Genetic Differentiation in Species of *Anopheles* from the Subgenera *Nyssorhynchus* Based on Mitochondrial DNA

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Abstract: Our group analyzed species of *Anopheles* of the subgenus *Nyssorhynchus* (*An. darlingi, An. marajoara, An. oswaldoi, An. benarrochi, An. triannulatus* and *An. rangeli*) and a single species of the subgenus *Anopheles* (*An. mattogrossensis*), originating in the Amazon, based on the mitochondrial DNA control region. The fragment had 381 pb and the nucleotide composition in A+T ranged from 85.8 to 89.5%. DNA polymorphism analysis of the species detected 15 haplotypes, with the presence of 81 polymorphic loci, 95 mutations, haplotypic divergence of 0.879, nucleotide diversity of 0.06507, nucleotide differences pair the pair mean of 23.1749, and the observed and expected variances of 113.840 and 8.33, respectively. The genetic distance among the *Anopheles* species varied from 0.06 to 1.37%. An. *oswaldoi* and An. *rangeli* were the most similar, with nucleotide divergence of 0.17%. The *An. benarrochi* populations of Ji-Paraná and Bolivia showed nucleotide divergence of 0.06%. *An. darlingi, An. marajoara* and *An. triannulatus* presented nucleotide divergences of 0.35% between *An. triannulatus* and *An. darlingiand* of 0.47% between *An. darlingi* and *An. marajoara*. This data showed ample interspecific nucleotide variation, though with low nucleotide divergence. The cladogram separated the species of the subgenus *Nyssorhynchus* and those of the subgenera *Cellia* and *Anopheles*, with 98% bootstrap. The region control data did not show strong phylogenetic support, as indicated by the transition/transversion mean rate (0.4643), which is necessary for increasing fragment size and using other more conservative genes for greater inference concerning the phylogeny of the *Anopheles* species of the subgenus *Nyssorhynchus*.

Keywords: Amazon, *Anopheles*, differentiation, genetic, mitochondrial DNA, *Nyssorhynchus*, region control.

INTRODUCTION

The *Anopheles* species has been studied regarding diverse aspects, since it is directly related to the transmission of malaria.

The genus *Anopheles* consists of 437 species, of which only a small number are important for malaria epidemiology. The genus is divided in six subgenera: *Anopheles, Cellia, Nyssorhynchus, Lophopodomyia, Stethomyia* and *Kerteszia* [1]. This classification has partially assisted in the analysis of morphological and phylogenetic similarities [2,3]. In Brazil, the principal vector species of human malaria pertains to the subgenus *Nyssorhynchus* that possess 29 species, among them *Anopheles darlingi*, which is considered the most important transmitter in the entire country, particularly in the Amazon region.

The use of techniques based on direct DNA analysis in genetic studies of the population has grown over the last decade, principally in the identification and genetic characterization of malaria vector species. Taxonomic studies of the subgenus *Nyssorhynchus* have revealed the existence of various complexes of the species [1]. Members of these species complexes vary in vector capacity, as well as in the nature of gene flow and genetic variation, both of which are critical for explaining disease epidemiology [4]. Among the molecular markers, mitochondrial DNA (mtDNA) can reveal historical and phylogeographic patterns, rates of gene flow and can also be used to define maternal gene genealogies within species [5-8]. mtDNA is also used in genetic inter and intraspecific studies due to the great number of copies per cell, shows a faster evolution rate compared with nuclear DNA, involves predominantly maternal inherence and is small in size and therefore does not undergo genetic recombination [9], which is suitable for inferring phylogenetic relations among populations and/or species with recent time divergences due to their high mutation rate [10].

The mitochondrial genome possesses a region denominated the "control region", a non-codifying region rich in A+T in invertebrates [11], which contains replicate initiation sites of heavy chain (H) and transcription promoters of light and heavy chain [12], whose function is to regulate the transcription and control of DNA replication [13]. This study focused on the control region of mtDNA analysis in the subgenus *Nyssorhynchus*. Our aim was to test the utility of this region according to its ability to recover anopheline relationships supported by previous studies. To achieve this, we estimated the intra and interspecific genetic differentiation based on the control region of the mtDNA.

