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Towards a Phylogenetic Approach to the Composition of Species Complexes in the North and Central American *Triatoma*, Vectors of Chagas Disease

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Abstract

Phylogenetic relationships of insect vectors of parasitic diseases are important for understanding the evolution of epidemiologically relevant traits, and may be useful in vector control. The subfamily Triatominae (Hemiptera:Reduviidae) includes ~140 extant species arranged in five tribes comprised of 15 genera. The genus *Triatoma* is the most species-rich and contains important vectors of *Trypanosoma cruzi*, the causative agent of Chagas disease. *Triatoma* species were grouped into complexes originally by morphology and more recently with the addition of information from molecular phylogenetics (the four-complex hypothesis); however, without a strict adherence to monophyly. To date, the validity of proposed species complexes has not been tested by statistical tests of topology. The goal of this study was to clarify the systematics of 19 *Triatoma* species from North and Central America. We inferred their evolutionary relatedness using two independent data sets: the complete nuclear Internal Transcribed Spacer-2 ribosomal DNA (ITS-2 rDNA) and head morphometrics. In addition, we used the Shimodaira-Hasegawa statistical test of topology to assess the fit of the data to a set of competing systematic hypotheses (topologies). An unconstrained topology inferred from the ITS-2 data was compared to topologies constrained based on the four-complex hypothesis or one inferred from our morphometry results. The unconstrained topology represents a statistically significant better fit of the molecular data than either the four-complex or the morphometric topology. We propose an update to the composition of species complexes in the North and Central American *Triatoma*, based on a

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phylogeny inferred from ITS-2 as a first step towards updating the phylogeny of the complexes based on monophyly and statistical tests of topologies.

Keywords

Triatominae; Chagas disease; Systematics; ITS-2; Morphometry; Species Complexes; Hypothesis Testing

1. Introduction

1.1 Background

The Triatominae are a sub-family of highly diverse Reduviidae that have evolved hematophagy of vertebrates. They can transmit *Trypanosoma cruzi*, a hemoflagellate protozoan and the causative agent of Chagas disease. Although there are approximately 140 extant species in five tribes within the sub-family, only a couple dozen species within three of the 15 genera (*Triatoma*, *Panstrongylus*, and *Rhodnius*) are associated with the majority of *T. cruzi* transmission to humans [1,2]. These three genera are from two tribes: *Triatoma* and *Panstrongylus* from Triatomini and *Rhodnius* from the Rhodniini. The most species-rich and widely distributed is the genus *Triatoma* with ~80 species found across South, Central, and North America including some islands of the Gulf of Mexico and three species found in the Old World.

Triatoma species are divided into three groups [2]. The Dispar group includes the high altitude species of the Andean region of South America, and the Infestans group includes all other South American species. The Rubrofasciata group includes the North and Central American species along with those from the Old World and islands of the Gulf of Mexico. Recent molecular work has revealed that the Rubrofasciata group form a well-supported clade, separate from the sister South American *Triatoma* [3].

The species complexes of the North and Central American *Triatoma*—Within the Rubrofasciata group, there are 29 species of *Triatoma* in North and Central America and the Islands of the Gulf of Mexico arranged in four species complexes that are defined “as species that share extensive morphological similarities” [4]. The species complex concept has been widely used in the Triatominae and has been a useful way to discuss related species. Molecular phylogenetic analyses support some complex assignments, showing complexes that are recovered as well-supported monophyletic clades, e.g. Brasiliensis, Infestans, Rubida, and the Phyllosoma complex [3,5–7]. In addition, molecular work has revealed that some “species” include cryptic species, so are best described as species complexes [8,9].

The currently accepted systematic hypothesis includes four complexes: Phyllosoma, Protracta, Lenticularia, and Flavida (Table 1). Assignment of species to complexes was initially based on morphology with later revisions based on phylogenetic inference. However, not all species assigned to a particular complex appear in monophyletic clades and

this four-complex systematic hypothesis has not been tested by statistical tests of topologies [2].

The Phyllosoma complex, of greatest epidemiological importance in North and Central America, is comprised mostly of species endemic to Mexico. These taxa have posed many difficult systematic problems. Originally, species of the Phyllosoma complex were recognized as sub-species of *Triatoma phyllosoma* [10] based on laboratory crosses in which fertile hybrids were reared [11]. However, Lent and Wygodzinsky [12] raised the sub-species to specific status rejecting the experimental design, scope, and interpretations of the crosses. ITS-2 (nuclear rDNA) and *cytochrome b* (mtDNA) sequence data have been used to clarify the relationships among the Phyllosoma [7]. Recent molecular and morphometric analyses, combined with data from experimental laboratory crosses have uncovered systematic intricacies of the Phyllosoma suggesting that many of the groups are morphotypes with chromatic and genetic variants, and might be more appropriately designated as sub-species [13]. In addition, placement of one species, *Triatoma ryckmani*, within the Phyllosoma is tentative as it is a poorly studied sylvatic species of Central America [14,15].

