Molecular evidence for a single taxon, *Anopheles nuneztovari* s.l., from two endemic malaria regions in Colombia

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To elucidate the Anopheles nuneztovari *s.l. taxonomic status at a microgeographic level in four malaria endemic localities from Antioquia and Córdoba, Colombia, fragments of the cytochrome oxidase subunit I (*COI*) and the* white *gene were used. The* COI *analysis showed low genetic differentiation with fixation index (F_{ST}) levels between -0.02-0.137 and Nm values between 3-∞, indicating the presence of high gene flow among* An. nuneztovari *s.l. populations from the four localities. The* COI *network showed a single most common haplotype, type 1 (n = 55), present in all localities, as the likely ancestral haplotype. Analysis of the* white *gene showed that* An. nuneztovari *s.l. populations from both departments grouped with haplotypes 19 and 20, which are part of lineage 3 reported previously. The results of the present study suggest that* An. nuneztovari *s.l. is a single taxon in the area of the present study.*

Key words: malaria - *Anopheles nuneztovari* - *white* gene - cytochrome oxidase subunit I - Colombia

Malaria is an important public health problem in Colombia, as demonstrated by the fact that, after Brazil, Colombia has the second highest number of malaria cases in Latin America (OPS 2009). In the past 10 years, the departments of Antioquia and Córdoba, where this investigation was carried out, have reported nearly 60% of the total cases of the country (Rodríguez et al. 2011). There are three main vectors, all in subgenus *Nyssorhynchus*, reported for Colombia present in endemic areas of these departments, including *Anopheles albimanus* Wiedemann, *Anopheles darlingi* Root and *Anopheles nuneztovari* Gabaldón (Olano et al. 2001).

An. nuneztovari s.l. is distributed from eastern Panamá to northern South America and Amazonia (Mirabello & Conn 2008) where it presents differences in behaviour (Montoya-Lerma et al. 2011). Brazilian collections of this species have detected natural infection with *Plasmodium* (Arruda et al. 1986, Tadei et al. 1998), but the role of *An. nuneztovari* s.l. in Brazil seems to be limited to local malaria transmission (Galardo et al. 2007). Indeed, populations from Brazil are mainly zoophilic and exophagic with a biting peak near sunset (Tadei & Thatcher 2000). In contrast, in Colombia and Venezuela, *An. nuneztovari* s.l. is a primary malaria vector (Lounibos & Conn 2000). Recent studies in northeastern Colombia showed that *An. nuneztovari* s.l. exhibits anthropophilic and endophagic behaviour, with a biting peak between 08:00-11:00 pm and can be naturally infected with *Plasmodium vivax* VK247 (Gutiérrez et al. 2009a). In Venezuela, the highest biting activity is close to midnight indoors and outdoors (Rubio-Palis & Curtis 1992).

Based on morphology, cytology, ecology, behavioural and molecular differences, several authors have suggested that *An. nuneztovari* s.l is a species complex (summarized in Calado et al. 2008, Montoya-Lerma et al. 2011). Recent *white* gene analyses described five lineages across its range, with one (lineage 3) present in Colombia (Mirabello & Conn 2008). *An. nuneztovari* s.l. is characterized by high intraspecific morphological variability and is thus easily misidentified or confounded with other members of the Oswaldoi Group (Calle et al. 2008, Cienfuegos et al. 2008, Gómez et al. 2010). A deeper dissection of this challenging variability resulted in the description of a new morphological form in the Brazilian Amazon (Bergo et al. 2007), later confirmed as *Anopheles goeldii* Rozeboom and Gabaldón by sequence analysis of internal transcribed spacer (ITS)2, cytochrome oxidase subunit I (*COI*) and *white* genes (Calado et al. 2008). Scarpassa and Conn (2011) recently determined that, based on a phylogenetic analysis of *COI* gene sequences, samples from the Amazon Basin could be assigned to two well-supported clades. They hypothesize that one of these clades represents *An. goeldii* and the second clade represents a previously undescribed lineage or taxon in *An. nuneztovari* s.l.