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MATERIALS AND METHODOLOGY

Mosquito Collection

Our group studied 30 individual for each species of *Anopheles* from different Amazonian localities (Table **1**). Females were captured in the field and brought to the INPA Laboratory of Malaria Vectors in Manaus and housed individually for oviposition. Following oviposition, the females, eggs and fourth stage larvae were identified as previously described [14].

Table 1. Taxa and Collection Sites of the Seven *Anopheles* Species Pertaining to the *Nyssorhynchus* and *Anopheles* Subgenera of the Amazon Region. RO: Rondônia, AP: Amapá, AM: Amazonas

Subgenera	Species	Collection Sites
Nyssorhynchus	*An. oswaldoi*	Ji Paraná-RO
	An. marajoara	Macapá-AP
	An. triannulatus	Pacoval-AP
	An. benarrochi	Guayará Mirim-Bolívia e Ji Paraná-RO
	An. rangeli	Ji Paraná-RO
	An. darlingi	Timbozinho-AM
Anopheles	*An. matogrossensis*	Janauari-AM

Molecular Analyzes

Genomic DNA was extracted individually from 4th-instar larvae, as previously described [15]. The methodology used in the maintenance of the eggs until adulthood was described by Santos *et al.* [16]. The primer sequences (forward and reverse) used according to a previously described method [17]. Another internal primer was designed (5' – CTA GAA TAA AAT AAT ATT AA – 3)' according to the program described by Xia and Xie [18]. The amplification was programmed for 30 cycles (25s at 94^{o}C, 1 min at 50 to 60^{o}C and 1 min 10s at 72^{o}C). Subsequently, polymerase chain reaction (PCR) products were checked on 1% agarose gel electrophoresis stained with ethidium bromide and analyzed under UV light. The PCR products were purified using the protocol developed by Sanger *et al.* [19]. The control region sequences of mtDNA of *An. gambiae* and *An. quadrimaculatus* were taken from the GenBank, under access numbers NC 002084 and Ue5793, representing the external group, along with sequences of *An. mattogrossensis* obtained in this study.

Statistical Analyses

DNA sequences were aligned and nucleotide composition was calculated using ClustalW [20], included in the Bioedit [21]. The DAMBE program [18] was used to verify the presence of saturations, PAUP version 4.0b10 [22] for verifying the phylogenetic signal and Modeltest version 3.06 [23] was used to select the nucleotide substitution model. The significance of the grouping was estimated by bootstrap analysis [24]. To estimate the phylogenetic relations among the sequenced fragments of the species, we used maximum parsimony, maximum likelihood, minimum evolution and neighbor-joining assisted by PAUP. The evolutionary model for maximum likelihood was HKY85+G, as proposed by Hasegawa *et al.* [25], assuming different substitution rates and with corrections for the gamma parameter and analysis of invariable sites.

RESULTS

Of the 210 sequences, we selected 42 due to the quality of the amplification, six sequences for each species. The analysis of the sequences showed 292 conserved sites of which 63 were informative for parsimony and 26 variables.

In the DNA polymorphism analysis of the species, 15 haplotypes were detected from among the 40 sequences obtained, with the presence of 81 polymorphic loci, 95 mutations, haplotypic divergence of 0.879, nucleotide diversity of 0.06507, nucleotide differences pair the pair mean of 23.1749, and observed and expected variances of 113.840 and 8.33, respectively.

The nucleotide composition of the control region showed 48.4% of adenine (A), 40.2% of thymine (T), 7.9% of cytosine (C) and 3.9% of guanine (G) (Table **2**). This table shows the most elevated percentages of adenine and thymine basis in all the species analyzed (88.6%). The highest percentage of adenine was detected in *An. marajoara*, while the lowest was observed in *An. mattogrossensis.* In relation to the thymine base, the highest percentage was verified in *An. darlingi.* For cytosine and thymine, the most elevated values were observed in *An. mattogrossensis*.

