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Research paper

Hypothesis testing clarifies the systematics of the main Central American Chagas disease vector, *Triatoma dimidiata* (Latreille, 1811), across its geographic range

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ABSTRACT

The widespread and diverse *Triatoma dimidiata* is the kissing bug species most important for Chagas disease transmission in Central America and a secondary vector in Mexico and northern South America. Its diversity may contribute to different Chagas disease prevalence in different localities and has led to conflicting systematic hypotheses describing various populations as subspecies or cryptic species. To resolve these conflicting hypotheses, we sequenced a nuclear (internal transcribed spacer 2, ITS-2) and mitochondrial gene (*cytochrome b*) from an extensive sampling of *T. dimidiata* across its geographic range. We evaluated the congruence of ITS-2 and *cyt* b phylogenies and tested the support for the previously proposed subspecies (inferred from ITS-2) by: (1) overlaying the ITS-2 subspecies assignments on a *cyt* b tree and, (2) assessing the statistical support for a *cyt* b topology constrained by the subspecies hypothesis. Unconstrained phylogenies inferred from ITS-2 and *cyt* b are congruent and reveal three clades including two putative cryptic species in addition to *T. dimidiata* sensu stricto. Neither the *cyt* b phylogeny nor hypothesis testing support the proposed subspecies inferred from ITS-2. Additionally, the two cryptic species are supported by phylogenies inferred from mitochondrially-encoded genes *cytochrome c* oxidase I and NADH dehydrogenase 4. In summary, our results reveal two cryptic species. Phylogenetic relationships indicate *T. dimidiata* sensu stricto is not subdivided into monophyletic clades consistent with subspecies. Based on increased support by hypothesis testing, we propose an updated systematic hypothesis for *T. dimidiata* based on extensive taxon sampling and analysis of both mitochondrial and nuclear genes.

1. Introduction

The Triatominae are a subfamily of the Reduviidae characterized by hematophagy of vertebrate hosts and the potential ability to transmit *Trypanosoma cruzi* (Chagas, 1909), the etiological agent of Chagas disease (Galvão et al., 2003). In Central America, the most widespread and important Chagas vector is *Triatoma dimidiata* (Latreille, 1811) (Ponce, 2007). Chagas disease is the parasitic disease causing the greatest economic burden on Latin America (World Health Organization, 2008), with approximately six million people infected with *T. cruzi* (World Health Organization, 2015) causing ~12,000 annual deaths (Pan American Health Organization, 2014).

The most effective approach to halting transmission of vector-borne diseases has been combating the arthropod vectors. The first step requires accurate identification of the most epidemiologically important taxa. Even within the same species, there are often populations whose traits differ; some of these traits (e.g. degree of association with humans, parasite infection prevalence, etc.) influence the epidemiological importance of populations (Dorn et al., 2007). Populations may even include cryptic (morphologically identical but genetically different) species e.g. (Panzera et al., 2015). And, as closely related taxa usually share similar traits, understanding the degree of relatedness among different taxa allows one to predict traits. Therefore, for accurate epidemiology and effective control it is essential to clarify the systematics of the major vectors.

T. dimidiata sensu lato (s.l.) has long been recognized as a polytypic species, consisting of populations that differ morphologically and by other characters, which has resulted in merging and splitting of the species multiple times reviewed in (Dorn et al., 2007). Historically,

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classifications have been based on phenotypes. In 1859, a Mexican morphotype, *Triatoma maculipennis*, was described at the northern end of its range (Stahl, 1859) followed by a Colombian morphotype, *Triatoma capitata*, at the southern end (Usinger, 1941). Usinger (1941) also described a "dimidiata group" variously comprised of three to four species or subspecies including: *hegneri*, *dimidiata*, *maculipennis*, and *capitata* (Usinger, 1941, 1944). The latter three were later synonymized into *T. dimidiata* by Lent and Wygodzinsky (Lent and Wygodzinsky, 1979) after a comprehensive review of 160 specimens from across its geographic distribution finding "*Triatoma dimidiata*… has not segregated into clearly separable allopatric populations" and that the variation was "roughly clinal in nature". These authors also noted that cave specimens differed from specimens collected outside the caves, similar to other arthropods, with "diminished pigmentation, reduction in size of eyes and ocelli, [and] longer head[s]"; however, a distinct taxonomic group was not suggested for the cave specimens as most of these are well-known environmentally-influenced modifications of cave dwellers. More recent work on antenna structure, head morphometry, and cuticular hydrocarbons has variously clustered *T. dimidiata* into two to four distinct taxa supporting the identification of unique cave populations, and identifying at least one cryptic species (by cuticular hydrocarbon analysis) (Bustamante et al., 2004; Calderón-Fernández et al., 2005; Calderón-Fernández et al., 2011; Catalá et al., 2005). So phenotypic results suggest one to four taxa.

As phenotypes contain both genetic and environmental influences (Dujardin et al., 2009), potentially explaining the conflict in these systematic hypotheses, researchers have turned to molecular genetic methods to resolve *T. dimidiata* systematics. Cytogenetics has revealed three distinct "cytotypes" of *T. dimidiata*, with cytotype 1 ranging from Central Mexico into northern South America, cytotype 2 present in Yucatán, Mexico, and cytotype 3 in Petén, Guatemala; the latter two diverged enough to be considered putative cryptic species (Panzera et al., 2006; Panzera et al., 2010).