Triatoma dimidiata, a highly polymorphic species of the Phyllosoma complex, is the most epidemiologically important species in Central America (reviewed in [16]). Multiple metrics have been used to assess variation across its geographic range; this taxon appears to be polytypic with an as yet unresolved number of cryptic species and/or subspecies [8,9,16–18]. *T. dimidiata* form a well-differentiated and supported clade within the Phyllosoma, which may include *T. hegneri*, an island variant [7,8,19]. This has led some to propose a Dimidiata sub-complex [2], although the definition of a sub-complex is unclear.

The Protracta complex, as described in the four-complex hypothesis, is comprised of species ranging from North to Central America including a few species of low to moderate epidemiological importance. The complex also includes some rare and consequently less studied sylvatic species such as *Triatoma incassata* and *Triatoma neotomae*. *Triatoma protracta* is a polymorphic species that is usually found in close association with wood rat (*Neotoma*) nests across the southwestern United States; due to habitat encroachment, human encounters are becoming more frequent [20]. Although only 23 cases of autochthonous transmission of *T. cruzi* have been reported in the United States [21], many more cases of allergic reactions including anaphylaxis from *T. protracta* salivary proteins have been reported in the southwestern United States [20]. While classically described as sylvatic species, human blood meals in *T. protracta* from California have been reported [22].

The Lenticularia complex includes species found in the southern United States and northern Mexico. With regards to epidemiological importance, *Triatoma gerstaeckeri*, *Triatoma sanguisuga*, and *Triatoma lenticularia* have been implicated as the vectors of autochthonous transmission in the United States [23–27]; and all three species are found in close association with homes in rural areas of Texas and Louisiana [28–31]. Human blood meals are common in *Triatoma rubida* from Arizona and California [22,32]. Many species within the Lenticularia complex show morphological variation across their broad geographic range including *T. rubida* and *T. sanguisuga*. Studies of species within the Lenticularia complex

are few, but phylogenetic inference using *cyt b* supported sub-specific status for populations of *T. rubida* [6]; and the population of *T. sanguisuga* responsible for the sixth case of autochthonous transmission in the United States was found to be hyper-variable by *cyt b* and 16S [31]. *Dipetalogaster maximus*, a species of the Baja peninsula, is the only species of its genus, separated due to extreme size as well as additional apomorphic characters [12]. *D. maximus* was sister to *T. lecticularia* by a fragment of the mitochondrial *cyt b* gene, but not by a phylogeny inferred by concatenated fragments of mitochondrial *cyt b* and nuclear ITS-2; the latter placed *D. maximus* in close affiliation with *T. barberi* and *T. protracta* [33].

The Flavida complex is comprised of species found only in Cuba and Jamaica. *Triatoma flavida* and *Triatoma bruneri*, both endemic to Cuba, were synonymized, but recent molecular data supports the elevation of each to specific status [34]. Included in this complex is *Triatoma obscura*, a poorly studied species of Jamaica.

1.2 The challenge: resolving competing systematic hypotheses

These complexes were originally proposed based on analysis of morphological characters [12]. The validity of some complexes has been supported using molecular phylogenetics; however, some complexes are not monophyletic and/or clades containing the complex members lack bootstrap or posterior probability support [5,7,8,19,33]. Furthermore, a recent update to the complexes lacks detail about assignment criteria [2]. A likelihood based statistical test of topology provides a means to test the support for the four-complex hypothesis [2] compared to different species complex compositions, inferred from new data.

The Shimodaira-Hasegawa (SH) test [35] is a non-parametric test of topologies that is suitable for testing *a posteriori* hypotheses, e.g. a maximum likelihood unconstrained phylogeny, against a set of competing hypotheses including *a priori* phylogenies, e.g. a constraint tree that forces monophyly based on the four-complex hypothesis [2,36]. This method has been widely used in other taxa (>2,400 citations on ISI Web of Science) but has not been used previously in the Triatominae. Ultimately, the SH assesses the power of the data to support a given topology relative to other systematic hypotheses and provides a means of testing the congruence of phylogenies originating from different molecular markers and metrics.

The purpose of this study is to further clarify the evolutionary relationships among the *Triatoma* found in North and Central America using a phylogenetic approach in concert with a well-established statistical method of testing systematic hypotheses. We use both a genetic marker, nuclear rDNA internal transcribed spacer region 2 (ITS-2), as well as phenotypic data, comparative morphometrics of the insect head [17]. ITS-2 represents the largest available comparative data set for triatomines, and is informative at the population, species, and genus level [37]. Using strict monophyly, we constrain ITS-2 topology to reflect competing systematic hypotheses arising from: (1) an unconstrained phylogeny of ITS-2, (2) the four-complex hypothesis (Table 1) and (3) test the congruence of head morphometry with ITS-2. We propose an update to the composition of complexes inferred from ITS-2 and a future direction for a complete revision using additional markers and statistical testing of systematic hypotheses.

2. Materials and Methods

2.1 Specimen Collection

All insect specimens (Tables 2 and 3) were collected in the field by personnel trained in the safe handling of infectious agents. Insects were stored in 95% ethanol, 5% glycerol at room temperature. Species were identified by morphology using a published key [12]. For morphometry, because it varies between males and females, only males were used as these were the most numerous.

