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SHORT NOTE

Intragenomic variation in ITS2 rDNA in the louse of humans, *Pediculus humanus*: ITS2 is not a suitable marker for population studies in this species

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Abstract

The two internal transcribed spacers (ITS) of ribosomal DNA are often used as markers of populations of insects. We studied the ITS2 of the head lice and body lice of humans, to determine whether this gene is a suitable marker of populations of these insects. ITS2 sequences were amplified by PCR from lice from four different countries: Australia, China, Japan and the USA. Direct cycle-sequencing of some of these PCR products gave equivocal nucleotide chromatograms. This indicated that some lice had more than one ITS2 sequence, so we cloned PCR products from these lice. Temperature gradient gel electrophoresis (TGGE) revealed that 50 of the 67 clones we screened had different nucleotide sequences. All lice had several ITS2 types, including those with unequivocal chromatograms. A phylogenetic tree of 15 different ITS2 sequences showed that the sequences from individual lice were not monophyletic. We conclude that the ITS2 is not a useful marker of populations for *Pediculus humanus*.

Keywords: *Pediculus humanus*, *Pediculus capitis*, lice, ITS2, concerted evolution.

Introduction

The two internal transcribed spacers of ribosomal DNA – ITS1 and ITS2 – are often used as markers of populations of insects (Clark *et al.*, 2001; Hackett *et al.*, 2000; Mukabayire *et al.*, 2001). Usually the nucleotide sequences of these spacers evolve in concert within a breeding population (Liao, 1999). The mechanism(s) of concerted evolution are not known; however, gene conversion, gene amplification and DNA recombination, repair and replication mechanisms have all been proposed (Liao, 1999).

Linnaeus first described the lice of humans as *Pediculus humanus* in the 10th edition of *Systema Naturae* (Linnaeus, 1956). In the 12th edition of *Systema Naturae*, he described lice from the hair and lice from the clothes as different varieties (cited in Ferris, 1935). Since then, there has not been a consensus in the scientific community on the specific status of these two 'varieties' of lice. Some workers think they are separate species, whereas others think they are subspecies of *P. humanus*.

We studied the ITS2 of the lice that infest the hair and clothes of humans, *Pediculus humanus capitis* and *Pediculus humanus humanus*, respectively, to determine whether this gene is a suitable marker for populations of these lice. Some nucleotide chromatograms indicated that more than one ITS2 sequence was present in an individual louse. This stimulated us to explore the extent of variation in ITS2 in these lice. We found: (i) high levels of intragenomic variation in the ITS2; (ii) that nucleotide variation was present, even in lice that had unequivocal nucleotide chromatograms; and (iii) that the ITS2 sequences in these lice were not monophyletic for individual lice.

Results

We found evidence of intragenomic variation in the ITS2 of all six lice. Indeed, 50 different types of ITS2 were found in 67 clones from the six lice that were compared with TGGE (Table 1, Fig. 1). Seventeen types of ITS2 were found at least twice by temperature gradient gel electrophoresis (TGGE). In each case, the identical types of ITS2 were found in the same individual louse.

Fifteen clones from four lice were sequenced – seven from an Australian louse (louse no. 1), three from a Chinese louse (louse no. 3), two from a Japanese louse (louse no. 5) and three from an American louse (louse no. 6) (Table 1, Fig. 2). These ITS2 sequences were 342–393 bp

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Table 1. Intragenomic variation in the six lice studied

Louse identifier no.	Louse type	Country of origin	No. of ITS2 clones sampled	No. of ITS2 types identified by TGGE (no. sequenced)
1	head	Australia	29	17 (7)
2	head	Australia	17	15 (0)
3	body	China	3	3 (3)
4	body	Japan	10	7 (0)
5	body	Japan	2	2 (2)
6	body	USA	6	6 (3)
Total			67	50 (15)

TGGE = temperature gradient gel electrophoresis. Numbers in brackets show the number of ITS2 types that were sequenced (see Fig. 3).