Table 2. Nucleotide Composition and Haplotypes of the Control Region of the Mitochondrial DNA of *Anopheles* Populations of the Subgenera *Nyssorhynchus* and *Anopheles* (A = Adenine, T = timine, C = Cytosine, G = Guanine). B = Bolivia, JP = Ji Paraná/Rondônia. + species from GenBank

Populations	T	C	A	G	Haplotypes
An (Nys.) oswaldoi	40.1	8.0	48.4	3.5	H1*
An. (Nys.) darlingi	41.2	7.5	48.2	3.0	H2 to H4*
An. (Nys.) marajoara	39.0	8.3	49.5	3.2	H5*
An. (Nys.) rangeli	40.9	7.2	48.4	3.5	H6 to H9*
An. (Nys.) triannulatus	41.1	7.5	48.4	3.0	H10 and H11*
An. (Nys.) benarrochi (B)	40.2	7.5	48.8	3.5	H12 and H13*
An. (Nys.) benarrochi (JP)	40.9	7.5	48.1	3.5	H14*
An. (Ano.) mattogrossensis	38.2	9.4	47.6	4.8	H15*
Mean of populations studied	40.2	7.9	48.4	3.9	
An. (Cel.) gambiae+	39.1	7.8	50.3	2.9	H16 and H17*
An. (Ano.) quadrimaculatus+	40.2	7.7	48.7	3.4	

* The sequence of these haplotypes is indicated in Figure 1, which refers to the alignment of nucleotide sequences.

Table **3** shows the genetic divergence values observed among the haplotypes analyzed. The nucleotide divergence varied from 0.6 to 44.2%, with the shortest distance between *An. rangeli* and *An. oswaldoi* (D = 0.025%) and the longest between *An. marajoara* and *An. gambiae* (D = 0.442%). The

Table 3. Matrix of Distances for the 381 pb of the Mitochondrial DNA Control Region Fragment, According to the Model HKY85+G

1- *An. oswaldoi* H1

2- *An. darlingi* H2 0.086

3- *An. darlingi* H3 0.080 0.003

4- *An. darlingi* H4 0.086 0.006 0.003

5- *An. albitarsis* H5 0.064 0.100 0.093 0.100

6- *An. rangeli* H6 0.028 0.082 0.076 0.082 0.053

7- *An. rangeli* H7 0.025 0.076 0.070 0.076 0.048 0.003

8- *An. rangeli* H8 0.028 0.080 0.075 0.080 0.052 0.003 0.003

9- *An. rangeli* H9 0.035 0.100 0.093 0.100 0.068 0.012 0.012 0.009

10- *An. triannulatus* H10 0.157 0.115 0.108 0.115 0.133 0.128 0.120 0.126 0.151

11- *An. triannulatus* H11 0.149 0.108 0.101 0.108 0.125 0.136 0.128 0.134 0.161 0.003

12- *An. benarrochi*/Bo H12 0.052 0.103 0.097 0.103 0.078 0.044 0.040 0.043 0.058 0.116 0.108

13- *An. benarrochi*/Bo H13 0.052 0.111 0.104 0.111 0.067 0.044 0.040 0.044 0.058 0.102 0.095 0.003

14- *An. benarrochi*/JI H14 0.062 0.103 0.096 0.103 0.078 0.053 0.049 0.053 0.068 0.116 0.108 0.006 0.009

15- *An. matogrossensis* H15 0.387 0.308 0.292 0.307 0.308 0.350 0.333 0.345 0.389 0.343 0.327 0.302 0.319 0.302

16- *An. gambiae* H16 0.259 0.357 0.340 0.357 0.235 0.272 0.258 0.268 0.314 0.442 0.421 0.286 0.288 0.317 0.519

17- *An. quadrimaculatus* H17 0.279 0.346 0.329 0.346 0.253 0.279 0.265 0.274 0.321 0.419 0.399 0.230 0.244 0.255 0.326 0.186

interpopulational nucleotide divergence observed within *An. benarrochi* was 0.06%. When compared with the nucleotide distance among the subgenera *Nyssorhynchus*, *Anopheles* and *Cellia*, the longest distance verified was between *An. gambiae* and *An. mattogrossensis* (D = 0.5l9) and the shortest between *An. quadrimaculatus* and *An. gambiae* (D = 0.186). The topologies produced by these phylogenetic methods were similar, indicating that there was an unresolved polytomous for the clade referring to the populations of *An. benarrochi* of Ji-Paraná and Bolivia; *An. oswaldoi* and *An. rangeli* and of *An. marajoara* and *An. darlingi*. However, the clade within the subgenus *Nyssorhynchus* is still formed with low bootstrap values. As expected, the separation of *Nyssorhynchus* and *Anopheles* shows robust support, indicated by the bootstrap value (Fig. **1**).

