



The first assess of the haplotypes from *COI* gene sequences in species of spittlebugs (Cicadomorpha: Hemiptera) and aquatic true bugs (Gerromorpha and Nepomorpha: Hemiptera) in Brazil

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Genet. Mol. Res. 12 (4): 5372-5381 (2013)

Received February 6, 2013

Accepted August 19, 2013

Published November 7, 2013

DOI <http://dx.doi.org/10.4238/2013.November.7.12>

ABSTRACT. We made the first analysis of the *COI* gene sequences of 22 species of spittlebugs and aquatic true bugs sampled in São Paulo State (Brazil) and used this information to determine the variability within these groups. Considering each codon position, we observed that the third base was the most variable, and the first base was the most conserved. Among species, *Mahanarva fimbriolata* and *Deois flavopicta* had the greatest genetic distance (0.181), and *Notozulia entreriana* and *Mahanarva* sp had the smallest distance (0.055), with an average variation of 0.119. In Gerromorpha, the

greatest distance occurred between *Halobatopsis platensis* and *Rhagovelia zela* (0.401), while between *Cylindrostethus palmaris* and *Brachymetra albinervis albinervis*, the distance was only 0.187; the average value observed for the Gerromorpha was 0.265. In the Nepomorpha, the species *Buenoa antigone antigone* and *Belostoma micantulum* had the greatest genetic distance (0.337), while *Martarega brasiliensis* and *B. a. antigone* had the smallest (0.154). The average value observed for Nepomorpha was 0.203. In Cicadomorpha (Auchenorrhyncha) and Nepomorpha (Heteroptera), the *COI* gene has been conserved; however, it is still useful for characterization of the different taxa. *COI* analysis was unable to resolve some of the Gerromorpha groups.

Key words: Heteroptera; Auchenorrhyncha; Gerromorpha; Nepomorpha; MtDNA

INTRODUCTION

The order Hemiptera is currently classified into four suborders: Auchenorrhyncha, Coleorrhyncha, Sternorrhyncha, and Heteroptera (Carver et al., 1991; Forero, 2008). The suborder Auchenorrhyncha (part of the paraphyletic clade Homoptera in former classifications) includes, among others, the spittlebugs, a group of hemipterans belonging to the superfamily Cercopoidea, which are important components of grass entomofauna. The family Cercopidae, an important member of this superfamily, is one of the largest groups of sucking insects, with representatives being phytophagous and feeding predominantly on xylem. Approximately 1500 species in 150 genera have so far been described in this family (Liang and Webb, 2002), where they are distributed mainly in tropical and subtropical regions. Of these, approximately 400 species are present in South America, with several important pests of forage grasses and sugar cane (Costes and Webb, 2004; Castanhole et al., 2010). In Brazil, these sharpshooters are also pests of sugar cane and pastures, and are therefore of great importance for biological control studies and systematics (Milanez et al., 1983; Zanol, 1996).

Heteroptera, or true bugs, is the largest and most diverse group of insects with incomplete metamorphosis. This suborder has seven infraorders, including Gerromorpha and Nepomorpha, and approximately 80 families, which can be found on all continents except Antarctica and on some islands (Schuh and Slater, 1995). In the São José do Rio Preto region of Brazil, Heteroptera families that are commonly found include Belostomatidae, Coreidae, Gelastocoridae, Gerridae, Lygaeidae, Notonectidae, Pentatomidae, Phyrrocoridae, Reduviidae, Rhopalidae and Veliidae. They can be either phytophagous, predatory or hematophagous. Specifically, these insects can live as parasites of birds and mammals, feed on plants and fungi or capture other arthropods in spider webs or water. Also, a few Heteroptera species reside in the ocean (Schuh and Slater, 1995).

Insects are extremely complex groups due to their great diversity of mitochondrial genomes with rearrangements and recombinations, related to substitution rates and nucleotide composition, suggesting the need for additional studies, including molecular approaches (Hua et al., 2008).