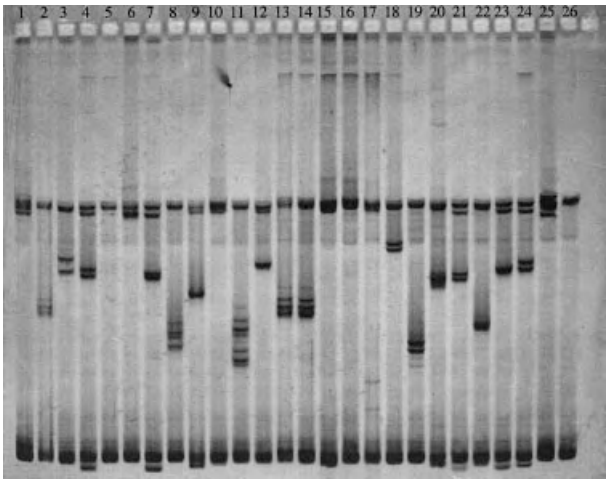

Figure 1. Temperature gradient gel electrophoresis (TGGE) of ITS2 fragments that had been cloned shows much intragenomic variation in nucleotide sequences of ITS2. Lane 1, clone from louse no. 1 from Australia; lanes 2–18 louse no. 2 from Australia; lanes 19–24, clones from the USA louse; and lanes 25–26, clones from louse no. 1 from Australia. Louse numbers refer to those given in Table 1.

long; the alignment was 430 bp (Fig. 2). Variation in the clone sequences corresponded to equivocal nucleotides in the chromatogram from the direct-cycle sequencing of PCR products. The alignment shows that some, but not all, sequences in lice from Australia and Japan share a 25 bp indel, and some sequences of other lice from Australia, China, and the USA share a 10 bp indel (Fig. 2). Since these indels may have been caused by one mutation event, they were each represented as a single base change in the phylogenetic analyses (see Fig. 3). A maximum parsimony (branch and bound) search was executed with PAUP 4.0b10 (Swofford, 2002) with gaps treated as a fifth base; this produced nine different trees. These trees, plus 1000 cycles of bootstrap resampling, indicated that sequences from individual lice were not monophyletic (Fig. 3).

Discussion

All the lice we studied had intragenomic variation of the ITS2, including those that had unequivocal nucleotide chromatograms from direct cycle-sequencing of PCR products. For example, the two lice from Japan had unequivocal chromatograms from direct cycle-sequencing of ITS2. Yet, nucleotide sequences of clones from one of these lice revealed an indel of 25 bp, and TGGE of 12 clones from both lice revealed nine different ITS2 sequences. A similar level of intragenomic variation was found in lice that had equivocal chromatograms from direct cycle-sequencing, and this variation corresponded to ambiguous sites in the nucleotide chromatograms from direct cycle-sequencing. Our results indicate that head and body lice have very high levels of intragenomic variation of the ITS2, and that this variation is not always discernible from the chromatograms of nucleotides from the direct cycle-sequencing of PCR products. This could occur if the PCR conditions favoured some ITS2 sequences over others.

Phylogenetic analysis revealed that sequences from an individual louse were not monophyletic (Fig. 3). One explanation for our results is that some of these sequences are from pseudogenes. However, the flanking sequences of about 150 bp of the 3′ end of the 5.8S and 100 bp of the 5′ end of the 28S, were identical in all sequences, except for a single nucleotide substitution in one clone from an Australian louse (louse no. 1). PCR error from some DNA polymerases has been shown to cause simple repetitive sequences of DNA to increase in length (Schlotterer & Tautz, 1991). Since our sequences show a wide spectrum of variation, from single nucleotide substitutions to 25 bp indels, it is unlikely that PCR error is responsible for the variation we observed. Another possible explanation is that the mutation rate is higher than the rate of homogenization at the ITS2 locus of these lice. Schlotterer & Tautz (1994) suggested that intrachromosomal recombination events occur at higher rates than recombination between homologous chromosomes in *Drosophila melanogaster*. It is possible that a similar situation exists in *Pediculus humanus*. Thus, there may be many different ITS2 types on different chromosomes that have not yet been homogenized among chromosomes. It is also possible that host blood or bacterial symbionts from the lice could have contaminated the PCR products, however, this seems unlikely, since the 5.8S sequence that flanked the ITS2 sequence matched most