The topology of the consensus tree (50% majority rule) by the consistency index parameters (CI = 0.7771), length in number of steps (L = 167) and the retention index (RI = 0167). The heuristic search indicated by the *branch-and-bound* manner, using equal weights for all nucleotide substitutions and including the repeated indels, generates three MP trees with 167 steps. The maximum parsimony analysis showed a separation of the subgenera *Nyssorhynchus* and *Anopheles* with moderate to strong support (≥80%). In the subgenus *Nyssorhynchus,* the populations of *An. benarrochi* of Ji-Paraná and Bolivia were separated with strong support (94%). *An. darlingi* and *An. triannulatus* were grouped in the same clade; however, this relationship was not well supported (56%) (Fig. **1A**).

The cladogram obtained by the grouping method of neighbors (Fig. **1B**), using matrixes of genetic distance calculated by the Kimura model - 2 - parameters, shows that in general tree topology, the populations were allocated in three clades: the first, was made up of *An. oswaldoi* and *An. rangeli*, the second, by populations of *An. benarrochi* and the third by *An. triannulatus* and *An. darlingi*. The generated topology also showed the separation of the subgenus *Nyssorhynchus* in relation to the subgenera *Anopheles* and *Cellia* with 66% bootstrap.

The cladogram obtained by the maximum likelihood (Fig. **1C**) showed, in the general tree typology, that the populations of *An. benarrochi* were also separated by 52% bootstrap support, as well as the separation of subgenus *Nyssorhynchus*, in relation to subgenera *Cellia* and *Anopheles*. The topology generated by the Minimum Evolution method showed that populations of *An. benarrochi* were separated by 66% bootstrap. *An. darlingi* and *An. triannulatus* formed a clade with 54% bootstrap and *An. rangeli* and *An. oswaldoi* formed a clade with 64% bootstrap. The separation of *Nyssorhynchus* from the other subgenera *Cellia* and *Anopheles* was 66% (Fig. **1D**).

DISCUSSION

The control region has been used in evolutionary studies due to its evolution rate, which varies among lineages [6, 26]. In insects, mutations occur involving nucleotide substi-