2.2 DNA extraction, PCR amplification, and Sequencing

DNA was extracted from 2–5 legs from each specimen (Table 2) using the DNeasy Blood and Tissue kit (Qiagen, Valencia, CA) following the manufacturer's instructions and stored at -80°C .

A 900 base pair fragment of nuclear ribosomal DNA containing a fragment of 5.8S, complete ITS-2, and a fragment of 28S DNA was amplified by PCR [19]. After verifying amplification of the correctly sized PCR product using agarose gel electrophoresis, PCR products were purified using the PCR Clean Up kit (Qiagen, Valencia, CA). All ITS-2 PCR products were cloned using the TOPO TA kit (Invitrogen, Carlsbad, CA) following the manufacturer's instructions. Plasmid DNA was extracted using Plasmid Pure Link Quick Plasmid Mini Prep Kit (Invitrogen, Carlsbad, CA). ITS-2 PCR products were cloned prior to sequencing due to difficulty in obtaining high quality sequence data directly from the PCR products. Both strands of all samples were sequenced (Eton Bioscience, San Diego, CA).

2.3 Sequence Alignment, Phylogenetic Analysis, and Hypothesis Testing

The quality of the DNA sequences was assessed, and the sequences edited and trimmed (MacVector v.12.0.6, Cary, N.C. and FinchTV Geospiza, Inc.; Seattle, WA). Haplotypes from ITS-2 sequence data were identified using DnaSP v. 5.0 [38] and named based on the nomenclature proposed by Bargues et al. [8]. Sequences were aligned for phylogenetic inference using MUSCLE [39] within Mega 5.05 [40]. The best-fit nucleotide substitution model was determined using jModelTest [41], based on the lowest Akaike Information Criterion (AIC) score.

Phylogenies were recovered using MrBayes 3.2 [42]. All phylogenies were rooted with *T. infestans* as the outgroup. The phylogenies inferred from the ITS-2 data set used a GTR+ Γ nucleotide substitution model, the best-fit model based on the AIC score inferred from jModelTest. For all phylogenies, an unconstrained search was conducted for 1,000,000 generations with four chains simultaneously traveling through parameter space with the default priors; trees were sampled every 100 iterations. The summaries of parameters and trees were generated following “burning in” the first 25% of iterations.

2.4 Morphometric Analyses

Traditional morphometric analysis was performed using the PAD module included in the CLIC package (<http://momedujardin.wordpress.com/clic-collection/clicmodules/pad/>) [43]. In total, we used nine log-transformed measurements of the head from 11 *Triatoma* species

from 183 male *Triatoma* specimens (Table 3 and Fig. 1). The analysis in PAD follows an isometry-free approach to remove the isometric component of changes [44]. The first step was to obtain nine size-free variables after subtracting the isometric component of size from each log-transformed measurement. Next, a principal components analysis was performed over the size-free variables to obtain the size-free components. In our analysis, we retained the first four size-free components, which represented 95% of the variability within the data. Lastly, a discriminant analysis was performed on the size-free components. Individuals were plotted in a factor map of the first two discriminant factors.

Mahalanobis distances among the 11 species included in the study were calculated using PAD. As an estimate of statistical significance for each Mahalanobis distance, the proportion of Mahalanobis distance values equal to or higher than those observed, relative to a total of 1,000 permutations, was used as an estimate of statistical significance. For each permutation, individuals were randomly interchanged among groups, and a discriminant analysis was performed after each permutation. The allometric residual was examined in PAD by performing a linear regression of isometric size on the first and second discriminant factors. A topology was constructed using TreeGen (<http://www.cbrg.ethz.ch/services/TreeGen>) from the Mahalanobis distance values.

2.5 Hypothesis Testing of Molecular Data Sets

We tested competing species complex compositions using strict monophyly. Topologies were constrained to force the monophyly of: (1) the deepest, well-supported (>90% BPP) monophyletic clades recovered in an unconstrained phylogenetic analysis of ITS-2 and (2) the four-complex hypothesis of Schofield and Galvão [2] (Table 1). These hypotheses will subsequently be referred to as the unconstrained phylogeny and the four-complex hypotheses, respectively. To assess the congruence between morphometry and ITS-2, we repeated the phylogenetic analysis using only the 11 species that were available for morphometry. The majority rule consensus tree from each analysis was imported into PAUP 4.0b10* [45,46] along with its corresponding alignment, and a one-tailed SH Test using 10,000 RELL bootstrap replicates was conducted to assess if the maximum likelihood topology significantly better fits the data than competing systematic hypotheses [35,36]. In all hypothesis testing a p-value < 0.05 was considered a statistically significant result.