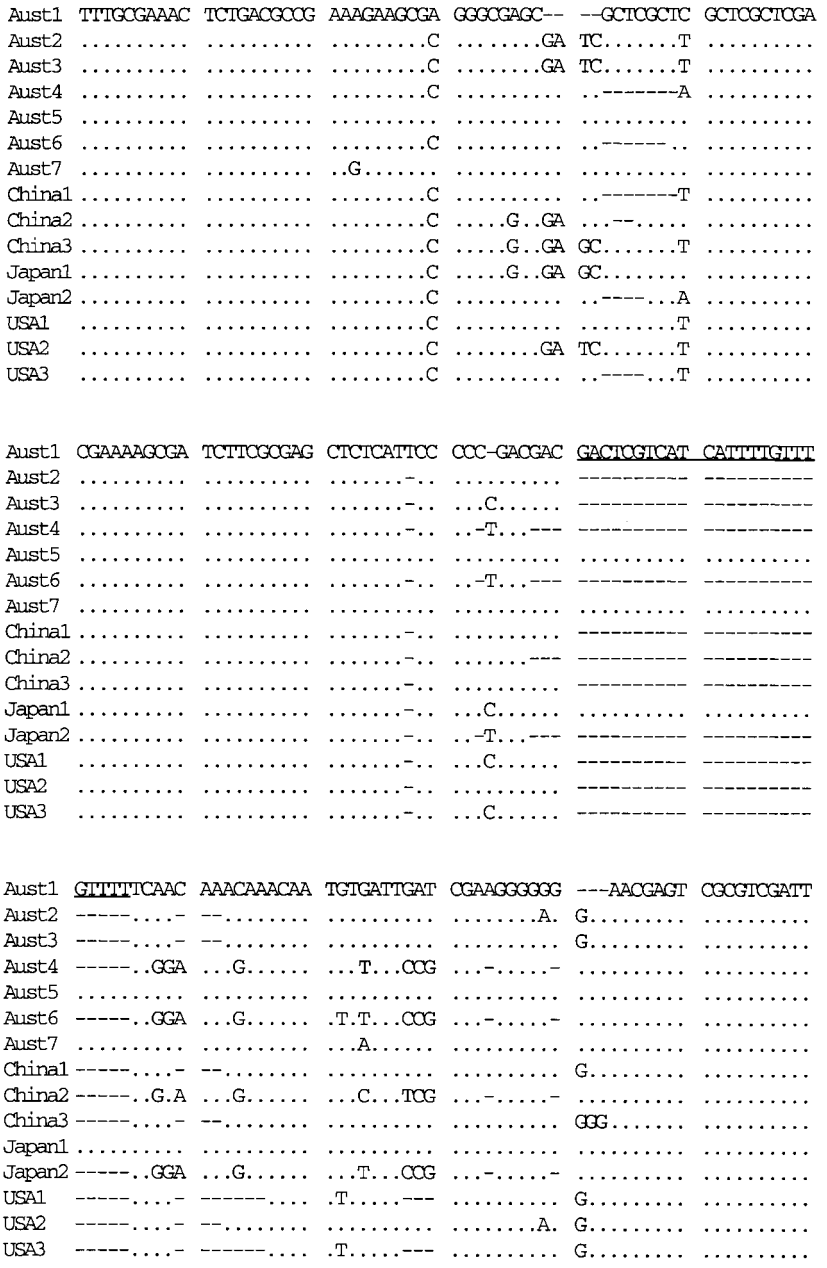

Figure 2. Alignment of the 15 ITS2 sequences of clones from four lice from four countries: one louse with seven sequences from Australia (louse no. 1), one louse with three sequences from China (louse no. 3), one louse with two sequences from Japan (louse no. 4) and one louse with three sequences from the USA (louse no. 6). Louse numbers refer to those given in Table 1. Dots '.' indicate identity with the first sequence; dashes '–' indicate no nucleotide at that position. Note that the 25 bp and 10 bp indels (underlined) were treated as a single nucleotide in the phylogenetic analyses.

closely to an insect in GenBank. The 28S sequence at the 3′ end of our PCR products was unusual in that it most closely matched a fish. However, the chance of contamination from this source was remote, and so our search of the database was probably confounded by the short (100 bp) 28S sequence. We conclude that the most likely explanation for why ITS2 is not phylogenetically informative for populations of *Pediculus humanus* is that the ITS2 copies do not evolve in concert.