Molecular analysis not only reveals the DNA sequence, but also polymorphisms

whose genetic basis and particular modes of transmission can be precisely determined (Avisé, 2004). Thus, the molecular data in several studies collected for different taxonomic groups have contributed to considerable advances in understanding the species' biology, ecology, behavior, genetics and evolution (Sunnucks, 2000; Avisé, 2004). Given the diverse characteristics of these suborders (Auchenorrhyncha and Heteroptera) and the evolutionary proximity of the groups, the use of molecular markers may be necessary for genetic characterization of these individuals. Such analyses could provide a better understanding of the group.

The objective of this study was to carry out the first assessment of different haplotypes for 22 species belonging to spittlebugs and aquatic true bugs (Hemiptera) sampled in Brazil, using mitochondrial cytochrome c oxidase subunit 1 gene (*COI*) sequences, and to use this information to determine the variability within this group of insects.

MATERIAL AND METHODS

Taxon sampling

A total of 22 species were used in this study, including four species belonging to the suborder Auchenorrhyncha (Cicadomorpha, Cercopidae) and 18 species of the aquatic and semi-aquatic infraorders (Gerromorpha and Nepomorpha) of the suborder Heteroptera (Table 1).

Table 1. Species used in this study, broken down by family, suborder and infraorder.

Suborder	Infraorder	Family	Species
Auchenorrhyncha	Cicadomorpha	Cercopidae	<i>Deois flavopicta</i> (Stål, 1954) <i>Mahanarva fimbriolata</i> (Stål, 1854) <i>Mahanarva</i> sp (Stål, 1854) <i>Notozulia enteriana</i> (Berg, 1879)
Heteroptera	Gerromorpha	Gerridae	<i>Brachymetra albinervis albinervis*</i> (Amyot and Serville, 1843) <i>Brachymetra albinervis albinervis**</i> (Amyot and Serville, 1843) <i>Cylindrostethus palmaris</i> (Drake and Harris, 1934) <i>Halobatopsis platensis</i> (Berg, 1879) <i>Limnogonus aduncus</i> (Drake and Harris, 1933) <i>Rheumatobates crassifemur crassifemur</i> (Esaki, 1926)
		Veliidae	<i>Microvelia longipes</i> (Uhler, 1894) <i>Rhagovelia robusta</i> (Gould, 1931) <i>Rhagovelia tenuipes</i> (Champion, 1898) <i>Rhagovelia zela</i> (Drake, 1959)
	Nepomorpha	Belostomatidae	<i>Belostoma micantulum</i> (Stål, 1858)
		Gelastocoridae	<i>Gelastocoris flavus flavus</i> (Guérin-Méneville, 1835)
		Notonectidae	<i>Buenoa amnigenus</i> (White, 1879) <i>Buenoa antigone antigone</i> (Kirkaldy, 1899) <i>Buenoa unguis</i> (Truxal, 1953) <i>Martarega brasiliensis</i> (Truxal, 1949) <i>Martarega membranacea</i> (White, 1879) <i>Martarega uruguayensis</i> (Berg, 1883)

*Population 1 collected in São José do Rio Preto; **Population 2 collected in Jales.

Auchenorrhyncha species were collected in Campo Grande, MS, Brazil (20°26'S, 54°38'W). Heteroptera species were collected in the cities of Américo de Campos (20°18'S, 49°44'W), Guaraci (20°30'S, 48°56'W), Jales (20°16'S, 50°32'W), Mirassol (20°49'S, 49°30'W), Sales (21°20'S, 49°29'W), and São José do Rio Preto (20°47'S, 49°21'W) in São

Paulo State, Brazil. Two populations of *Brachymetra albinervis albinervis*, population 1 (*) and population 2 (**), were collected in São José do Rio Preto and Jales, respectively. All samples were stored at -20°C in absolute ethanol at the Laboratory of Cytogenetic and Molecular of Insects (LACIMI), Instituto de Biociências, Letras e Ciências Exatas, Universidade Estadual Paulista (UNESP), Campus São José do Rio Preto, SP, Brazil.