Few studies have attempted to document the taxonomic status and population structure of *An. nuneztovari* s.l. in Colombia. One study evaluated phenotypic variation in specimens from western and eastern Colombia and detected high levels of wing spot variation, a key character that is often difficult to assess for proper identification (Ramos et al. 2008). Similarly, morpho-

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metric analysis of female specimens of *An. nuneztovari* s.l. from Montelíbano (MLT), Córdoba, found high intraspecific variability (Gómez et al. 2010).

Studies using molecular markers have also attempted to clarify the taxonomic status and population structure of *An. nuneztovari* s.l. from northern and western Colombia. Using random amplified polymorphic DNA (RAPD), Posso et al. (2003) found low levels of genetic differentiation among three populations. Analysis of ITS2 sequences concluded that in Colombia, *An. nuneztovari* s.l. from both sides of the Andes mountains comprised a single genetic species (Sierra et al. 2004). Given the wide distribution of *An. nuneztovari* s.l. in Antioquia and Córdoba, this study was conducted to test the hypothesis that a single taxon is present in this region, to assess the genetic variability at a microgeographic level and to identify the *white* gene lineage(s) present.

MATERIALS AND METHODS

Mosquito collection and processing - Adult female specimens of *An. nuneztovari* s.l. were collected between November 2007-February 2010 in El Bagre (BAG), Zaragoza (ZAR) and San Pedro de Urabá (SPU), in the department of Antioquia and in MTL and Puerto Libertador (PTL), in Córdoba (Fig. 1, Table I). The collection protocols and informed consent were reviewed and approved by a Bioethics Committee for Human Research at the University of Antioquia. Mosquitoes were identified by morphological keys (Faran 1980, Faran & Linthicum 1981, González & Carrejo 2009). A subset of specimens collected from each field trip is maintained in the University collection. For morphological reference, we retain the wings and legs fixed on slides of a percentage of the specimens. We also maintain photographic records of most specimens that have been processed for molecular analyses.

Molecular analyses - Total DNA was extracted from individual mosquito abdomens by a standard protocol (Rosero et al. 2010) used to confirm species identification following a polymerase chain reaction (PCR)-restriction fragment length polymorphism (RFLP)-ITS2 assay developed by our group (Zapata et al. 2007, Cienfuegos et al. 2008, 2011) and then amplified for the *COI* and *white* gene fragments as outlined below. A 1,300 bp fragment of the mtDNA *COI* gene was amplified by PCR using 10 µM primers UEA3/UEA10 (Lunt et al. 1996) in a 25 µL final reaction volume as previously described (Gutiérrez et al. 2010). PCR products were purified using Wizard SV Gel and PCR Clean-up System (Promega, Madison, WI) following the protocol recommended by the manufacturer and sequenced in both directions.

An 800 bp region of the nuclear *white* gene was amplified using primers WF and W2R in a 25 µL PCR reaction with conditions previously described by Mirabello and Conn (2008). Because of allelic variation (Mirabello & Conn 2008), *white* gene PCR products were cloned as described in Onyabe and Conn (1999). Five clones corresponding to five specimens from each locality were sequenced. Sequences were edited, assembled by pairwise alignment and then multiple alignments with CLUSTALW were performed using the Geneious software 5.3.4 (Drummond et al. 2011).

Descriptive statistics and population differentiation test - Haplotype and nucleotide diversities of mtDNA *COI* sequences were obtained using DnaSP version 5.00.07 (Rozas et al. 2003) and Arlequin version 3.11 software (Excoffier et al. 2005). Analysis of molecular variance (AMOVA) was used to evaluate within and among population variation using Arlequin software. The *COI* haplotype distribution was evaluated by a statistical parsimony network with 95% support (Clement et al. 2000).

