Size of gene and NCR in each minichromosome (bp)	GenBank accession number
cob-A-W-F-* ^{P}*nad6*-NCR*	929-69-77-78-93-115	OQ834926
*cox1**	610	OQ834927
NCR*-^{P}*nad5*-NCR-*Y-cox2-E*-NCR*	200-207-120-70-684-70-200	OQ834928
*cox3**-NCR*	375-130	OQ834929
NCR*-*T-nad1**	132-75-510	OQ834930
S_2-*R-nad4L-M-G-nad3*-NCR	76-67-282-77-73-342-3095	OQ834931
nad4-C*-NCR*	101-72-87	OQ834932
NCR*-L_2-*rrnS*-NCR*	200-74-845-200	OQ834933
rrnL-V*-NCR*	390-72-120	OQ834934

Note: Gene names are *cob* for cytochrome b, *cox1-3* for cytochrome c oxidase subunits 1–3, *nad1-5* and *nad4L* for NADH (nicotinamide adenine dinucleotide + hydro) dehydrogenase subunits 1–5 and 4L, *rrnS* and *rrnL* for small and large subunits of ribosomal RNA, and single-letter names corresponding to amino acids are for tRNAs (*A* for alanine, *C* for cysteine, *E* for glutamic acid, *F* for phenylalanine, *G* for glycine, L_2 for leucine (anticodon TAG), *M* for methionine, *R* for arginine, S_2 for serine (anticodon TGA), *T* for threonine, *V* for valine, *W* for tryptophan and *Y* for tyrosine). NCR is for large non-coding region where no genes can be found. Genes and NCR labelled with * are partially sequenced. Genes and NCR not labelled with * are fully sequenced. ^{P}*nad5* is for a pseudo *nad5* gene with 76.6% pairwise identity to a middle section of *nad5* of the Asian elephant louse reported in Shao et al. (2015). ^{P}*nad6* is for a pseudo *nad6* gene with 55.9% pairwise identity to the 5′-end section of *nad6* of the Asian elephant louse reported in Shao et al. (2015).

Asian elephant louse reported in Shao et al. (2015) (Table 1). Of the 37 mt genes typical of animals, 14 genes were not found: *atp6*, *atp8*, *nad2*, *nad5*, *nad6*, *D*, *H*, *I*, *K*, L_1, *N*, *P*, *Q* and S_1. The S_2-*R-nad4L-M-G-nad3* minichromosome, 4,062 bp in size, was fully sequenced, whereas the other eight minichromosomes were partially sequenced (Supplementary Material S3 and Table 1). The majority of the S_2-*R-nad4L-M-G-nad3* minichromosome is the large non-coding region (NCR), 3,095 bp in size, in contrast to a much shorter region that contains genes, 967 bp in size (Supplementary Material S3). Both the *Y-cox2-E* minichromosome (~5150 bp) and the L_2-*rrnS* minichromosome (~5050 bp) were amplified at full length and sequenced. The regions that contain genes were assembled in full length for these two minichromosomes (Table 1). However, the large NCRs of these two minichromosomes could not be assembled at full length reliably despite our repeated attempts with varying assembly parameters, apparently due to the length of these large NCRs (>4100 bp), the long repeats in these regions and the short read length of Illumina platforms.

African and Asian elephant lice differ by >23% in mitochondrial gene sequences

Pairwise alignments of the mt gene sequences of African and Asian elephant lice revealed >23% sequence divergence for the 23 mt genes combined and ~17% sequence divergence for *cox1* gene (Supplementary Material S4). Of the 23 genes, *trnM* had the lowest divergence, 9.09%, whereas *nad4* had the highest divergence, ~38%, between African and Asian elephant lice (Supplementary Material S4). Among the three Asian elephant lice, the sequence divergence was <0.5% for *cox1* gene alone and <1% for 33 mt genes combined (Supplementary Material S5). Of the 33 genes, 12 tRNA genes had identical sequences among the three Asian elephant lice with the highest divergence being 4.11% for *trnV*. We also compared the mt gene sequence divergence between the African savanna elephant and the Asian elephant. The sequence divergence between these two elephant species was 4.52% for *cox1* gene and slightly >5% for the entire mt genomes regardless of whether the D-loop region was included in the analysis (Supplementary Material S6).