A wide range of levels of intragenomic variation in the ITS regions have been found in insects such as aphids (Fenton *et al*., 1998), *Anopheles nuneztovari* mosquitoes (Onyabe & Conn, 1999), black flies (Tang *et al*., 1996) and tiger beetles (Vogler & DeSalle, 1994). However, our results show an unprecedented level of intragenomic variation for the ITS2, a gene that is usually more conserved than ITS1 (El *et al*., 2001; Navajas *et al*., 1999; Tang *et al*., 1996). Rich *et al*. (1997) sequenced eight ITS2 clones that differed by up to 4.5%, from one *Ixodes dammini* tick, and Wesson *et al*. (1992) found that 10 clones of the ITS2 from one *Aedes simpsoni* mosquito varied by 0.46%. We found 50 different sequences from 67 clones, from which 148 positions were variable in a 430-bp alignment (Fig. 2). Vogler & DeSalle (1994) found intragenomic variation in the ITS1 of the tiger beetle *Cicindela dorsalis* at a level comparable to our results. They found 42 different sequences from 50 clones,

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Aust1   CAGAAAAATA AAAAAAAAAA A---TCTCAT T--------- ACGAAGCGCG AACGATGACG
Aust2   ......G.A. ........-- -......... .TCGATTTTT T......... .........
Aust3   ......G.A. .......... -......... .TCGATTTTT T......... .........
Aust4   ......G... .......... .......... .......... .......... .........
Aust5   .......... .......... .A........ .......... .......... .........
Aust6   ......G... .......... .A........ .......... .......... .........
Aust7   .......... .......... .AA....... .......... .......... .........
China1  A.....G.A. .......... .......... .TCGATTTTT T......... .........
China2  ......G... .........- -......... .......... .......... .........
China3  ......G.A. .......... -......... .TCGATTTTT T......... .........
Japan1  ......G... .........- -......... .......... .......... .........
Japan2  ......G... .......... .A........ .......... .......... .........
USA1    ......G.A. .......... .AAA...... .TCGATTTTT T......... .........
USA2    ......G.A. .........- -......... .TCGATTTTT T......... .........
USA3    ......G.A. .........- -......... .TCGATTTTT T......... .........

Aust1   ACGACG---- -----AACCG AAATCATCGT GCGCGCTCTC TCTGAAAAGA AAC-----GA
Aust2   ......GCG. .......... .......... .......... .......C.. ...GAAGC..
Aust3   ......ACG. .......T.. .......... .......... ...A...C.. G..GAAAC..
Aust4   .......... .......... .......... .......... .......... .........
Aust5   .......... .......... .......... .......... .......... .........
Aust6   ......ACG. .......... .......... .......... .......... G........
Aust7   .......... .......... .......... .......... .......... .........
China1  ......ACGA CG........ .......... .......... .......C.. .........
China2  ......ACGA CGACG..... .......... .......... .......... .........
China3  ......ACG. .......... .......... .......... .......C.. .........
Japan1  ......ACG. .......... .......... .......... .......C.. .........
Japan2  .......... .......... .......... .......... .......... .........
USA1    ......ACG. .......... .......... .......... .......C.. ...GAAAC..
USA2    ......ACG. .......... .......... .......... .......C.. ...GAAGC..
USA3    ......ACG. .......... .......... .......... .......C.. ...GAAAC..

Aust1   GAGAGAGAG- -CCGCCGAGG GATCCGGGGA AAAAGCAACT GCGACGACGA CTACGTTTTC
Aust2   .......--. .......... .......... .........- --......-- ----......
Aust3   .......--. .......... .......... .........- .-.-.----- ----......
Aust4   .........A G......... .......... .......... .......... .........
Aust5   .......... .......... .......... .......... .......... .........
Aust6   .........A G......... .......... .......... .......... .........
Aust7   .......... .......... .......... .......... .......... .........
China1  .......---. G......... .......... .........- --........ .---.....
China2  ........-. G......... .......... ......---- --........ .........
China3  .......---. G......... .......... .........- .-.-.----- ----......
Japan1  .........A G......... .......... .......... .......... .........
Japan2  .........A G......... .......... .......... .......... .........
USA1    .......---. G......... .......... .........- .-.-.----- ----......
USA2    .......---. G......... .......... .........- --......-- ----......
USA3    .......---. G......... .......... .........- .-.-.----- ----......
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Figure 2. *continued*

of which 112 positions were variable in a 301 bp alignment of these sequences.