3. Results

3.1 ITS-2 Sequence Analysis and Unconstrained Phylogeny

We sequenced the complete ITS-2 from 19 specimens and obtained 18 unique sequences. This represented eight new haplotypes (indicated by “*” Table 2) from six species. Unique haplotypes obtained in this study were combined with representative haplotypes of the North and Central American *Triatoma* from GenBank ($n = 24$) for a total of 32 haplotypes representing 19 species. Multiple ITS-2 haplotypes were recovered for nine species: *T. lecticularia* ($n = 3$), *T. sanguisuga* ($n = 3$; JX872265, JX872264, JX890270), *T. mazzottii* ($n = 2$), *T. longipennis* ($n = 2$), *T. hegneri* ($n = 2$), *T. mexicana* ($n = 2$), *T. nitida* ($n = 2$; JX872260), *T. ryckmani* ($n = 2$; JX872262), and *T. dimidiata* ($n = 4$; these include proposed geographic sub-species and a cryptic species [8]). The remaining 10 species are represented

by a single haplotype including: *T. barberi* (JX872261), *T. protracta* (JX872260), and *T. pallidipennis* (JX872262). ITS-2 length varied from 483–515 bp, and percentages of AT ranged from 74.3% to 78.7%. Of the 577 characters in the ITS-2 data set, 140 (24%) are parsimony informative, 341 are invariable, and 96 sites contained gaps.

In the unconstrained phylogeny, all species represented by two or more ITS-2 haplotypes were monophyletic with Bayesian Posterior Probability (BPP) support 99% with the exception of *T. hegneri*, which is sister with *T. dimidiata* (Fig. 2). Disregarding polyspecific clades with BPP support < 90%, ITS-2 supports three major clades among the *Triatoma* found in North and Central America (Fig. 2). Species are color coded by the four-complex hypothesis (Table 1) revealing a lack of congruence with proposed complexes based on monophyly and BPP support. *T. flavida* forms its own clade. The first well-supported clade (A, 100% BPP) is comprised of *T. nitida*, *T. ryckmani*, *T. bolivari*, and *T. rubida*, with a well-supported (98% BPP) sister relationship between *T. nitida* and *T. ryckmani* / *T. bolivari*. This clade contains species of the Protracta complex (orange), Phyllosoma complex (green) and the Lenticularia complex (blue). A clade containing *D. maximus*, *T. lecticularia*, *T. barberi* and *T. protracta* is only weakly supported (87% BPP), and ITS-2 sequence data is unable to resolve this relationship among these species with high confidence. Therefore, the second designated clade is clade B (100% BPP) containing *T. protracta* and *T. barberi*, both species in the Protracta complex, the only monophyletic clade from the previously described four-complex hypothesis. The third clade, clade C, shows moderate support (C, 92% BPP) and is comprised of species of the Lenticularia complex (blue, *T. gerstaeckeri*, *T. sanguisuga*) and the Phyllosoma complex (green, *T. pallidipennis*, *T. mazzottii*, *T. longipennis*, *T. bassolsae*, *T. mexicana*, *T. dimidiata*, and *T. hegneri*).

3.2 Morphometric Analysis

The discriminant analysis based on the size-free components revealed significant head shape differences among all the species. The probabilities of obtaining Mahalanobis distance values between group centroids equal or larger than the ones observed by chance alone was very low based on 1,000 permutations ($P < 0.001$; Table 4). The smallest pairwise distance observed was between *T. pallidipennis* and *T. longipennis* (2.44), while the largest distance was between *T. ryckmani* and *T. barberi* (11.25). A factor map of all the insects on discriminant factors I and II shows the relative positions of the species and their group centroids in the morphometric space (Fig. 3a). Most of the differentiation among species occurs along Factor I. We found a significant allometric residual in Factor I because it had a 71.7% correlation with isometric size, and the regression of isometric size on Factor I yielded an R^2 of 0.514 with a slope significantly different from zero ($t=13.84$, 181 d.f.). This indicates that there is a significant size effect and that the difference observed among groups in the morphometric space is largely due to size differences.

We constructed a tree based on the Mahalanobis distances, which indicated three major clusters (Fig. 3b). These clusters became our morphometry-based hypothesis of species complexes. The first cluster includes *T. rubida*, *T. protracta*, *T. nitida* and *T. barberi*; the latter two are most similar. The second cluster includes *T. pallidipennis* and *T. longipennis*; it also includes *T. ryckmani*, although with relatively large dissimilarity from the other two.

The third cluster includes *T. dimidiata* and *T. hegneri* as the most similar, and also *T. mexicana* and *T. sanguisuga*.

There are two differences in the morphometric clusters compared to the four-complex hypothesis (complexes color-coded): a lack of support for the Lenticularia complex and the inclusion of *T. rubida* in the Protracta complex (Fig. 3b vs. 4b). There are also differences with the unconstrained ITS-2 phylogeny (Fig. 3b vs. Fig. 2); most notably, the placement of *T. ryckmani* within the Phyllosoma by morphometry.

3.4 Systematic Hypothesis Testing

In order to determine the best-supported species complex compositions, we determined the statistical support for the ITS-2 data constrained into topologies reflecting: (1) well-supported (>90% BPP) clades recovered from the unconstrained phylogeny (Fig. 4a), (2) the four-complex hypothesis (Table 1 and Fig. 4b), and (3) the morphometric species complex hypothesis (Fig. 4c). Collapsed clades and the red “100” BPP values indicate the monophyletic constraints imposed on the trees. The unconstrained phylogenetic inference (i.e., the ITS-2 tree) (Fig. 4a) was the best fit of the data indicated by the lowest likelihood score (Table 5). We used the SH test to determine if the unconstrained topology represents a significantly better fit. After 10,000 RELL bootstrap replicates, the estimated probability of observing a difference in log likelihood scores between the best-fit tree and competing topologies was $p < 0.001$ (Table 5). Therefore, we can reject the four-complex hypothesis as a reasonable fit of the data.

