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Research Article

Phylogenetic incongruence inferred with two mitochondrial genes in *Mepraia* spp. and *Triatoma eratyrusiformis* (Hemiptera, Reduviidae)

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Abstract

Mitochondrial DNA (mtDNA) is widely used to clarify phylogenetic relationships among and within species, and to determine population structure. Due to the linked nature of mtDNA genes it is expected that different genes will show similar results. Phylogenetic incongruence using mtDNA genes may result from processes such as heteroplasmy, nuclear integration of mitochondrial genes, polymerase errors, contamination, and recombination. In this study we used sequences from two mitochondrial genes (*cytochrome b* and *cytochrome oxidase subunit I*) from the wild vectors of Chagas disease, *Triatoma eratyrusiformis* and *Mepraia* species to test for topological congruence. The results showed some cases of phylogenetic incongruence due to misplacement of four haplotypes of four individuals. We discuss the possible causes of such incongruence and suggest that the explanation is an intra-individual variation likely due to heteroplasmy. This phenomenon is an independent evidence of common ancestry between these taxa.

Keywords: heteroplasmy, sylvatic vectors, Chagas disease, *gajardoi*, *spinolai*, cytochrome.

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Introduction

The simplicity of maternal inheritance without biparental recombination, high rates of variability and large copy numbers are key features that make mitochondrial DNA (mtDNA) highly useful in resolving questions related to phylogenetic and phylogeographic relationships (Avise *et al.*, 1987; Zhang *et al.*, 1995). However, certain processes such as heteroplasmy, nuclear integration of mitochondrial genes, polymerase errors, DNA contamination, ancestral polymorphisms and recombination may lead to incongruence using independent loci. For example, heteroplasmy, the presence of more than one haplotype within a single organism (Boyce *et al.*, 1989; Frey and Frey, 2004) may result in incongruence of phylogenetic topologies and hence negatively impact the resolution of evolutionary relationships of organisms. Heteroplasmy has been reported in insects and other arthropods (Frey and Frey, 2004; Fontaine *et al.*, 2007; Magnacca and Brown, 2010; Nunes *et al.*, 2013). Another source of potential phylogenetic ambiguity is the nuclear integration of mitochondrial gene fragments, which has been suggested to occur in Triatominae (Dotson and Beard, 2001) and other arthropods (Zhang and Hewitt, 1996a; Parfait *et al.*, 1998; Bensasson *et al.*, 2001).

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Triatomines of the genus *Mepraia* (Mazza *et al.*, 1940) are blood-sucking insects that play an important role in the transmission of *Trypanosoma cruzi*, the etiologic agent of Chagas disease in the sylvatic cycle (Rozas *et al.*, 2007; Botto-Mahan *et al.*, 2008). *Mepraia* is endemic to semiarid and arid regions, and is distributed in coastal and interior valleys of northern and central Chile (Frías *et al.*, 1998; Campos *et al.*, 2013a). Their distribution in wild and peridomestic habitats, their opportunistic feeding behavior and human settlement in risk areas are features of high epidemiological significance as potential vectors for humans (Cattan *et al.*, 2002; Toledo *et al.*, 2013). Three species are currently included in the genus: *M. spinolai* Porter 1943, *M. gajardoi* and *M. parapatrica* (Frías *et al.*, 1998; Frías, 2010). *M. parapatrica* has been recently described based on morphology (Frías, 2010). Mitochondrial gene sequences also support three lineages congruent with the three described species (Campos *et al.*, 2013a) However, the specific status of *M. parapatrica* remains controversial, given the widely recognized morphological plasticity within the subfamily Triatominae (Dujardin *et al.*, 1999) and the presumptive introgression/hybridization processes acting within *Mepraia* (Calleros *et al.*, 2010; Campos *et al.*, 2011). The genus *Mepraia* belongs to the *spinolai* complex together with *Triatoma eratyrusiformis* Del Ponte 1929 and *Triatoma breyeri* Del Ponte 1929. The last two taxa are geographically separated from *Mepraia* species by the An-

des Range (Lent and Wygodzinsky, 1979). *T. eratyrusiformis* is closely related to *Mepraia* (Lent and Wygodzinsky, 1979; Hypa *et al.*, 2002; Moreno *et al.*, 2006; Frías, 2010; Campos *et al.*, 2013b). The monophyly of the *spinolai* complex is supported by mitochondrial gene sequences (Campos *et al.*, 2013b; Justi *et al.*, 2014), and their divergence on both sides of the Andes from the common ancestor probably occurred after the uplift of the Andes during the Miocene (Moreno *et al.*, 2006; Frías, 2010; Campos *et al.*, 2013a,b). Although several studies have reported heteroplasmy in arthropods, this phenomenon seems to be underestimated because conventional (automated) sequencing may fail to detect it (Dos *et al.*, 2008; Magnacca and Brown, 2010). In this study, we found incongruence in phylogenies inferred with two mitochondrial markers, cytochrome b (*cyt b*) and cytochrome oxidase subunit-I (*COI*) in *Mepraia* species and *T. eratyrusiformis* and discuss the origin that may have produced this pattern.