Tang *et al*. (1996) suggested that concerted evolution of ITS1 had occurred in each of the isolated black fly populations they studied, but subsequent interbreeding between these isolated populations led to the presence of more than one ITS1 sequence in individual flies. Intragenomic variation of the ITS2 in the parasitic nematode *Haemonchus contortus* was also interpreted by the researchers as a result of interbreeding populations (Gasser *et al*., 1998). Vogler & DeSalle (1994) noted that the species of insects reported to have heterogeneous ITS1 were all from complexes of sibling species. This is interesting, since some people consider head and body lice to be different species, *P. capitis* and *P. humanus*, whereas other people argue that head and body lice belong to the same biological species. The latter view head and body lice as different varieties or subspecies of one species, *P. humanus*. It is possible that louse populations are, or have been, isolated ecologically and/or geographically and that this isolation led to the divergence of ITS2 sequences. Indeed, populations of head and body lice from different countries were found to differ significantly in the frequencies of cytochrome oxidase I (COI) haplotypes (Leo *et al*., 2002). Recent migration among these populations may explain why there are so many copies of ITS2 in *P. humanus*. We conclude that the ITS2 is not a useful marker of populations for *Pediculus humanus*, and advise caution to those considering the use of this gene for any population studies.

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Aust1  TCGTCGTTTT TTTT--CCAA GTCGCCGCCG CCGCATCGAG CGCGAATGAT AAT---ACAC
Aust2  .......... ....T..... ....T..... .......... .......A.. ..........
Aust3  .......... ....T..... ....T..... .......... .......A.. ..........
Aust4  .......... ....T..... T...T..... .......... .......A.. ........T.
Aust5  .......... .......... ....T..... .......... .......... ....AC....
Aust6  .......... ....T..... ....T..T.. .......... .......... ......--..
Aust7  .......... .......... ....T..... .......... .......... ..........
China1 .......... ....T..... ....T..... .......... .......A.. ..........
China2 .......... ....T..... T...T..... .......... .......A.. ..........
China3 ....A..... .......... T...T..... .......... .......A.. ...AAT....
Japan1 .......... ....T..... T...T..... .......... .......A.. ..........
Japan2 .......... ....T..... T...T..... .......... .......A.. ........T.
USA1   .......... ....TT.... ....T..... .......... .......A.. ..........
USA2   .......... ....T..... ....T..... .......... .......A.. ..........
USA3   .......... ....T..... ...---.... .......... .......A.. .........T

Aust1  ACACACACAC
Aust2  ....T.----
Aust3  ....T.----
Aust4  ....T.T.--
Aust5  ..........
Aust6  ....------
Aust7  ..........
China1 ....T.----
China2 ....T.----
China3 ....T.----
Japan1 ....G.----
Japan2 ....T.T.--
USA1   -.-.------
USA2   ....T.----
USA3   -.-.------
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Figure 2. *continued*

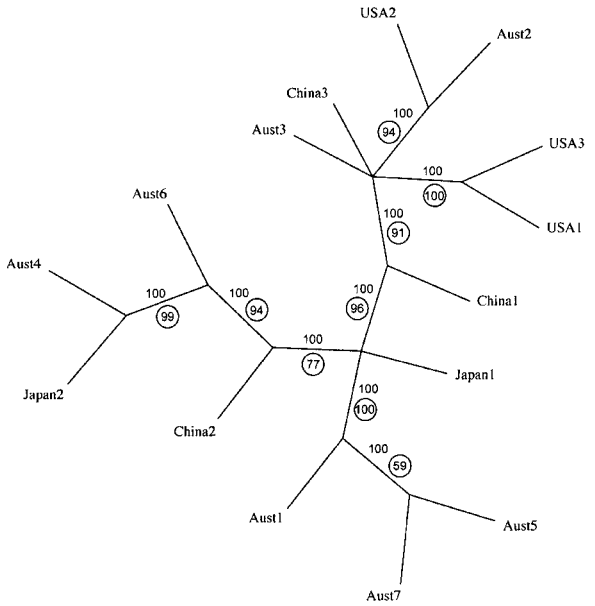

Figure 3. An unrooted semistrict consensus tree from the nine trees generated by a maximum parsimony (branch and bound) search of the 15 sequences of Fig. 2, in PAUP 4.0b10 (Swofford, 2002). Regular numbers show the percentage of the nine trees that shared a branch (the group frequencies). Bootstrap values are encircled and are shown as a percentage of 1000 replicates. Louse numbers refer to those given in Table 1.