Molecular data

mtDNA was extracted using the protocol described by Tartarotti (2002) and the Genomic DNA Extraction kit protocol from Machery Nagel Tissue. To obtain the sequence of the *COI* gene, we used two primers (1 and 2, Table 2) to first amplify the gene. The gene was amplified by PCR with the following conditions: denaturation at 94°C for 1 min; 35 cycles of denaturation (94°C for 30 s), annealing (temperature gradient 48 - 55°C for 1 min), extension (72°C for 1 min); and extension at 72°C for 7 min. The reaction consisted of 0.3 µL nucleotides (ATCG), 11.6 µL ddH₂O, 0.44 µL MgCl₂, 1.5 µL PCR buffer, 0.06 µL DNA Platinum Taq polymerase, and 0.3 µL primers (10 mM).

Table 2. Primers used for the amplification and sequencing of the *COI* gene.

Primers	Forward	Reverse	Measure (+/-)	Reference
1	TTTCAACAAATCATAAAGATATTGG	TAAACTTCAGGGTGACCAAAAAATCA	700 bp	Folmer et al. (1994)
2	CAACATTATTTTGATTTTTGG	GAATACTGCTCTATGGATA	500 bp	Simon et al. (1994); Damgaard and Sperling (2001)

After the amplification reactions, sequencing was performed, a total of 22 sequences were used for the species of the suborders Auchenorrhyncha (infraorder Cicadomorpha) and Heteroptera (infraorders Gerromorpha and Nepomorpha). The primers used for sequencing annealed to the 5' portion of the *COI* gene (primer 1) and the middle of the gene (primer 2). Sequencing using primers 1 and 2 generated fragments of 650 and 350 bp, respectively. These fragments were used to construct the distance matrices and dendrograms and for analysis of nucleotide composition.

The samples were purified using a purification kit from GE Healthcare. BigDye sequencing was performed by the Center for Human Genome Studies at the University of São Paulo-USP, Brazil, according to a standard protocol (<http://genoma.ib.usp.br>).

The analysis of sequence alignments and determination of nucleotide composition and distance matrix learning were performed using BioEdit (Hall, 1999) and MEGA 5 (Tamura et al., 2011). For the distance matrix analysis and in order to obtain the dendrograms using the neighbor-joining method (NJ), Kimura-2-Parameter (K2P, nucleotide substitution model) and the support branches estimated from 1000 bootstrap replicates, using the program MEGA 5. *Neomaskellia andropogonis* Corbett 1926 (Aleyrodidae), which belongs to the order Hemiptera, suborder Sternorrhyncha, was used as an outgroup. The complete sequence of the *N. andropogonis* mitochondrial genome can be found in the NCBI database (<http://www.ncbi.nlm.nih.gov/>). The choice of this species was based on the work of Maddison (1995), who reported that the suborders Auchenorrhyncha and Heteroptera are derived from Sternorrhyncha.

The sequences were submitted to NCBI with the following GenBank accession numbers: JN202451- JN202471, JN681122-JN681148, and JN689483-JN689505.

RESULTS AND DISCUSSION

Variability analysis

The greatest genetic diversity for the *COI* sequences among the Cicadomorpha (Auchenorrhyncha) species was found between *Mahanarva fimbriolata* and *Deois flavopicta* (0.181), whereas the least variation occurred between *Notozulia entreriana* and *Mahanarva* sp. (0.055). Overall, the mean variation for the infraorder Cicadomorpha was 0.119.

For the infraorder Gerromorpha (Heteroptera), the largest distance was observed between *H. platensis* and *R. zela* (0.401), while the smallest was between *C. palmaris* and *B. a. albinervis*** (0.187). Overall, the mean distance value was 0.265 for Gerromorpha. For the infraorder Nepomorpha (Heteroptera), the average value was 0.203, and the greatest variation was between *B. a. antigone* and *B. micantulum* (0.337). *M. brasiliensis* and *B. a. antigone* showed the lowest variation (0.154) (Table 3). These results indicate that, on the basis of the *COI* marker, the species of Cicadomorpha (Auchenorrhyncha) have greater genetic similarity than those of the infraorders Gerromorpha and Nepomorpha.