Divergence in *cox1* sequence between closely related congeneric species of parasitic lice

We compared *cox1* sequence divergence between eight pairs of closely related congeneric species of parasitic lice available to date in the GenBank database (Supplementary Material S2). These congeneric species of parasitic lice were from four parvorders: Amblycera, Anoplura, Ischnocera and Trichdectera. The *cox1* sequence divergence varied from 6% to 27% among the eight pairs of congeneric species. The lowest *cox1* sequence divergence (6%) was between the wild pig louse (*Haematopinus suis*) and the domestic pig louse (*Haematopinus apri*). The highest *cox1* sequence divergence (27%) was between the sheep biting louse (*Bovicola ovis*) and the goat-biting louse (*Bovicola caprae*). The *cox1* sequence divergence observed in the current study between African and Asian elephant lice (~17%) was very close to the divergence: (1) between *Franciscoloa pallida* and *Franciscoloa funerei* (16%), (2) between *Pthirus pubis* and *Pthirus gorilla* (16%) and (3) between *Pediculus humanus* and *Pediculus schaeffi* (20%) (Supplementary Material S2).

DISCUSSION

Our results of mt gene sequence comparison indicate that the elephant louse *H. elephantis* (Piaget, 1869) contains cryptic species. African savanna elephant lice are very likely a different species from Asian

elephant lice. The ~17% divergence in *cox1* gene sequence between African and Asian elephant lice (Supplementary Material S4) is far higher than that observed within an animal species, which is usually <2% (Ratnasingham & Hebert, 2013) such as the <0.5% *cox1* sequence divergence observed in the current study among the three Asian elephant lice (Supplementary Material S5). Most animal species have >4% *cox1* sequence divergence from their closest sister species (Hajibabaei et al., 2006; Hebert et al., 2003), for example, 8.69% divergence between humans (GenBank accession number NC_012920) and chimpanzees (NC_001643). This is also true for the parasitic lice that have been studied to date. The *cox1* sequence divergence varied from 6% to 27% among the eight pairs of closely related congeneric species of parasitic lice from four parvorders (Supplementary Material S2). In addition to *cox1*, the >23% sequence divergence of 23 mt genes combined between African and Asian elephant lice also supports the notion that they are substantially different (Supplementary Material S4), which is in stark contrast to the <1% sequence divergence of 33 mt genes combined among the three Asian elephant lice (Supplementary Material S5). The observation that two pseudogenes (^{P}*nad5* and ^{P}*nad6*) are present in the African elephant louse (Table 1) but absent in the Asian elephant louse (Shao et al., 2015) is consistent with mt gene sequence divergence comparison, supporting African and Asian elephant lice being substantially different genetically.

Parasitic lice are wingless insects, and there are many documented cases of parasitic lice co-diverging and co-evolving with their hosts (Kim, 1985; Light et al., 2010; Price et al., 2003; Reed et al., 2004). This is most likely the case for the elephant lice. The African savanna elephant (*L. africana*) and the Asian elephant (*E. maximus*) are geographically separated and distinct from one another in morphology, thus being classified into different genera in the family Elephantidae (Shoshani, 1998). According to TimeTree (http://www.timetree.org/), *L. africana* and *E. maximus* diverged 8.4 million years ago (MYA, median time) (Kumar et al., 2017). The divergence time between *L. africana* and *E. maximus* is long enough for their parasitic lice to evolve into different species. A comparison can be made with the parasitic lice of humans (*Homo sapiens*) and chimpanzees (*Pan troglodytes*). *H. sapiens* and *P. troglodytes* diverged 6.4 MYA (median time according to the TimeTree). Human lice (*P. humanus*) and chimpanzee lice (*P. schaeffi*) are distinctly different species with a divergence of 20% in *cox1* sequence (Supplementary Material S2).