Materials and Methods

Sample collection

We used 66 mitochondrial gene sequences of *COI* and *cyt b* of *Mepraia* (GenBank accession numbers KC236913-KC236978, Campos *et al.*, 2013a). Ten additional sequences of both genes were also included: *M. spinolai* from Til Til, Metropolitan Region (33°06'19'' S; 70°55'53'' W, N = 2) and Los Andes, Valparaíso Region (32°01'33' S; 70°04'16'' W; N = 2); *T. eratyrusiformis* from Salinas de Bustos, Departmento of Independencia, Province of La Rioja, Argentina (N = 4); *Triatoma infestans* (N = 2) was used as outgroup, (accession numbers KM258433-KM258442).

Insects were manually collected by trained people as follows: a person wearing safety clothes and carrying collecting tools (forceps, brush, plastic can) was used as bait, waiting 15-20 min in areas with ecological attributes to harbor kissing-bugs; if no kissing-bug appeared, the investigator moved to another sampling site. Bugs were transported to the laboratory; the limbs were dissected and kept in 70% ethanol at -20 °C.

Mitochondrial DNA extraction, amplification, and sequencing

Genomic DNA from legs was extracted using the DNA extraction kit E.Z.N.A. Tissue DNA® (Omega Biotec, Georgia) according to manufacturer's instructions. A 636-bp fragment of the mitochondrial cytochrome oxidase subunit-I (*COI*) gene, and a 682-bp fragment of the cytochrome b (*cyt b*) gene were amplified via polymerase chain reaction (PCR) using Platinum® *Taq* DNA polymerase (Invitrogen, Brazil) and the primers 7432 (forward) (5'-GGACGWGGWATTTATTATGGATC-3') and 7433 (reverse) (5'-GCWCCAATTCARGTTARTAA-3') for cyt b (Monteiro *et al.*, 2003) and the primers LCO1490 (forward) (5'-GGTCAACAAATCATAAAGATATTGG-3') and HCO2198 (reverse) (5'-TAAACTTCAGGGTGAC CAAAAAATCA-3') for *COI* (Folmer *et al.*, 1994). The following conditions were used to amplify both *cyt b* and *COI*: an initial denaturation at 94 °C for 3 min, 30 cycles of 1 min at 94 °C, 45 °C for 1 min and 72 °C for 1 min, followed by a final extension of 10 min. Verification of successful amplification was assessed by 2% agarose gel electrophoresis.

Sequencing reactions were conducted by Macrogen Inc. (South Korea) using the same PCR primers. Sequences were edited using Bioedit 7.0.8.0 (Hall, 1999) and aligned using Clustal W (Thompson *et al.*, 1994) as implemented in Bioedit (Hall, 1999). After the alignments, sites that showed nucleotide substitutions were re-examined by visual inspection of each individual's raw chromatogram. For phylogenetic reconstruction (see below) ambiguous bases were coded using the nucleotide ambiguity code (IUPAC). Non-synonymous substitutions and stop codons were checked using DnaSp 5.1 (Librado and Rozas, 2009). The resulting COI data was 508 bp in length and *cyt b* was 514 bp.

Phylogenetic analyses

Phylogenetic reconstructions were performed separately for each mitochondrial gene by maximum likelihood (ML) and maximum parsimony (MP). ML analyses were performed using the online platform PhyML 3.0 (Guindon *et al.*, 2010). The best-fitting model of nucleotide substitution was selected using the Akaike information criterion (Akaike, 1974) on *cyt* b (TrN +I, I: 0.66) and COI (HKY+G, G: 0.068) implemented in the program jmodelTest 0.1.1 (Posada, 2008). MP analyses were performed using PAUP* 4.0b10 (Swofford, 2002) with the heuristic search option and tree bisection reconnection (TBR) branch swapping. Nodal supports were estimated by the bootstrap method (Felsenstein, 1985) with 1000 replicates. We considered branches receiving > 70% bootstrap support to be well-supported (Hillis *et al.*, 1993; Wilcox *et al.*, 2002). Trees were visualized using the FigTree v. 1.1.2 program. *Triatoma infestans*, was used as outgroup based on its phylogenetic proximity to the *spinolai* complex (Hypa *et al.*, 2002; de Paula *et al.*, 2005; Campos *et al.*, 2013b). This research was undertaken with approval from the Bioethics Committee of the Pontificia Universidad Católica de Valparaíso, Chile.