Table 3. Highest and lowest values of interspecific and intraspecific distance and mean values for the three groups analyzed using the K2P model of nucleotide substitution.

Group	Distance			
	High	Low	Mean	Intragenetic Mean
Cicadomorpha	0.181 (<i>M. fimbriolata</i> / <i>D. flavopicta</i>)	0.055 (<i>N. entreriana</i> / <i>Mahanarva</i> sp)	0.119	0.100 - <i>Mahanarva</i>
Gerromorpha	0.401 (<i>R. zela</i> / <i>H. platensis</i>)	0.187 (<i>C. palmaris</i> / <i>B. a. albinervis</i> **)	0.265	0.291 - <i>Rhagovelia</i>
Nepomorpha	0.337 (<i>B. micantulum</i> / <i>B. a. antigone</i>)	0.154 (<i>M. brasiliensis</i> / <i>B. a. antigone</i>)	0.203	0.122 - <i>Buenoa</i> 0.119 - <i>Martarega</i>

Taking into account intragenetic variation, the genus *Rhagovelia* had an average distance value of 0.291, indicating that this genus has twice the genetic diversity of species of *Mahanarva* (Cicadomorpha, 0.1), *Buenoa* (Nepomorpha, 0.122), and *Martarega* (Nepomorpha, 0.119) (Table 3).

Specimens of *B. a. albinervis** and *B. a. albinervis*** had an intraspecific value of 0.087, whereas this was 0.055 for species of the genus *Notozulia* and *Mahanarva* of Cicadomorpha, showing the variation that exists within these groups (Tables S1, S2, and S3).

Overall, despite the variation observed, the distance values are low in the three groups studied. Sosa-Gómez et al. (2005) used the RAPD genetic marker and found that for other Heteroptera neotropical species (*Nezara viridula* Linnaeus 1758, Pentatomidae), this type of genetic similarity was associated with a high gene flow in the populations evaluated.

Park et al. (2011) and Jung et al. (2011) conducted a large study of insects of the suborder Heteroptera and observed different patterns of intraspecific and interspecific divergence using *COI* as a marker gene. In these studies, intraspecific variations were not uncommon within the suborder. The authors concluded that intraspecific divergence could be due to ancestral polymorphisms or introgression, but did not rule out the possibility of a cryptic species divergence. This observation may explain why the observed genetic distance

values for the species *B. a. albinervis* were above the lower values observed for different species of the family Cercopidae.

Figure 1A-C depicts the dendrograms formed from the genetic distance measurements. For Cicadomorpha (Auchenorrhyncha), the species *M. fimbriolata* and *Mahanarva* sp. were sister groups with a bootstrap value of 94%, and the species *N. entreriana* was more external with a 100% bootstrap value. The species *D. flavopicta* was external to the other groups (Figure 1A). In Nepomorpha, the species of the genera *Buenoa* and *Martarega* belonging to the same family (Notonectidae) were grouped together with bootstrap values of 89 and 80%, respectively. In addition, the species *B. micantulum* (Belostomatidae) and *G. f. flavus* (Gelastocoridae) were more external to the family Notonectidae (Figure 1B). Some of the Gerromorpha species belonging to the same family had unresolved clusters. For example, *M. longipes* (Veliidae) and *R. c. crassifemur* (Gerridae) were grouped together as sisters, and the between-group in relation to species of the same genus, with *R. zela* grouped outside the genus *Rhagovelia* and all other species studied. However, the grouping *B. a. albinervis** and *B. a. albinervis*** (Gerridae) showed a bootstrap value of 98% (Figure 1C).