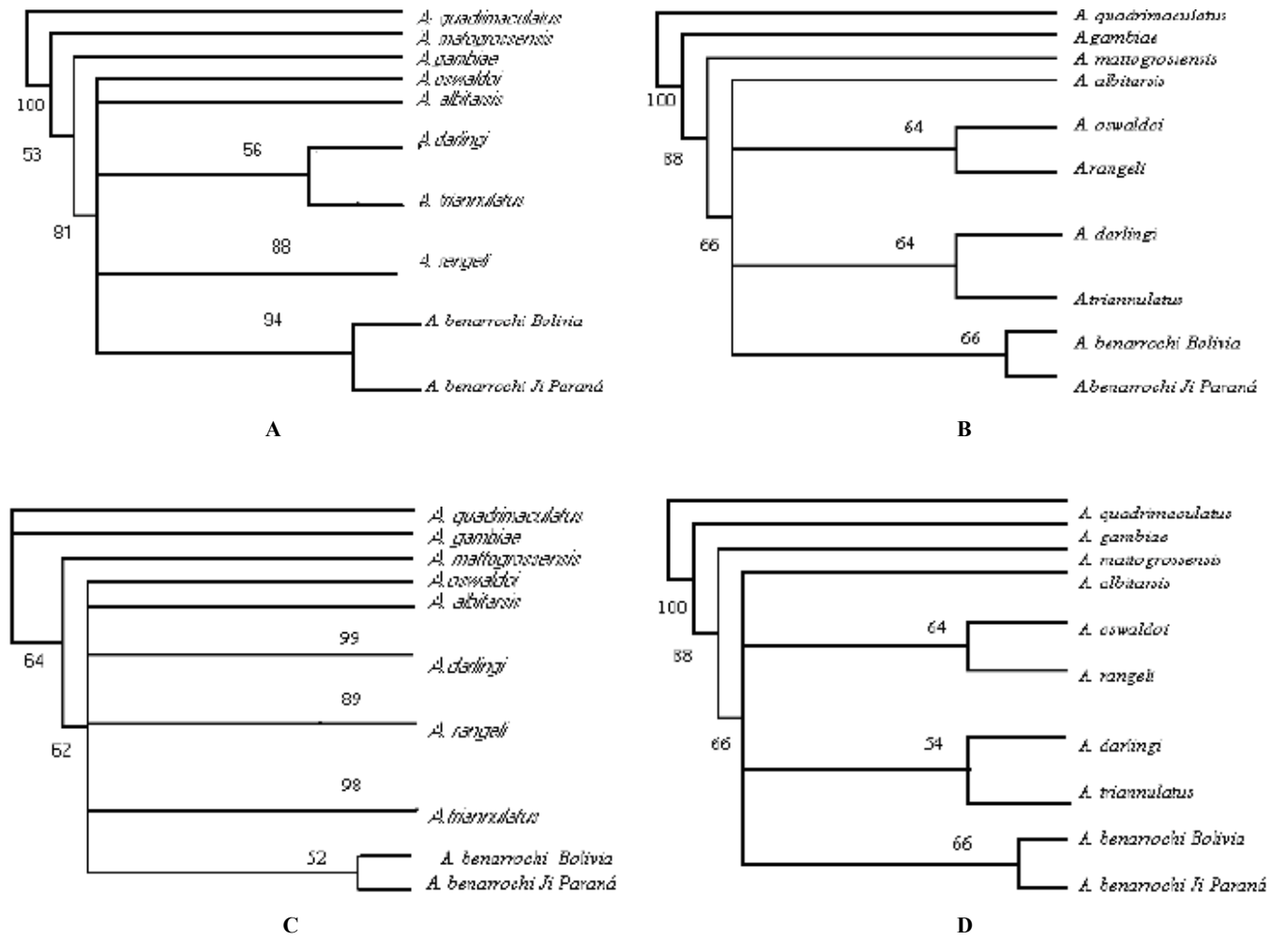

Fig. (1). Cladograms obtained by the Maximum-Parsimony (**A**), Neighbor-Joining (**B**), Maximum Likelihood (**C**), and Minimum Evolution (**D**), from nucleotide sequences of the control region, in the subgenus *Nyssorhynchus* species. The numbers are bootstrap values for 1000 replicates.

tutions, minor deletions/inversions and variations in the number of copies of repetition in tandem. Regarding the variability of the control region, it has been observed that variability is reduced in this group [27]. Thus, the data indicates that the control region in this group of mosquitos may evolve more slowly than other codification regions of the mitochondrial and nuclear DNA.

The elevated A+T rate (88.6%) determined for the subgenus *Nyssorhynchus* species reflects the greater polymorphism in the members of this subgenus. Moreno *et al.* reported that the nucleotide composition (A + T) of this region had shown variation [6], assuming a value of 78.1% in *An. darlingi*. Data obtained by Mitchell *et al.* [28] for *An. quadrimaculatus* populations of this subgenus show that the control region of the mitochondrial DNA constituted 94% A + T.

Concerning the size of the control region, literature data shows variations in the different taxons. In *An. gambiae* and *An. quadrimaculatus*, this region was 520 and 625 pb, respectively [6, 28, 29]. The small size of the fragment obtained in the *Anopheles* species of this work and presence of regions in *tandem*, may have contributed to inconsistent phylogenetic inferences. Such a hypothesis was raised by Taylor *et al.* when they detected 96% A + T in fragments of 300 pb in butterfly species [30]. Meanwhile, such results may be used in polymorphism and population structure studies of *An. darlingi*, because of the high quantity of A + T [6].

The monophyletism of *Nyssorhynchus* presented low consistency across trees previously generated by methods based on characters: Maximum Parsimony (MP) and Neighbor Joining (NJ), with low bootstrap values. These results partially corroborate those observed by Krzywinski *et al.* [31, 32] using molecular characters. Within the subgenus *Nyssorhynchus*, the relations remain unclear, even though Sallum *et al.* [2, 33] confirmed the paraphyly of the *Argyritarsis* sections, which in this work are contained in *An. darlingi* and *An. marajoara*, and of the *Albimanus* section, where *An. benarrochi, An. rangeli, An. triannulatus* and *An. oswaldoi* are allocated.