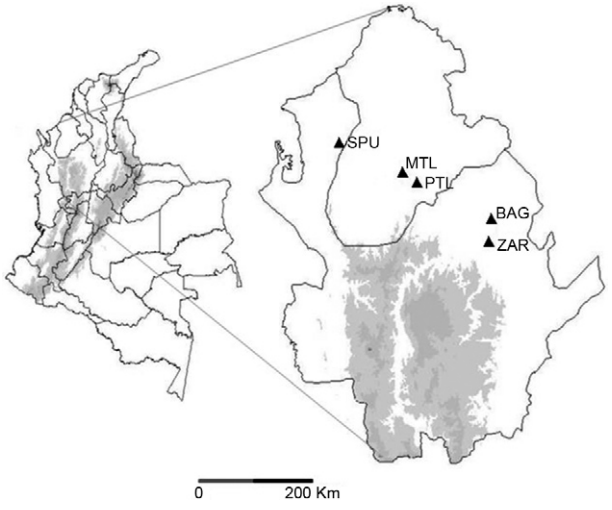

Fig. 1: distribution of collection sites for *Anopheles nuneztovari* s.l. in Antioquia and Córdoba, Colombia. BAG: El Bagre; MTL: Montelíbano; PTL: Puerto Libertador; SPU: San Pedro de Urabá; ZAR: Zaragoza.

TABLE I

Study sites, dates of collection, coordinates and sample sizes (n) of *Anopheles nuneztovari* s.l. collected from Antioquia and Córdoba, Colombia

Municipality	Year	Month (number of days)	Coordinates	n
Montelíbano	2007	July (5) August (2)	7º59'N 75º25'W	29
Puerto Libertador	2007	January (6)	07º43'N 75º51'W	32
El Bagre	2009	March (3) May (3)	07º35'N 74º49'W	41
Zaragoza	2008	January (1) March (3) June (1)	07º29'N 74º51'W	8
San Pedro de Urabá	2010	February (5)	06º28'N 75º33'W	37
Total				147

Phylogenetic relatedness of An. nuneztovari s.l. populations from Antioquia and Córdoba with populations from South America using white gene sequences - Split networks are successful tools for visualizing incompatible and ambiguous signals and have been used previously for phylogenetics studies (Gutiérrez et al. 2009b, 2010). Therefore, a neighbour-net network was estimated using SplitsTree4 version 4.10 (Huson & Steel 2004, Huson & Bryant 2006) for *An. nuneztovari* s.l. *white* gene sequences obtained in this study and for those in GenBank corresponding to specimens from Brazil, Bolivia, Surinam, Venezuela and Colombia (accessions EU500727-EU500758). Bayesian analysis was carried out using Mr Bayes v3.1 (Huelsenbeck & Ronquist 2001), available in Geneious software 5.3.4 (Drummond et al. 2011), using the default HKY85 model and gamma distributions. The settings for the Markov Chain Monte Carlo were four chains running every 100,000 generations.

RESULTS

An. nuneztovari s.l. genetic variation at the intra-population level - Out of 2,490 *Anopheles* mosquitoes collected in Antioquia and Córdoba, Colombia, 147 *An. nuneztovari* s.l. specimens were chosen (Fig. 1, Table I). A 1,180 bp sequence of the *COI* gene was analyzed. The aligned sequences showed 23 variable sites and 11 that were parsimony informative. The highest nucleotide diversity was detected in SPU (0.00249+/-0.001315) and moderate to lowest diversity was detected in PTL (0.00172+/-0.001120). The haplotype diversity (Hd) was highest for SPU (0.8134/-0.0418) and lowest for PTL (0.709+/-0.0578) (Table II). To compare the population pairs using the *COI* gene, samples from BAG and ZAR were combined. These localities are 11 km apart and Nm detected infinite migrants between them. There were 20 unique haplotypes (GenBank accessions JN255752-JN255771), all of which connected parsimoniously. Four haplotypes were shared among all localities. The most widespread haplotype (type 1), showed a frequency of 38% (55/147) and was found in all localities. Three other haplotypes were distributed among all localities: type 2 (21%), type 3 (12%) and type 4 (10%). Haplotype 3 was not found in SPU (Table II). There were few private haplotypes (Fig. 2).