Results

Ambiguous bases in the chromatograms were observed in the samples Tera1 and 53Til for the *COI* gene, and in the Tera2 sample for the *cyt b* gene. Samples Tera1 and Tera2 were reamplified and sequenced for both genes. The sample 53Til was not again amplified due to limited genomic DNA availability. The second round of sequencing for the *cyt b* gene did not show differences with the first

round for the Tera2 sample, however some nucleotide differences were observed in the Tera1 sample. The second round of sequencing for the *COI* gene amplification did not show differences in the Tera2 sample, while six sites showed nucleotide differences in the Tera1 sample. All nucleotide differences between the first and the second round of sequencing for the Tera1 and Tera2 samples were observed in sites with ambiguous bases (Figure 1). Analyses of the *cyt b* and *COI* genes showed no stop codons. The *cyt b* and *COI* alignments showed 155 and 125 variable polymorphic sites, respectively.

Tree topologies performed with *cyt b* and *COI genes* are shown in the Figure 2. Both phylogenetic topologies showed similar results, although incongruence was observed with four haplotypes intermingled among lineages. The cladogram reconstructed with the *COI* gene sequence (Figure 2B) showed two haplotypes of *M. spinolai* (53Til and 21LA) grouped within the *M. gajardoi* lineage, and two haplotypes of *T. eratyrusiformis* (Tera1 and Tera2) were also included within the *M. gajardoi* lineage (81% bootstrap support). These haplotypes were grouped as expected according to their taxonomy and geography in the cladogram reconstructed with *cyt b*, leading to conclude that the samples Tera1 and Tera2 represent sister species of *Mepraia* and the samples 53Til and 21LA belong to *M. spinolai* (Figure 2A). In the incongruent sequences (53Til, 21LA, Tera1, and Tera2) non-synonymous mutations were not observed in the *COI* gene.

Discussion

The extent of topological incongruence between two molecular markers depends both on evolutionary and methodological processes. For example, incongruence of mitochondrial and nuclear gene trees may be due to differential lineage sorting, introgression or heteroplasmy (Sota and Vogler, 2001; Magnacca and Brown, 2010; Barnabé and Brenière, 2012; Messenger *et al.*, 2012). Different substitution rates might also lead to incongruence (Sasaki *et al.*, 2005). However, due to the linked nature of mtDNA genes it is expected that different mitochondrial genes show similar topologies. Our phylogenetic analyses using two mtDNA genes (*cyt b* and *COI*) showed incongruent topologies that were evident for four haplotypes placed in different lineages. This unexpected result may be explained by: i) contamination of samples with foreign or neighboring DNA; ii) *Taq* polymerase may yield errors during the amplification process due to lack of fidelity; and iii) nucleotide intra-individual variation.

In this study, samples were extracted and amplified in different periods and were processed as groups of the same species, which rules out contamination errors. Similarly, it is highly unlikely that polymerase errors would be the primary source of the phylogenetic incongruence, as we used the same conditions for the Platinum® *Taq* DNA high fidelity polymerase in all our PCR reactions (Gyllensten, 1989; Frey and Frey, 2004). Hence, it is highly probable that the main cause of the incongruence is related to nucleotide intra-individual variation.

Nuclear integration of mitochondrial gene fragments has been reported in Triatominae (Dotson and Beard, 2001) and other arthropods (Zhang and Hewitt, 1996a; Parfait *et al.*, 1998; Bensasson *et al.*, 2001), but this process seems not to represent a major issue due to the larger number of mtDNA copies (Lightowlers *et al.*, 1997; Scheffler, 2001). However, the presence of multiple nuclear copies of *COI* and other mitochondrial genes has been reported in aphids