Separation among members of the *Nyssorhynchus* subgenus and the *Cellia* and *Anopheles* subgenera, based on the mitochondrial DNA, was also verified by Sallum *et al.* [2] while studying the phylogeny of the *Anophelinae* subfamily. Very little evidence was verified concerning the molecular data, because of the elevated number of homoplasies

presented by the species. Homoplasies were also observed in this work. Another study by Sallum *et al.* [33] also confirms the data observed in our study, in which we verified the low phylogenetic signal by analyzing the mitochondrial DNA. When studying the variation in mitochondrial DNA of *An. rangeli* and *An. trinkae*, Conn *et al.* detected a low phylogenetic signal, despite having observed the monophyletic origin of these species [34]. While studying members of the *Anopheles punctulatus* group using mitochondrial genomes, Beebe *et al.* observed little phylogenetic information [35].

Thus, the existence of so little phylogeny data that shows good phylogenetic signs in the *Nyssorhynchus* subgenus based on morphological, isoenzyme and DNA characters [36], allied with the almost total absence of fossil registers, has made studying the evolutional history and phylogenetic reconstruction in this subgenus difficult. Thus the data presented, based on the control region of mitochondrial DNA, is important to minimize the existing conflicts in the systematic of the group and seems promising for resolving questions relative to alpha taxonomy, beyond determining that the species studied is the result of a recent genetic divergence. However, its contribution is still minimal regarding stricter phylogenetic relations among members of the *Nyssorhynchus* subgenus. Similar information was reported by Foley *et al.* [37], who studied the phylogenetic relations and origin of the *Cellia* subgenus members based on the variation of sequences of the COII gene. They also found it difficult to resolve the phylogenetic relations of this subgenus. As for the *Anopheles* subgenus, its monophyletic group within the genus still remains obscure, due in part to the discordance among authors [2], even though Krzywinski *et al.* suggested the monophyly of the group [31, 32], which could corroborate data in our study.

The results generated by the distance matrix showed an elevated genetic divergence, separating the species in two large groups represented by the subgenera *Nyssorhynchus* and *Anopheles*, corroborating data obtained in several studies [2, 34, 38]. Considering the genetic distance for all species of the *Nyssorhynchus* subgenus, we verified that *An. rangeli* and *An. oswaldoi* were the most similar. *An. gambiae* and *An. mattogrossensis* presented an elevated genetic divergence in relation the species of the *Nyssorhynchus* subgenus. The four clusters formed by the genetic distance values are in agreement with Sallum *et al.* [33], who used molecular characters which form the group *An. darlingi* and *An. triannulatus* and separated these from *An. marajoara*. Meanwhile, previous results obtained by Sallum *et al.* [2] based only on morphological characters assigned the *An. marajoara* and *An. triannulatus* groups to the same clade, placing *An. darlingi* in a different clade.

In relation to *An. intermedius* and *An. mattogrossensis*, which were separated from the species of the *Nyssorhynchus* subgenus, these results partially corroborate data presented by Sallum *et al.* [33], on the basis of molecular characters.

The cladograms generated for the seven *Anopheles* species of the *Nyssorhynchus* and *Anopheles* subgenera are in partially agreement with cladograms of these and other anophelines species, based on morphological and molecular characters [33, 39]. In general, the anophelines present an evolution with low morphological differentiation, but frequently possess genetic, physiological and chromosomic differences [40] that permit their characterization, serving as a diagnostic tool for identifying cryptic species that are very common in these groups of mosquitoes.

CONCLUSION

This data showed ample interspecific nucleotide variation, with low nucleotide divergence. The cladogram separated the species of the subgenus *Nyssorhynchus* and those of the subgenera *Cellia* and *Anopheles*. The region control data did not show strong phylogenetic support, which is necessary for using more conservative genes for greater inference concerning the phylogeny of species of the subgenus *Nyssorhynchus.*

CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest.

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