In addition, we tested the congruence of the morphometric data and ITS-2 phylogeny using a reduced data set comprised of the 11 species available for morphometry. We determined the statistical support for: (1) the unconstrained phylogeny (Fig. 5a) and (2) the three clusters inferred from the morphometry data set (Fig 5b). Again, the unconstrained phylogeny significantly better fit the data than the morphometry species complex hypotheses (Table 6).

4. Discussion

Our results show the currently accepted four-complex arrangement of the North and Central American *Triatoma* requires reconsideration. Specifically, ITS-2 phylogeny does not support the monophyly of the four complexes, and an unconstrained analysis of the data significantly better fits the data. The “four-complex” Protracta and Lenticularia complexes include members that are split between two clades in the ITS-2 topology and the Phyllosoma complex includes additional members.

The three-clade topology inferred by ITS-2 data supports a monophyletic clade containing: *T. bolivari*, *T. nitida*, *T. rubida*, and *T. ryckmani*. *T. ryckmani* was initially not placed in any species complex [12]. The tentative placement of *T. ryckmani* in the Phyllosoma complex [2,15] can be rejected, consistent with results of Bargues et al. [8]. To accommodate this finding, we propose that the species complexes are updated to include a “Rubida” complex (Table 6). Although previous literature includes a “Rubida complex,” based on analysis of *cyt b*, it did not include multiple species, rather only of subspecies of *rubida* [47].

The Lenticularia complex, as constituted in the four-complex hypothesis, is not supported. *T. lecticularia* was never recovered in a well-supported clade with *T. gerstaeckeri* or *T. sanguisuga*; rather these two species are found within the Phyllosoma. This leaves *T. recurva* and *T. indictiva* as the only other members of a Lenticularia complex, a hypothesis we were unable to test as these were not available. (Espinoza, et al. [33] published ITS-2 sequence of *T. recurva*, however, it is only 400 bp long, and the lack of information on the variable sites in the 3' end made it unusable for this analysis). However, since *T. recurva* clusters with the phyllosoma by *cyt b* [33], and *T. indictiva* was previously classified as a subspecies of *T. sanguisuga* [12] these species likely to cluster within Clade C, rather than with *T. lecticularia*. Interestingly, *T. lecticularia* is the only North American *Triatoma* that has the undivided X chromosome, whereas most South American *Triatoma* have a divided X [48], further evidence for its separation from other North American *Triatoma*.

In addition, this study supports the previously described Protracta complex including *T. protracta* and *T. barberi*, however, with the exclusion of *T. nitida*, in congruence with results of Espinoza et al. using a shorter segment of ITS-2 [33]. Inference using a fragment of the mitochondrial *cyt b* sequence also supports this Protracta complex, however, places *D. maximus* and *T. lecticularia* as sister species to *T. protracta* and *T. barberi* [33]. Due to weak BPP support (87%), there is insufficient statistical support for inclusion of *D. maximus* and *T. lecticularia* within Protracta, which remain unresolved.

Although sequence data for the sister taxa of *T. flavida* within the Flavida complex was not available, in a previous study *T. flavida* and *T. bruneri* formed monophyletic clade with 100% bootstrap support inferred from 16S mtDNA sequence data [49]. Therefore it is likely these two species constitute a clade, but the inclusion of *T. obscura* still needs to be tested.

4.1 Morphometric Analysis

Congruence of molecular phylogenies and morphometric data have been reported in the Triatominae [50] and head morphometry has been used to distinguish species of *Triatoma* successfully [17]. However, morphometric data has also shown an environmental influence in insect shape variation, e.g. cave-adaptations seen in arthropods [12]. A morphometric study addresses the question of what groups (species) have the most similar shape (in this case, head shape) among those compared. Furthermore, morphometry attempts to separate the genetic and evolutionary components from the environmental influence on shape, which is represented by the isometric size, given that size can be affected by environmental factors like temperature. The analysis also assumes there is isometric growth, and that all morphometric distances grow at the same rate with overall insect body size. Here we found that size removal was not entirely possible thus the morphometry-based hypothesis includes an environmental component, and this was probably due to the fact that morphometric distances do not grow similarly with body size in all species.

In the present study, we observed the morphometry-based hypothesis of species complexes had some agreement with the unconstrained molecular phylogeny (Figs. 2 & 3b). For instance, the morphometry-based hypothesis supported head shape similarities between *T. dimidiata* - *T. hegneri* and *T. longipennis* - *T. pallidipennis*. Morphometry also showed similarity between *T. sanguisuga* and the Phyllosoma complex in congruence with the

unconstrained phylogeny. These results support a genetic component in the morphometric results. Morphometry also agrees with the unconstrained phylogeny in the lack of support for a Lenticularia complex.