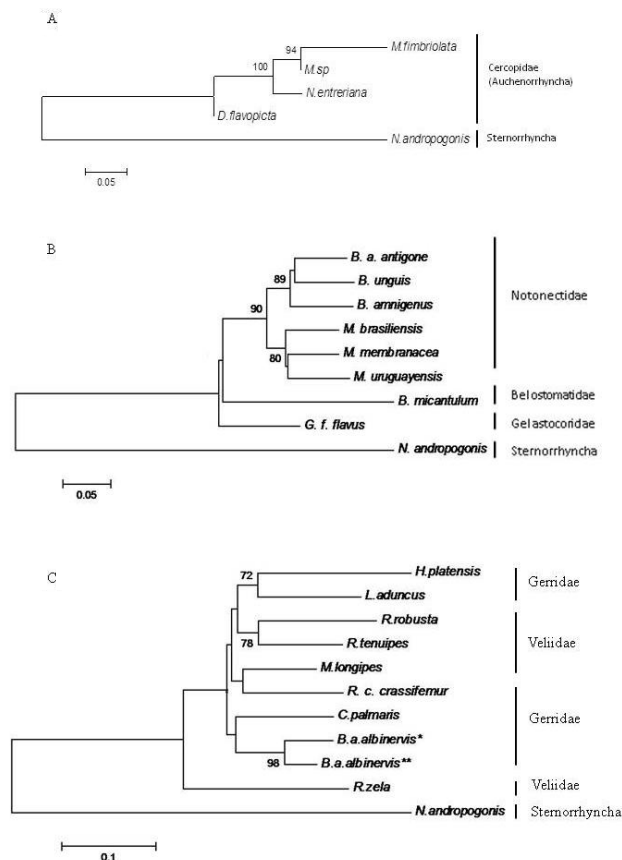


Figure 1. Dendrograms based on the neighbor joining method and the K2P model for the taxa of: **A.** Cicadomorpha (Auchenorrhyncha); **B.** Nepomorpha; **C.** Gerromorpha (Heteroptera). The numbers at the nodes of the branches refer to the bootstrap values.

Thus, we found that the clusters generated for Cicadomorpha were satisfactory because they formed congeneric groups (*Mahanarva*). The same result was obtained for the infraorder Nepomorpha genera *Buenoa* and *Martarega*, which constitute a group within the family Notonectidae and differ slightly more from the families Belostomatidae (*B. micantulum*) and Gelastocoridae (*G. flavus flavus*). Unlike the results that were previously reported for Cicadomorpha (Auchenorrhyncha) and Nepomorpha (Heteroptera), the close relationships between specimens belonging to the infraorder Gerromorpha were presented as an unresolved mixture of terminal taxa. Two of the taxa belonging to the genus *Rhagovelia* (*R. robusta* + *R. tenuipes*) were closely related, yet *R. zela* was paraphyletic. Damgaard (2008) showed that their studies differed most significantly by rejecting the family Veliidae and the superfamilies “Gerroidea” (comprising Gerridae + Veliidae + Hermatobatidae) and “Hydrometroidea” (comprising Hydrometridae + Macroveliidae + Paraphrynoveliidae) as paraphyletic entities, compared to earlier studies of global gerromorphan diversity (Andersen, 1982; Andersen and Weir, 2004). In addition, species traditionally grouped in different families resulted as sister-groups. Such is the case of *M. longipes* (Veliidae), which resulted as sister-group of *R. c. crassifemur* (Gerridae). This result corroborates the paraphyletic condition of the family Veliidae presented by Damgaard (2008), in which *Microvelia* and other Microveliinae genera are more closely related to the Gerridae than to other Veliidae. The confidence values generated by bootstrap in this analysis ranged from 72 to 98%.

The diversification observed for *R. zela* could be a natural characteristic of this species. Alternatively, this diversity could be due to variation at the collection site (Américo de Campos, SP, Brazil), which was different from other similar groups of *Rhagovelia* (Mirassol, SP, Brazil), and was therefore liable to undergo differential selective pressure. Garcia et al. (2003) noted that the differences observed in *Triatoma infestans* (Reduviidae, Heteroptera) are not necessarily related to gene flow in these populations, but they could be eventually attributed to local selection pressure or genetic drift. However, additional studies are needed, including population analysis of *R. zela* collected in different regions to determine if this kind of diversification is natural or if the population is subject to different selective forces.