Genetic structure and demographic inference - The fixation index (F_{ST}) values estimated for *COI* sequences from localities in Antioquia and Córdoba indicated low to moderate genetic differentiation, with values ranging between -0.02-0.137. The number of migrants per generation, Nm, was between 3-∞, indicating gene flow among all *An. nuneztovari* s.l. tested (Table III). AMOVA, based on *COI* haplotype frequencies and conducted with all

TABLE II

Shared cytochrome oxidase subunit I (*COI*) haplotypes and genetic polymorphism statistics for *Anopheles nuneztovari* s.l. collected in localities of Antioquia and Córdoba, Colombia

Locality	n	*COI* haplotype	Segregating sites (n)	Haplotype (h)	Haplotype diversity (SD)	Nucleotide diversity (SD)
Montelíbano	29	1(11), 2(5), 3(2), 4(5), 6(2), 8(2)	9	8	0.807+/-0.5240	0.00189+/-0.001199
Puerto Libertador	32	1(15), 2(8), 3(1), 4(5), 6(2), 8(2)	7	7	0.709+/-0.0578	0.00172+/-0.001120
Total Córdoba	61	1(26), 2(13), 3(13), 4(10), 6(4)	10	10	0.758+/-0.0397	0.00180+/-0.001128
San Pedro de Urabá	37	1(10), 2(13), 4(2), 5(6), 6(2), 9(2)	15	10	0.8134/-0.0418	0.00249+/-0.001315
El Bagre and Zaragoza	49	1(21), 2(5), 3(15), 4(3), 7(2)	9	8	0.720+/-0.0441	0.00237+/-0.001417
Total Antioquia	86	1(29), 2(17), 3(15), 4(2), 5(6)	19	13	0.773+/-0.0280	0.00271+/-0.001445
Overall Total	147	1(55), 2(230), 3(28), 4(12), 5(6), 6(4)	23	20	0.781+/-0.0302	0.00234+/-0.001288

numbers inside parentheses are the frequency of haplotypes by locality. All haplotypes are shared among populations of Antioquia and Córdoba. SD: standard deviation.

TABLE III

Pairwise genetic differentiation - fixation index (F_{ST}) and gene flow among *Anopheles nuneztovari* s.l. collected from Antioquia and Córdoba, Colombia

	Cytochrome oxidase subunit I			
Localities	Puerto Libertador	Montelíbano	El Bagre and Zaragoza	San Pedro de Urabá
Puerto Libertador	-	∞	6.02	4.09
Montelíbano	-0.02020	-	6.88	3.13
El Bagre and Zaragoza	0.07657	0.06767	-	3.12
San Pedro de Urabá	0.10872	0.13743	0.13778	-

above diagonal: Nm value; below diagonal: F_{ST} values; ∞: infinity. $p < 0.05$.

localities (populations), as one group showed that 12.49% (p = 0.18) of the variance, could be attributed to among localities (populations) and 87.51% within (Table IV).

The *COI* network showed a single most common haplotype (type 1, n = 55), which was present in all the localities. There were only 16 private haplotypes, which demonstrates high demographic stability and the existence of gene flow among populations (Fig. 2). After removing the intron sequence, a 630 bp fragment of the nuclear *white* gene was analyzed. From 20 sequences obtained, the consensus sequence was submitted to GenBank with accession JN255772. Phylogenetic neighbour-net network based on *white* gene sequences showed that *An. nuneztovari* s.l. specimens from Antioquia and Córdoba grouped with haplotypes 19 and 20, which are part of lineage 3, as reported by Mirabello and Conn (2008). However, the Bayesian analysis was completely unresolved and uninformative; whereas the neighbour-net network had an area of boxes, which indicated the need for larger sample sizes from these regions.