Experimental procedures

DNA analyses

We studied two head lice from Australia, one body louse from China, two body lice from Japan and one body louse from a colony originating from the USA (Table 1). DNA was extracted from the whole lice by either a phenol-chloroform method (Sambrook *et al.*, 1989) or with chelex beads (Bio-Rad, USA). The latter involved crushing a louse with a micropestle in a tissue grinding tube partially immersed in liquid nitrogen. One millilitre of boiling 5% chelex beads in 1× TE buffer, with RNaseA (1 μl of 25 mg/ml RNaseA for every 100 ml of chelex in 1× TE buffer), was added to the tube and then put in boiling water for 15 min. Tubes were cooled for 10 min at –20 °C and then spun in a microcentrifuge at 12 000 ***g*** for 10 min. Three microlitres of the top layer was used in each 25 μl PCR reaction. The primer 3sa, for the 5.8S rDNA (forward: 5′-CTAAGCGGTGGATCACTCGG-3′) and the primer 28sra, for the coding region for the large ribosomal subunit (5′-CTTTTCGCTCGCCGTTACT-3′) were used to amplify the entire ITS2 plus 149 bp of the 5.8S and 102 bp of the 28S rDNA. PCR reactions contained: 1–3 μl of DNA, 2.5 μl of Reaction Buffer IV at 10× concentration (AB Gene, UK), 2.25 μl of $MgCl_2$ (25 mM), 1.1 μl of dNTPs (5 mM), 0.3 μl of each primer (10 μM), 0.2 μl of Red Hot *Taq* polymerase (5 units/μl) (AB Gene, UK) and MilliQ water to a final volume of 25 μl. The cycling conditions were: 94 °C for 1 min; 35 cycles of 30 s at 94 °C, 30 s at 50 °C and 60 s at 68 °C; and a final extension time of 5 min at 68 °C. PCR products were visualized under UV light after electrophoresis in an ethidium bromide-stained agarose gel. PCR products were purified with QIAquick columns (Qiagen, Netherlands) and sequenced directly (DyeDeoxy Terminator; PE Applied Biosystems, USA) with both PCR primers (above), using an ABI 377 gene sequencer.

The nucleotide chromatograms from the direct cycle-sequencing of some PCR products of ITS2 were equivocal, i.e. there were multiple peaks at some nucleotide sites. When these direct cycle-sequencing experiments were repeated up to four times, the same sites were equivocal. This indicated to us that some lice had more than one ITS2 sequence. So we cloned the ITS2 PCR products from these lice, and from lice with unequivocal chromatograms, into a p-Gem T vector, as recommended by the manufacturer (Promega, USA). Individual clones were then sequenced after PCR amplification of the insert, as above.

Nucleotide sequence analyses

The 5′ and 3′ ends of ITS2 were identified by alignment with the 3′ end of the 5.8S rDNA of arthropods (Wesson & Collins, 1992) and the 5′ end of the 28S rDNA of a range of vertebrates and invertebrates (Qu *et al*., 1988). The sequences were aligned by eye in a Sequencher 3.1.1 (Gene Codes Corporation, USA), then the partial 5.8S (150 bp) and 28S (100 bp) sequences were compared to sequences in GenBank to check that the ITS2 of the lice had been amplified. We then executed a maximum parsimony (branch and bound) search in Paup 4.0b10 (Swofford, 2002) to find the most parsimonious (shortest) tree(s) for the 15 ITS2 sequences. The robustness of the branches in these trees was tested with 1000 cycles of bootstrap resampling.

Temperature gradient gel electrophoresis (TGGE) analyses

Temperature gradient gel electrophoresis (TGGE) was used to screen 67 clones. Polyacrylamide gels (36 ml of 10 M urea, 0.9 ml 50× ME Buffer, 2.25 ml of 40% glycerol, and 7.5 ml of 30% acrylamide with 136 μl of 10% ammonium persulphate and 75 μl TEMED) were poured into a TGGE glass mould, and then allowed to set for 60 min. The gel was then placed on a ceramic plate with a temperature gradient ranging from 35 °C at the negatively charged end to 55 °C at the positively charged end. PCR fragments from the clones were heteroduplexed with a reference clone, a clone from the Chinese body louse. This was done by placing 5–20 ng of DNA from a clone and 5–20 ng of DNA from the reference clone in a tube with 3.2 μl of 10 M urea and 0.8 μl of 10× ME buffer and dye (0.05% Bromophenol Blue and 0.05% Xylene Cyanol FF). MilliQ water was added to a total volume of 6 μl. The tube was then placed on a heating block with the following conditions: 94 °C for 1 min, then a decrease in temperature in increments of 0.7 °C until roughly 35 °C was reached. Then the tube was removed, spun down and allowed to cool to room temperature. The heteroduplexed samples were run on the gel at 300 V for 3 h 40 min. The gel was silver-stained, and the clones were identified by the pattern of bands.

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