For Cicadomorpha (Auchenorrhyncha) and Nepomorpha (Heteroptera), the dendrograms and distance matrices suggest that the *COI* gene had been conserved, yet it was efficient for the characterization of the different taxa analyzed. *COI* analysis was unable to resolve the groups in Gerromorpha. However, this diversification may be a natural characteristic of some species analyzed, or it may be related to the environment.

Nucleotide composition

The nucleotide composition, or AT%, for the species of the infraorder Cicadomorpha (Auchenorrhyncha, Group 1) studied ranged 60.41-72.10% with an average of 68.15%. The species of Heteroptera of the infraorder Gerromorpha (Group 2) had an AT% ranging 64.34-70.51% and an average of 67.56%. Overall, the species of the infraorder Nepomorpha (Group 3) had an average AT% of 67.77%, and AT% ranged 63.62-69.71% for the individual species.

The AT% was high for all three groups, but the highest value was observed in Group 1 (68.15%), and the lowest value was found in Group 2 (67.56%) (Table 4). These results revealed that among the different haplotypes of the *COI* gene, the nucleotide composition was above average, with an increased frequency of adenines and thymines. In line with these results,

Hua et al. (2008) analyzed the mitochondrial genome of Pentatomomorpha (Heteroptera) and noted that the nucleotide composition was 68.86 to 77.8%. This study also found that gene rearrangements were not correlated with rates of nucleotide substitution and that different evolutionary patterns between genes were due to variation in GC content.

Table 4. Total amino acid and DNA sites analyzed, total amino acids and polymorphic sites, ratio of transition and transversions (Ti/Tv) and average frequency of A + T of the three groups for the suborder Auchenorrhyncha (1) and the infraorder Gerromorpha (2) and Nepomorpha (3) of the suborder Heteroptera.

Groups	Sites		Aminoacids		Nucleotide composition	
	Total	Polymorphics	Total	Polymorphics	Ti/Tv	%AT
1	1010	42	336	1	1.271	68.15
2	1015	264	338	88	0.983	67.56
3	1013	191	337	27	0.818	67.77

The total number of sites analyzed for Groups 1, 2 and 3 were 1010, 1015 and 1013, respectively. Of these, Group 2 showed the highest number of polymorphic sites (264), followed by Group 3 with 191 sites. The total number of these polymorphic sites that caused an amino acid change was relatively small. For Group 1, only DNA polymorphism resulted in an amino acid change. This result was different from what was observed for Gerromorpha and Nepomorpha infraorders, which had 88 and 27 polymorphic amino acids, respectively (Table 4).

Analysis of the ratio between the number of transitions (Ti) and transversions (Tv) showed that Ti occur more often, which was indicated by a shorter genetic distance. In regards to this, the shortest distance was observed for Group 3 (0.818), and the largest distance was found for Group 1 (1.271) (Table 4).

When considering the potential information for each codon position, we observed that for all of the groups, the third base in a codon was the most variable, and the first base was the most conserved. The third base position for the infraorder Nepomorpha was the most variable (229 polymorphisms), and it was the least variable (126) in Auchenorrhyncha. The infraorder Gerromorpha showed the third base with more nucleotide information (142) (Table 5). This is a characteristic that has been described by Hebert et al. (2003), who found that for the *COI* gene, there is great variation in the base in the third position of its codons, although changes in the *COI* amino acid sequence occur more slowly than for other mitochondrial genes. The *COI* gene, when compared with other genes, has a slower rate of protein evolution.

Table 5. Number of variable sites based on the parsimonious nucleotide in a codon.