The genetic component in the morphometric results was less evident with respect to the Protracta and Rubida complexes illustrated by the placement of *T. ryckmani* within the Phyllosoma complex in the morphometric topology (Fig. 3b). *T. ryckmani* is found in low-altitude (360–400 meters) semiarid regions of Guatemala (average temperature 31–36°C, 30–65% relative humidity) and probably similar habitats in the rest of Central America [51,52]. To the best of our knowledge, its distribution in Guatemala does not overlap with that of any of the other species studied. The head shape similarity of *T. ryckmani* with the Phyllosoma complex does not appear to be due to geographic proximity; and it would be necessary to investigate whether the particularly dry and hot environment in which this species develops modulates head shape and explains the convergence with *T. hegneri*, the species with which it has the smallest Mahalanobis distance (4.99, Table 4).

In summary, morphometry produced some results that agree with the unconstrained phylogeny and support a genetic basis for most head shape similarities. However, the placement of *T. ryckmani* and the relations among the species of the Protracta and Rubida complexes were not in agreement. This could be due to a residual environmental component.

4.2 The Strength of Hypothesis Testing in Molecular Phylogeny

Adhering to strict monophyly provides a mechanism for testing competing species complex compositions, and testing the congruence of independent molecular phylogenies (Fig. 4a–b, Table 5). Using the SH test, we showed that likelihood based tests of topology overwhelmingly supported the unconstrained topology suggesting a three-clade hypothesis. The only remaining uncertainty in the three-clade hypothesis is the affiliation of *T. lecticularia* and *D. maximus* with the Protracta complex due to weak BPP support (87%, Fig. 2). Additional sequence data from independent genes is necessary to resolve this.

Given our and published results on North and Central America *Triatoma* molecular phylogenies, we present an update to the species complex compositions, based on the best-supported systematic hypothesis using ITS-2 (Table 6). We propose a framework for future studies to test systematic hypotheses with the aim of incorporating strict monophyly and hypothesis testing as a means to resolve species complex composition. Future studies with additional DNA markers, and using statistical tests of topologies will allow investigators to refine these groups concordant with evolutionary history. A well-resolved, well-supported phylogeny will permit more relevant inferences about ecology/feeding behavior/niche, and epidemiologically relevant traits, which might have a common evolutionary origin for taxa in the same complex.

4.3 Conclusion

In summary, these results suggests the following: (1) the Lenticularia complex is not tenable; rather *T. sanguisuga* and *T. gerstaeckeri* are associated with the Phyllosoma complex; (2) the Phyllosoma complex is well supported; (3) the Protracta complex is

supported with the exclusion of *T. nitida*, and future studies should include other species that were not available and are currently placed in the Protracta complex; (4) we propose a new Rubida complex comprised of *T. nitida*, *T. rubida*, *T. ryckmani*, and *T. bolivari* and present an updated table of species complexes based on these results (Table 6). It will be important to resolve the position of *D. maximus* and *T. lecticularia* as well as the remaining eight unstudied species and to test this systematic hypothesis with additional molecular markers, using statistical tests of topologies to select amongst competing hypotheses.

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Highlights

- We determine ITS-2 sequence and morphometry on *Triatoma* of N. and C. America
- We infer phylogenetic relationships among the species based on these two data sets
- We determine statistical support for current hypothesis using SH tests of topologies
- We propose a new composition of species complexes

Fig. 1.
Schematic representation of a triatomine head (viewed from above). Fourteen landmarks were placed on head images. Nine interlandmark distances were used in the morphometric analysis: 1–2, 1–6, 1–12, 2–4, 3–10, 3–13, 4–5, 4–6, 6–7.

Fig. 2.

Majority rule consensus ITS-2 phylogeny of the North and Central American *Triatoma*. H=Haplotype (Table 2). Numbers represent Bayesian posterior probability (BPP); scale represents genetic distance. Haplotypes are color-coded by the four-complex species complex hypothesis (Red = Flavida Complex, Orange = Protracta Complex, Green = Phyllosoma, and Blue = Lenticularia).

Fig. 3.

Morphometric analysis results. (a) Factor map of species and their group centroids on the first two discriminant factors; Factor I contained 55% of variation and Factor II contained 55% of variation in the head shape data. (b) Topology based on Mahalanobis distances, showing three major branches that represent our morphometry-based hypothesis of species complexes. Species are color-coded by the four-complex species complex hypothesis (Orange = Protracta Complex, Green = Phyllosoma, and Blue = Lenticularia).

Fig. 4.

a–b. Constraint trees representing two competing systematic hypotheses based on ITS-2 phylogeny. Numbers at nodes represent BPP, and red “100” indicates a monophyletic constraint. Species are color-coded by four-complex species hypothesis (Red = Flavida Complex, Orange = Protracta Complex, Green = Phyllosoma, and Blue = Lenticularia). H=Haplotype (Table 2). (a) ITS-2 topology of the North and Central American *Triatoma* constrained by the three-complex hypothesis (Fig. 2), (b) ITS-2 topology constrained by the four-complex hypothesis.