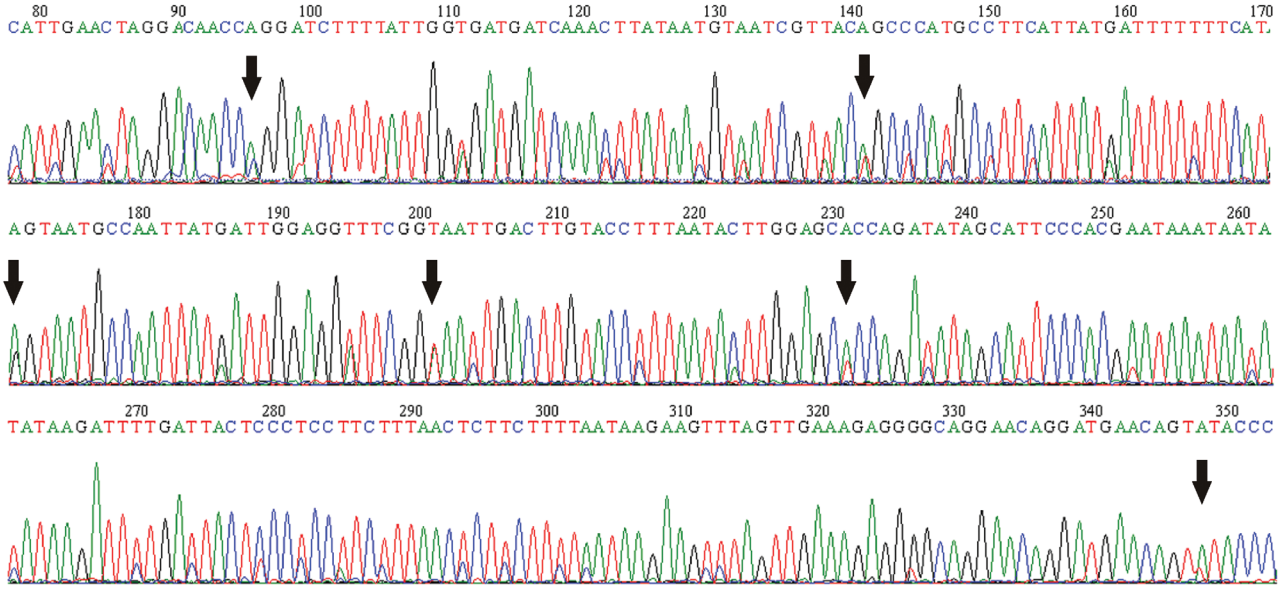

Figure 1 - Chromatogram of a segment of the *COI* gene after the second sequencing round for Tera1. Arrows indicate the sites with differences compared with the first sequencing round.