With respect to DNA markers, phylogenies inferred from the nuclear ribosomal DNA internal transcribed spacer 2 (ITS-2) sequence also suggested a highly divergent taxon in the Yucatán peninsula, Mexico (Marcilla et al., 2001; Tamay-Segovia et al., 2008), Petén, Guatemala (Dorn et al., 2007), and Cayo, Belize (Dorn et al., 2009), corroborating the cytogenetic data and supporting the identification of a cryptic species. However, in contrast to the cytogenetic results, ITS-2 sequences of specimens from Yucatán, Mexico and Peten, Guatemala were monotypic (Dorn et al., 2007). Further phylogenetic inference based on ITS-2 divided *T. dimidiata* into four clades: ITS-2 Groups 1A, 1B, 2, and 3 (Bargues et al., 2008); however, Group 1A is not monophyletic (Bargues et al., 2008). The authors revived the geographic subspecies classifications: *T. dimidiata dimidiata* (ITS-2 group 1A), *T. dimidiata capitata* (ITS-2 group 1B), and *T. dimidiata maculipennis* (ITS-2 group 2), and named the putative cryptic species found in the Yucatán, Mexico, *T.* species affinis *dimidiata* (ITS-2 group 3). Since different individuals and geographic ranges were studied with different markers, it is difficult to relate the data and resolve the conflicts.

In our previous studies, to assess the phylogenetic support of putative cryptic species and subspecies within *T. dimidiata*, we sequenced both mitochondrial *cytochrome b* (*cyt* b) and nuclear DNA (ITS-2) in the same individual of a limited number of specimens. Our results confirmed the putative cryptic species, *T.* sp. aff. *dimidiata* (Bargues et al., 2008) found in particular regions of Mexico, Guatemala and now Belize, as a divergent, well-supported monophyletic clade, by both markers (Dorn et al., 2009). However, our results did not support the subspecies hypothesis. The proposed subspecies (*T. d. maculipennis*, *T. d. dimidiata*, and *T. d. capitata*) (Bargues et al., 2008) were not monophyletic in phylogenies inferred from *cyt* b and ITS-2 (Dorn et al., 2009). Specifically, individuals typed as *T. d. maculipennis* by ITS-2 were found in two different clades of the *cyt* b phylogeny. A more recent study rejects inferences based on ITS-2 as "too conserved" and divides *T. dimidiata* into four separate species, based on monophyletic clades inferred from two mitochondrial genes, *cyt* b and NADH dehydrogenase subunit 4 (ND4, presented concatenated with *cyt* b), and the percent sequence divergence used in past studies including a new cryptic species in Belize (Monteiro et al., 2013).

So how many cryptic species (one to four) and how many subspecies (zero to three) are present under the *T. dimidiata* umbrella? A long-standing difficulty in systematics in general and the Triatominae in particular is the difficulty of building on previous work. Rather than set aside previous work, what is needed is an assessment of the relative statistical support for previous systematic hypotheses compared to each new systematic hypothesis (i.e., best, most parsimonious tree) proposed with every new gene or specimen analyzed. The use of a single marker (or concatenated data from the same mitochondrial genome when two markers were used) on each individual has been particularly problematic. In the general field of systematics, one statistical method for comparing phylogenetic trees, the Shimodaira-Hasegawa Test (Shimodaira and Hasegawa, 1999), has been used extensively (>2700 citations in ISI Web of Knowledge, May 2016) including several vectors and pathogens (de la Rua et al., 2014; Dyer et al., 2011; Gaunt and Miles, 2002; Hamilton et al., 2007; Outlaw and Ricklefs, 2011).

In this study, using extensive geographic sampling, we investigate how many taxa are included under the *T. dimidiata* umbrella. Specifically, we test whether or not cryptic species and subspecies are congruent monophyletic clades inferred from both mitochondrial and nuclear genes and determine the statistical support for the subspecies systematic hypothesis (Fig. 1). Our results uncover a richer picture of the diversity present in *T. dimidiata* s.l.

2. Methods

2.1. Study design

The phylogeny of *T. dimidiata* from across its geographic range was inferred separately from 65 and 141 haplotypes of ITS-2 and *cyt* b, respectively and the congruence of the trees assessed. For 99 specimens we determined both ITS-2 and *cyt* b haplotypes from the same individual. The subspecies hypothesis, which is that *T. dimidiata* sensu stricto is subdivided into three subspecies (*T. dimidiata maculipennis*, *T. dimidiata dimidiata*, and *T. dimidiata capitata* (Bargues et al., 2008), Fig. 1) was then tested two ways: (1) by overlaying the proposed ITS-2 subspecies classification on the unconstrained *cyt* b tree (sequence of both genes with the same DNA isolated from a single individual); and (2), by constraining *cyt* b trees into monophyletic clades for each proposed subspecies and assessing the statistical support of the constrained tree compared to an unconstrained tree. This second method has been shown to provide strong statistical criteria to distinguish between alternative evolutionary hypotheses (Whelan et al., 2001). We then assessed additional support for putative cryptic taxa by phylogenetic inference using ND4 and mitochondrial cytochrome oxidase I (COI) and network analysis of ITS-2 and *cyt* b data.

2.2. Specimen collection and DNA isolation

Professionals trained in the safe handling of infectious agents collected 197 adult *T. dimidiata* from eight countries across its geographic range from 2007 to 2015 (Table 1). Specimens collected in protected areas of Guatemala were collected under collection permit 00994 and research permit 00201B from the Consejo Nacional de Areas Protegidas (CONAP) and from Belize by collecting permit #CD/60/3/15(28). All specimens were identified to species using the key of Lent and Wygodzinsky (Lent and Wygodzinsky, 1979). A voucher specimen of the proposed *T.* spp. aff. *dimidiata* (cave) was deposited in the Entomology Research Museum at the University of California, Riverside. Legs were removed from specimens and stored at −4 °C in a 95% ethanol/5% glycerol solution. DNA was isolated from two to five legs per