Fig. 5.

a–b. Constraint trees representing two competing systematic hypotheses inferred from ITS-2 sequence variation using the reduced dataset. Species are color-coded by the four-complex species hypothesis (Orange = Protracta Complex, Green = Phyllosoma, and Blue = Lenticularia). (a) ITS-2 phylogenetic inference of the North and Central American *Triatoma* with *Triatoma infestans* as the outgroup constrained by the three-complex hypothesis (Fig. 2). Numbers at nodes represent support values (BPP), and red “100” indicates a monophyletic constraint. (b) ITS-2 phylogenetic inference of the North and Central American *Triatoma* with *Triatoma infestans* as the outgroup constrained by the Morphometric clusters.

Table 1

Currently Accepted “Four-complex hypothesis” of Species Complexes of North and Central American *Triatoma*

Complex	Species
Phyllosoma	<i>T. dimidiata</i> , <i>T. hegneri</i> , <i>T. brailoviskyi</i> , <i>T. gomeznunezi</i> <i>T. bassolsae</i> , <i>T. bolivari</i> , <i>T. longipennis</i> , <i>T. mazzottii</i> , <i>T. mexicana</i> , <i>T. pallidipennis</i> , <i>T. phyllosoma</i> , <i>T. picturata</i> , <i>T. ryckmani</i>
Flavida	<i>T. flavida</i> , <i>T. bruneri</i> , <i>T. obscura</i>
Protracta	<i>T. barberi</i> , <i>T. incrassata</i> , <i>T. neotomae</i> , <i>T. nitida</i> , <i>T. peninsularis</i> , <i>T. protracta</i> , <i>T. sinaloensis</i>
Lecticularia	<i>T. gerstaeckeri</i> , <i>T. indictiva</i> , <i>T. lecticularia</i> , <i>T. recurva</i> , <i>T. rubida</i> , <i>T. sanguisuga</i>

Adapted from Schofield and Galvão [2].

Table 2

Triatoma species studied. For each specimen, collection location, ITS-2 haplotype, sequence length, AT composition, and GenBank accession numbers are given.

Species	Sampling Location	ITS-2 Haplotype	Sequence Length (nt)	%AT	Accession Number
FLAVIDA COMPLEX					
<i>T. flavida</i>	Peninsula of Guanahacabibes, Cuba	T.flav.H1	493	78.7	AM286732
PHYLLSOMA COMPLEX					
<i>T. bassolsae</i>	Puebla, Mexico	T.bas.H1	489	76.9	AY860402
<i>T. bolivari</i>	Oaxaca, Mexico	T.bol.H1	501	76.8	AM286725
<i>T. dimidiata</i>	La Brea, Guatemala	T.dim.H1	497	76.0	AM286693
<i>T. dimidiata</i>	Lachua, Guatemala	T.dim.H1	497	76.0	AM286694
<i>T. dimidiata</i>	Santa Fe, Panama	T.dim.H16	497	76.0	AM286708
<i>T. dimidiata</i>	Santa Fe, Panama	T.dm.H16	497	76.0	AM286709
<i>T. dimidiata</i>	Izabal, Guatemala	T.dim.H18	497	75.8	AM286710
<i>T. dimidiata</i>	Quiché, Guatemala	T.dim.H37	496	75.8	FJ197151
<i>T. dimidiata</i>	Quiché, Guatemala	T.dim.H18	496	75.8	AM286710
<i>T. dimidiata</i>	Petén, Guatemala	T.dim.H36	494	75.7	FJ197151
<i>T. hegneri</i>	Cedral, Cozumel isle., Quintana Roo	T.heg.H1	496	75.8	AM286726
<i>T. hegneri</i>	Cedral, Cozumel isle., Quintana Roo	T.heg.H2	496	76.0	AM286727
<i>T. longipennis</i>	Nayarit, Mexico	T.lon.H1	491	76.5	AY860397
<i>T. longipennis</i>	Nayarit, Mexico	T.lon.H2	491	76.7	AY860398
<i>T. mazzottii</i>	Guerrero, Mexico	T.maz.H1	490	76.5	AY860392
<i>T. mazzottii</i>	Guerrero, Mexico	T.maz.H2	488	76.4	AY860393
<i>T. mexicana</i>	Hidalgo, Mexico	T.mex.H1	492	75.6	AM286728
<i>T. mexicana</i>	Guanajuato, Mexico	T.mex.H2	487	75.0	AM286728
<i>T. pallidipennis</i>	Mexico	T.pal.H1 *	491	76.8	JX872262
<i>T. ryckmani</i>	El Progreso, Guatemala	T.ryc.H1	500	76.0	AM286731
<i>T. ryckmani</i>	Guatemala	T.ryc.H2 *	501	76.0	JX872266