It has been reported in previous studies that in the cases of co-evolution between parasitic lice and their hosts, mt genes evolve much faster in lice than in their hosts, likely due to elevated mutation rate, short generation time, small population size of lice and founder events in louse transmission to new hosts (Johnson et al., 2014; Page et al., 1998). In bird lice, *cob* gene evolves 2–3 times faster in lice than in their hosts (Page et al., 1998). In the lice of humans and chimpanzees, *cob* and *cox1* evolve 2.3 times faster in lice than in their hosts, and all mt protein-coding genes combined evolve 2.9 times faster in lice than in their hosts (Johnson et al., 2014; Reed et al., 2004). Discrepancies in the rate of mt gene evolution are also observed between elephant lice and their hosts. The sequence divergence is 4.52% for *cox1* alone and >5% for all mt genes combined between African savanna and Asian elephants (Supplementary Materials S6). The *cox1* divergence between African and Asian elephant lice, however, is ~17% (Supplementary Material S4), which is 3.76 times higher than that between the African and Asian elephants. The divergence of 23 mt genes combined between African and Asian elephant lice is >23% (Supplementary Material S4), which is ~4.6 times higher than that of all mt genes combined between the African and Asian elephants. These discrepancies in mt gene evolution indicate that African savanna and Asian elephant lice may have co-diverged and co-evolved with their respective hosts.

In light of the genetic evidence presented in the current study, we suggest that the taxonomy and classification of elephant lice should be reviewed and revised to recognise one species on the African savanna elephants and another species on the Asian elephants. This should involve detailed morphological comparisons between lice from both elephant species. It is unknown currently whether any lice parasitize the African forest elephant (*L. cyclotis*). If parasitic lice are found on *L. cyclotis*, their taxonomic status should also be determined based on both genetic and morphological evidence.

AUTHOR CONTRIBUTIONS

Sarah Kelly: Conceptualization; methodology; data curation; formal analysis; investigation; writing – original draft; validation. **Yalun Dong:** Software; validation. **Wei Wang:** Methodology. **Sonja Matthee:** Writing – review and editing; resources. **Jeanette M. Wentzel:** Resources. **Lance A. Durden:** Conceptualization; resources; writing – review and editing. **Renfu Shao:** Conceptualization; formal analysis; validation; supervision; funding acquisition; writing – review and editing; writing – original draft; data curation; project administration.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

Annotated mitochondrial genome sequences of the African savanna elephant lice produced in the current study are available in GenBank (accession numbers OQ834926-OQ834934; https://www.ncbi.nlm.nih.gov/genbank/); raw Illumina sequence data are available in the NCBI Sequence Read Archive (SRA) database (BioProject accession number PRJNA1021748). Genomic sequence reads of an Asian elephant louse (SRR5308122) and transcriptomic sequence reads of an Asian elephant louse (SRR2051491) are available in the SRA database (https://www.ncbi.nlm.nih.gov/sra).

ETHICS STATEMENT

The protocol for collecting lice from African elephants received Ethics Approval from Stellenbosch University (ACU-2019-13247). Lice were

collected under Mpumalanga province Research permit number MPB. 5756, and DALRDD permit number 12/11/1/1/6/1562(HP).

ORCID

Renfu Shao https://orcid.org/0000-0002-1686-4968

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

Supplementary Material S1. PCR primers used to amplify the mitochondrial genes or minichromosomes of the African savanna elephant louse.

Supplementary Material S2. Mitochondrial *cox1* gene sequence divergence between closely related congeneric species of parasitic lice.

Supplementary Material S3. The fully sequenced *S_2-R-nad4L-M-G-nad3* mitochondrial minichromosome of African savanna elephant louse (RS460). *trnG*, *trnM*, *trnR* and *trnS_2* are tRNA genes for amino acids glycine, methionine, arginine and serine respectively. *nad3* and *nad4L* are for NADH dehydrogenase subunits 3 and 4L. SKnad4LFO2 and SKnad4LRO2 are the primer pair that amplifies the entire *S_2-R-nad4L-M-G-nad3* minichromosome.

Supplementary Material S4. Mitochondrial gene sequence divergence between African (RS460) and Asian elephant lice (B1567, SRR5308122 and SRR2051491).

Supplementary Material S5. Mitochondrial gene sequence divergence among Asian elephant lice (B1567, SRR5308122 and SRR2051491).

Supplementary Material S6. Mitochondrial *cox1* gene and genome sequence divergence between African savanna elephant (*Loxodonta africana*, GenBank accession number NC_000934) and Asian elephant (*Elephas maximus*, NC_005129).

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