Suborder	Infraorder	Sites					
		Variables			Parsimonious		
		1st	2nd	3rd	1st	2nd	3rd
Auchenorrhyncha	Cicadomorpha	36.00	6.00	126.00	6.00	1.00	35.00
Heteroptera	Gerromorpha	139.02	62.60	198.00	85.00	36.60	142.00
	Nepomorpha	80.75	22.00	229.00	48.00	9.00	134.00

These analyses provide important information in studies such as strand asymmetry, which help in understanding the variations of selection on amino acid sequences and mutation during replication and transcription of mitochondrial DNA for evolution studies (Wei et al., 2010).

Studies analyzing the diversity of Hemiptera are rare but extremely important for understanding the genetic characteristics of this group, including the nucleotide composition and the variation of genes within and between species and related groups. In this study, we observed that the mitochondrial *COI* gene region (1 kb) appears to be a good marker. These findings are consistent with what had been described for other species of Hemiptera.

Additional studies and analyses with different markers should be conducted to confirm the observations of this study; meanwhile, these results contribute to taxonomic, systematic and genetic variability studies of the populations of the entomologic community.

ACKNOWLEDGMENTS

Research supported by FAPESP and FUNDUNESP. The identification of aquatic insects was performed by the research group of Prof. Dr. Jorge Luiz Nessimian (Contributor, UFRJ, RJ), and the Auchenorrhyncha samples were supplied by Prof. Dr. Jose Raul Valerio (EMBRAPA-CENARGEN, Campo Grande, MS).

[Supplementary material](#)

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SUPPLEMENTARY MATERIAL

Table S1. Distance matrices utilizing K2P for the infraorder Cicadomorpha (Auchenorrhyncha). In bold the highest and lowest values.

Espécies	1	2	3	4
1 Neomaskellia andropogonis				
2 Deois flavopicta	0.629			
3 Mahanarva fimbriolata	0.897	0.181		
4 Mahanarva spectabilis	0.768	0.105	0.100	
5 Notozulia entreriana	0.756	0.098	0.179	0.055

Table S2. Distance matrices utilizing K2P for the infraorder Gerromorpha (Heteroptera). In bold the highest and lowest values.

Espécies	1	2	3	4	5	6	7	8	9	10
1 Neomaskellia andropogonis										
2 Brachymetra albinervis albinervis*	0.801									
3 Brachymetra albinervis albinervis**	0.726	0.087								
4 Cyllindrostethus palmaris	0.778	0.214	0.187							
5 Halobatopsis platensis	0.918	0.306	0.299	0.303						
6 Limnogonus aduncus	0.804	0.260	0.262	0.245	0.279					
7 Microvelia longipes	0.757	0.210	0.196	0.206	0.273	0.211				
8 Rheumatobates crassifemur	0.772	0.217	0.204	0.264	0.296	0.280	0.189			
9 Rhagovelia robusta	0.848	0.293	0.245	0.259	0.334	0.264	0.247	0.258		
10 Rhagovelia tenuipes	0.802	0.224	0.224	0.228	0.297	0.244	0.208	0.243	0.216	
11 Rhagovelia zela	0.777	0.353	0.342	0.336	0.401	0.377	0.317	0.349	0.357	0.302

Table S3. Distance matrices utilizing K2P for the infraorder Nepomorpha (Heteroptera). In bold the highest and lowest values.

Espécies	1	2	3	4	5	6	7	8
1 Neomaskellia andropogonis								
2 Buena amnigenus	0.814							
3 Buena antigone antigone	0.730	0.122						
4 Belostoma micantulum	0.813	0.294	0.337					
5 Buena unguis	0.786	0.131	0.115	0.286				
6 Gelastocoris flavus	0.707	0.242	0.214	0.271	0.218			
7 Martarega brasiliensis	0.720	0.176	0.154	0.310	0.178	0.200		
8 Martarega membranacea	0.736	0.157	0.171	0.307	0.174	0.216	0.111	
9 Martarega uruguayensis	0.774	0.166	0.160	0.323	0.177	0.225	0.127	0.120