Species	Sampling Location	ITS-2 Haplotype	Sequence Length (nt)	%AT	Accession Number
LECTICULARIA COMPLEX					
<i>T. gerstaeckeri</i>	San Luis Potosí, Mexico	T.ger.H1	483	76.8	AM286734
<i>T. lecticularia</i>	Nuevo León, Mexico	T.lec.H1	490	75.3	AY860405
<i>T. lecticularia</i>	Nuevo León, Mexico	T.lec.H2	489	75.3	AY860406
<i>T. lecticularia</i>	Nuevo León, Mexico	T.lec.H3	488	75.2	AY860407
<i>T. sanguisuga</i>	New Orleans, Louisiana, U.S.A.	T.san.H1*	514	76.8	JX872265
<i>T. sanguisuga</i>	New Orleans, Louisiana, U.S.A.	T.san.H2*	510	76.0	JX872264
<i>T. sanguisuga</i>	St. Gabriel, Louisiana, U.S.A	T.san.H3*	515	76.9	JX890270
<i>T. rubida</i>	Arizona, U.S.A.	T.rub.H1	512	77.7	AY860390
PROTRACTA COMPLEX					
<i>T. barberi</i>	Mexico	T.bar.H1*	494	74.3	JX872261
<i>T. nitida</i>	Guatemala	T.nit.H2*	491	76.3	JX872260
<i>T. nitida</i>	El Progreso, El Progreso Guatemala	T.nit.H1	490	76.3	AM286733
<i>T. protracta</i>	Arizona, U.S.A.	T.pro.H1*	489	75.9	JX872263
INFESTANS COMPLEX					
<i>T. infestans</i>	Not Available	T.inf.H1	467	78.3	AY860387
DIPETALOGASTER					
<i>D. maximus</i>	Baja, Mexico	D.max.H1	475	76.4	AJ286887

* New haplotypes identified this study

Table 3 Male *Triatoma* specimens used for morphometric analysis

Species	Municipality	Department or State	Country	n
<i>Triatoma barberi</i>			Guatemala	11
<i>Triatoma dimidiata</i>		Jutiapa	Guatemala	6
	Campeche		Guatemala	4
			Panama	7
<i>Triatoma hegneri</i>		Cozumel	Mexico	7
<i>Triatoma longipennis</i>			Mexico	19
<i>Triatoma mexicana</i>			Mexico	18
<i>Triatoma nitida</i>			Guatemala	18
<i>Triatoma pallidipennis</i>			Mexico	22
<i>Triatoma protracta</i>			Arizona	17
<i>Triatoma rubida</i>			Arizona	28
<i>Triatoma ryckmani</i>			Guatemala	11
<i>Triatoma sanguisuga</i>	New Orleans	Louisiana	United States of America	15
Total				183

Table 4

Mahalanobis distances among group centroids after a discriminant analysis over the size-components of head shape. P-values from permutation tests (1,000 permutation rounds) for Mahalanobis distances among groups were all $P < 0.00091$, indicating significant differences.

	T. barberi	T. nitida	T. protracta	T. rubida	T. sanguisuga	T. mexicana	T. hegneri	T. dimidiata	T. ryckmani	T. pallidipennis	T. longipennis
<i>T. barberi</i>	0										
<i>T. nitida</i>	2.54	0									
<i>T. protracta</i>	3.94	5.58	0								
<i>T. rubida</i>	6.41	4.69	6.91	0							
<i>T. sanguisuga</i>	7.86	7.71	6.28	5.45	0						
<i>T. mexicana</i>	9.15	8.93	7.93	6.98	2.75	0					
<i>T. hegneri</i>	10.94	10.54	9.09	7.44	3.64	3.06	0				
<i>T. dimidiata</i>	10.99	10.42	9.74	7.39	3.51	2.83	2.55	0			
<i>T. ryckmani</i>	11.25	10.94	8.71	7.66	6.25	7.21	4.99	7.14	0		
<i>T. pallidipennis</i>	10.03	10.34	7.86	9.26	7.1	6.38	6.3	8.35	6.2	0	
<i>T. longipennis</i>	8.3	8.41	6.51	7.21	4.96	4.32	4.9	6.51	5.91	2.44	0

Table 5

Summary of the topology test statistics.

	- ln L	Difference	P-Value
Unconstrained	2925.3	Best	
Four-complex	3022.8	97.4	<0.001

Table 6

Summary of topology test statistics for reduced data set.

	- ln L	Difference	P-Value
Unconstrained	2171.9	Best	
Morphometric	2211.7	39.7	<0.01

Table 7

Updated table of species complexes based on ITS-2 phylogenetic inference and statistical tests of topologies

Group	Complex	Species
Rubrofasciata	Phyllosoma	<i>T. dimidiata</i> , <i>T. hegneri</i> , <i>T. bassolsae</i> , <i>T. gerstaeckeri</i> , <i>T. longipennis</i> , <i>T. pallidipennis</i> , <i>T. phyllosoma</i> , <i>T. picturata</i> , <i>T. mazzottii</i> , <i>T. mexicana</i> , <i>T. recurva</i> , <i>T. sanguisuga</i>
	Protracta	<i>T. protracta</i> , <i>T. barberi</i> ,
	<u>Rubida</u>	<i>T. bolivari</i> , <i>T. nitida</i> , <i>T. rubida</i> , <i>T. ryckmani</i>
	Flavida	<i>T. flavida</i> , <i>T. bruneri</i> , <i>T. obscura</i> [#]
	Unassigned	<i>T. indictiva</i> [#] , <i>T. lecticularia</i> , <i>T. brailovskyi</i> [#] , <i>T. gomeznunezi</i> [#] , <i>T. incrassata</i> [#] , <i>T. neotomae</i> [#] , <i>T. peninsularis</i> [#] , <i>T. sinaloensis</i> [#]

Underlined species moved to new complexes (this study).

[#] Species not tested.