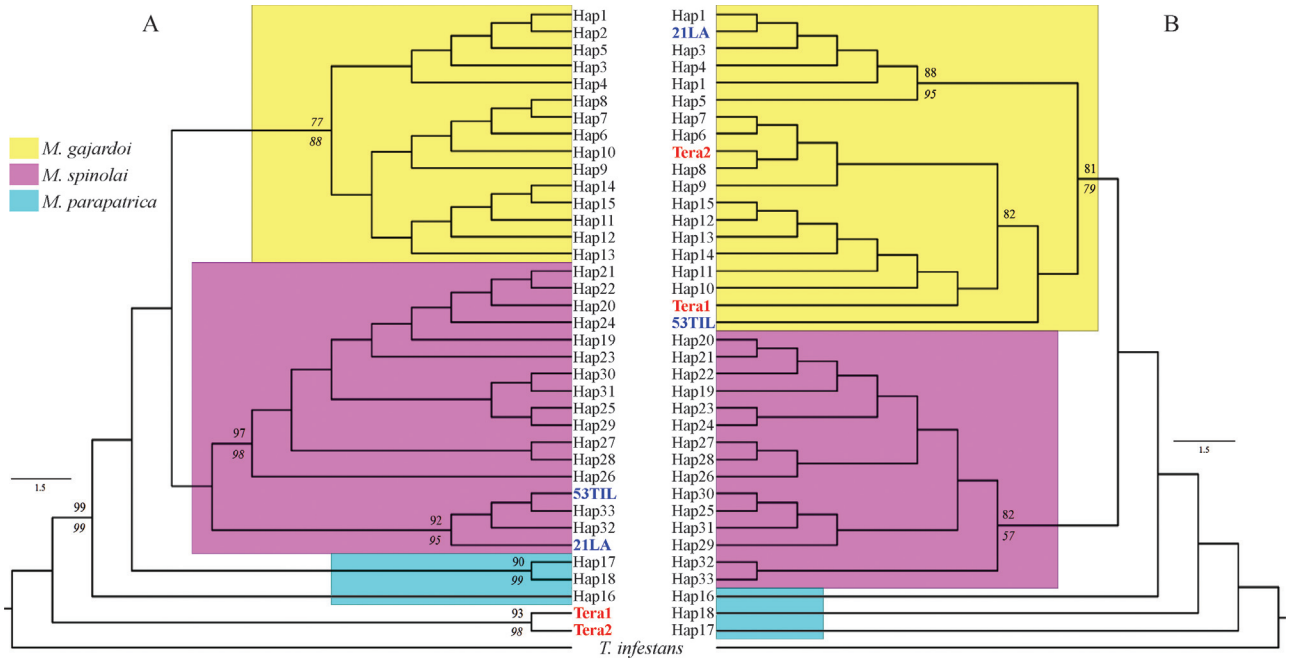

Figure 2 - Maximum likelihood phylogenetic trees of *Mepraia* and *Triatoma eratirusiformis*. (A) *cyt b* gene sequences (514 bp) inferred with the model TrN +I, I: 0.66; (B) *COI* gene sequences (508 bp) model HKY+G, G: 0.068. Numbers above the branches are the support values derived from bootstrap resampling with 1000 pseudoreplicates and numbers under the branches are parsimony bootstrap values. Colors of nodes represent lineages. 21LA, 53Til in blue are the incongruent haplotypes of *M. spinolai*. Tera1, Tera2 in red are the incongruent haplotypes of *T. eratyrusiformis*. The bar represents the number of nucleotide substitutions per site.

and other insects (Gellissen and Michaelis, 1987; Sunnucks and Hales, 1996; Bensasson *et al.*, 2000), and therefore we do not rule out this phenomenon until further analyses are performed. These copies behave as pseudogenes that are not correctly expressed, because they accumulate nonsense mutations resulting in stop codons in the reading frame and a greater number of non-synonymous changes, thus tending to diverge from the homologous mitochondrial genes (Zhang and Hewitt, 1996a; Frey and Frey, 2004). We found no sequences with stop codons in the two genes, and found no non-synonymous mutations in the *COI* gene. Finally, the co-amplification of more than one mitochondrial haplotype (*i.e.* heteroplasmy; Solignac *et al.*, 1986; Boyce *et al.*, 1989), may explain our results. In the samples Tera1 Tera2 and 53Til we observed ambiguous bases after the nucleotide amplification (Figure 1); the sample 21LA did not show ambiguous bases.

The retention of ancestral polymorphisms through incomplete division of lineages may also explain the misplacement of the *T. eratyrusiformis* and *M. spinolai* haplotype. However, due to the linked nature of mitochondrial genes, the same results (incongruence) should have been observed in both topologies, contrary to our results. In addition, incomplete lineage sorting has a higher chance to occur in recently diverged groups and with high population sizes (Maddison and Knowles, 2006), which contrasts with the lineages in our study that are highly structured and show deep divergences (Campos *et al.*, 2013a). Therefore, we suggest that heteroplasmy is the most parsimonious explanation for the incongruence observed in our study.

Heteroplasmy of mtDNA may result from two or more haplotypes within a single mitochondrion, or different mitochondria with different haplotypes, either within one cell or in different cells (Lightowlers *et al.*, 1997; Frey and Frey, 2004). Processes that may explain the latter are paternal leakage, recombination, and segregating mutations (White *et al.*, 2008). Paternal leakage or inheritance of mitochondrial DNA has been described in insects including Hemiptera (Kondo *et al.*, 1990; Fontaine *et al.*, 2007; Nunes *et al.*, 2013; Wolff *et al.*, 2013), and may have occurred between *T. etratyrusiformis* and *Mepraia.* This incongruence between mitochondrial genes due to heteroplasmy may lead to erroneous phylogenetic hypotheses resulting in wrong taxonomic classification and population genetic structure (Zhang and Hewitt, 1996b; White *et al.*, 2008). The presumptive phenomenon of heteroplasmy suggests that mtDNA from *T. eratyrusiformis* can be found within the *Mepriaia* genome, which supports their close systematic relationship and is independent evidence of common ancestry. Due to the non-overlaping distribution of *Mepraia* and *T. eratyrusiformis* (separated by the Andes), the processes that may have caused the heteroplasmy seen within the *spinolai* complex probably occurred a long time ago, before or at early periods during the uplifting of the Andes Ranges, and reveal that heteroplasmy may persist in populations even though new lineages are subsequently formed.

In conclusion, after performing phylogenetic reconstructions with two mitochondrial genes, we found incongruent topologies for some haplotypes of *M. spinolai* and *T. eratyrusiformis*. We suggest as explanation of our results that there is intra-individual variation likely due to heteroplasmy. The mitochondrial incongruence within the *spinolai* complex requires further investigation to determine more accurately the extent and probable causes of this pattern.

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Internet Resources

FigTree v1.1.2 program, http://tree.bio.ed.ac.uk/software/figtree/ (accessed: March 20, 2013).

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