DISCUSSION

The results obtained with *COI* sequences of specimens *An. nuneztovari* s.l. collected in localities of high malaria transmission of Antioquia and Córdoba showed low genetic differentiation and gene flow among these localities. In general, levels of *COI* nucleotide diversity were higher for the populations of Antioquia than for populations of Córdoba. Further, most haplotypes were shared between both regions, suggesting that these populations have a common demographic history (Castelloe & Templeton 1994) (Fig. 2). Avise (2000) proposed that populations with high nucleotide and Hd are more stable, constant in size and have higher effective population sizes. In this study, *An. nuneztovari* s.l. from SPU had the highest nucleotide and Hd, indicating that this population is the most stable among the populations studied. F_{ST} and Nm values obtained suggest that *An. nuneztovari* s.l. from Antioquia and Córdoba may actually comprise a single population. Nm values in malaria vectors are of relevance because they can be used to predict gene spread, e.g. of those genes important for insecticide resistance or parasite refractoriness. An understanding of gene flow is essential for the introduction of transgenic mosquitoes carrying genes for *Plasmodium* resistance.

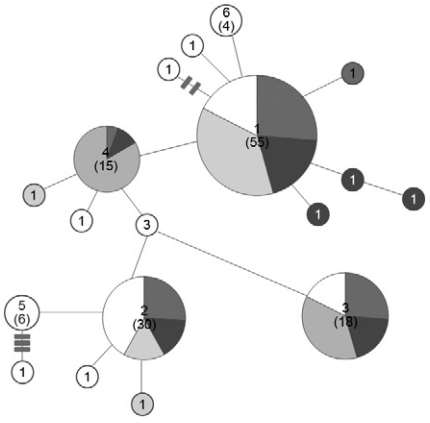

Fig. 2: parsimony based haplotype network of 20 cytochrome oxidase subunit I haplotypes of 147 *Anopheles nuneztovari* s.l. specimens. Each locality is represented by a distinct shade. Numbers out of the parentheses represent the haplotype designation and those inside the parentheses indicate the number of sequences included in each haplotype. The size of the circle is proportional to the frequency of detected haplotype. BAG: El Bagre; MTL: Montelíbano; PTL: Puerto Libertador; SPU: San Pedro de Urabá; ZAR: Zaragoza.

White gene phylogenetic analyses of samples from Antioquia and Córdoba together with other South American populations produced mixed results. Our sequences were identical to haplotypes 19 and 20 reported by Mirabello and Conn (2008), which corresponded to their lineage 3. The area of boxes in the neighbour-net network combined with the unresolved Bayesian analysis, suggest that a broader sampling of this species complex with analyses of *white* gene and other nuclear genes should be undertaken to more formally test the five lineages proposed in Mirabello and Conn (2008). Nevertheless, our results are consistent with previous studies with various markers that detected differentiation between *An. nuneztovari* s.l. populations from Colombia and Venezuela west of the Andes with those in other regions of South America (Elliott 1972, Kitzmiller et al. 1973, Conn et al. 1993, Fritz et al. 1994, Scarpassa et al. 1996, 1999, 2000, Mirabello & Conn 2008, Scarpassa & Conn 2011).

In comparison with the results presented here, a study using the rDNA ITS2 suggested low differentiation between *An. nuneztovari* s.l. populations located on opposite sides of the Andes mountains (northwest and northeast Colombia), indicating that *An. nuneztovari* s.l. is conspecific in Colombia (Sierra et al. 2004). Further, a study using RAPD markers showed low levels of genetic differentiation for *An. nuneztovari* s.l. populations from northwest and northeast Colombia, with higher levels of gene flow among western populations (Posso et al. 2003). Although Posso et al. (2003) used RAPD for discrimination of populations in western Colombia their results are in agreement with the ones in the present study with *COI*, also in western Colombia. Collectively, results of these studies with *An. nuneztovari* s.l. populations from northwest and northeast Colombia are consistent in the detection of low genetic differentiation among the populations evaluated.

Localities in the present study are placed in the Magdalena biogeographic province (Morrone 2006),

TABLE IV

Analysis of molecular variance of *Anopheles nuneztovari* s.l. based on cytochrome oxidase subunit I haplotypes

Source of variation	Variance components	Variation (%)	Fixation index
Among populations	0.1896	12.49	0.12[a]
Within populations	1.3234	87.51	-

a: not significant (p > 0.05).

which encompasses western Venezuela and northwest Colombia. The Magdalena province is characterized by temperatures above 24ºC, plains with altitudes no greater than 500 m, fragmented forest, presence of livestock and agriculture and active mining (IGAC 2002). The concurrence of similar ecologic and geographic conditions in this biogeographic region may influence *An. nuneztovari* s.l. populations, favouring the low genetic differentiation observed. The location of *An. nuneztovari* s.l. cytotypes B and C coincide with the Magdalena biogeographic province, while cytotype A was reported in the Brazilian Amazon, south of the country. Given that *An. nuneztovari* s.l. is broadly distributed in Colombia, including regions in the south in close proximity to the Brazilian Amazon, genetic analyses of *An. nuneztovari* s.l. should be expanded to include specimens from the southern regions such as the Orinoquía and the Amazonia, and other markers such as microsatellites to detect recent mutational events.

Previous work by our group evaluated microgeographic genetic variation of *An. darlingi*, another important Colombian malaria vector, in Antioquia and Córdoba. Those results were based on microsatellites and *COI* analysis and also showed that *An. darlingi* populations were highly homogeneous and presented high levels of gene flow (Gutiérrez et al. 2010). In the report presented here, microsatellite analysis was not performed because at the time of our collections and analyses, these markers had not been available for *An. nuneztovari* (Cardoza et al. 2011). It is possible that microsatellite analysis of *An. nuneztovari*, as with *An. darlingi*, would have revealed low genetic differentiation and high gene flow. In particular, analyses of *An. darlingi* in Brazil at the microgeographic level (152 km apart) showed low levels of genetic differentiation (F_{ST} = 0.001-0.095) and Nm values between 4.7-363.8, indicating that at this distance and within similar ecological and geographic conditions, anopheline differentiation may be low (Scarpassa & Conn 2007). However, differentiation at the microgeographic level has been documented for other important malaria vectors such as *Anopheles gambiae* (McLain et al. 1989). Specifically, in seven Kenyan localities less than 10 km apart, the authors found significant differences in the genetic distribution of this important African vector, indicating the absence of gene flow, probably due to poor dispersal abilities of these populations (McLain et al. 1989).

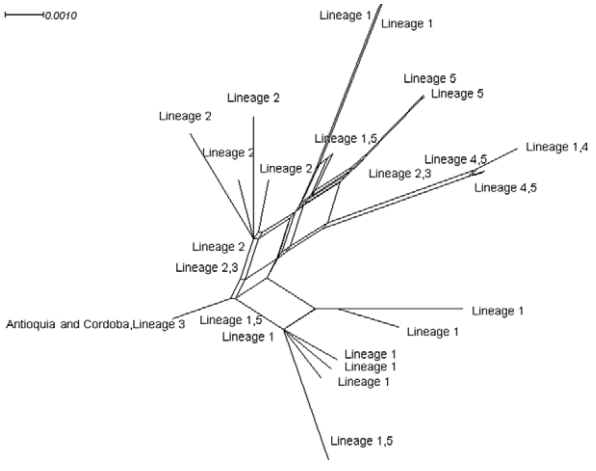

Fig. 3: neighbour-net network of *Anopheles nuneztovari* s.l. *white* gene lineages from Antioquia and Córdoba compared with other *An. nuneztovari* s.l from South America.

In summary, the results obtained in the present study provide critical data for understanding the primary malaria vector *An. nuneztovari* s.l. in localities with the highest annual reports of malaria cases in Colombia (Rodríguez et al. 2011). In particular, the presence of low genetic differentiation and high gene flow for important malaria vector species, such as *An. darlingi* and *An. nuneztovari s.l.*, indicate that malaria control programs could apply the same strategies, for example insecticide spraying, in these localities of Antioquia and Córdoba, as has been previously suggested (Gutiérrez et al. 2010).

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