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Morphogenetic characterisation, date of divergence, and evolutionary relationships of malaria vectors *Anopheles cruzii* and *Anopheles homunculus*

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ABSTRACT

The mosquito species *Anopheles cruzii* and *Anopheles homunculus* are co-occurring vectors for etiological agents of malaria in southeastern Brazil, a region known to be a major epidemic spot for malaria outside Amazon region. We sought to better understand the biology of these species in order to contribute to future control efforts by (1) improving species identification, which is complicated by the fact that the females are very similar, (2) investigating genetic composition and morphological differences between the species, (3) inferring their phylogenetic histories in comparison with those of other Anophelinae, and (4) dating the evolutionary divergence of the two species. To characterise the species we used wing geometry and mitochondrial cytochrome oxidase subunit I (COI) gene as morphological and genetic markers, respectively. We also used the genes *white*, 28S, ITS2, Cytb, and COI in our phylogenetic and dating analyses. A comparative analysis of wing thin-plate splines revealed species-specific wing venation patterns, and the species *An. cruzii* showed greater morphological diversity (8.74) than *An. homunculus* (5.58). Concerning the COI gene, *An. cruzii* was more polymorphic and also showed higher haplotype diversity than *An. homunculus*, with many rare haplotypes that were displayed by only a few specimens. Phylogenetic analyses revealed that all tree topologies converged and showed [*Anopheles bellator* + *An. homunculus*] and [*Anopheles laneanus* + *An. cruzii*] as sister clades. Diversification within the subgenus *Kerteszia* occurred 2–14.2 million years ago. The landmark data associated with wing shape were consistent with the molecular phylogeny, indicating that this character can distinguish higher level phylogenetic relationships within the *Anopheles* group. Despite their morphological similarities and co-occurrence, *An. cruzii* and *An. homunculus* show consistent differences. Phylogenetic analysis revealed that the species are not sister-groups but species that recently diverged within the *Kerteszia* group, perhaps concomitantly with the radiation of bromeliads in South America or during the Pleistocene climate oscillations.

1. Introduction

The mosquito species *Anopheles cruzii* Dyar & Knab and *Anopheles homunculus* Komp co-occur in southeastern Brazil and are the primary and secondary vectors, respectively, for etiological agents of malaria (*Plasmodium* spp.) in this region (Smith, 1952). There are other examples of sympatric *Anopheles* species that transmit the same disease, such as within the *Anopheles gambiae* complex in Africa (Levine et al., 2004). It is important to correctly identify vector species and distinguish between co-occurring ones in order to implement better control measures and eliminate disease. In Brazil, *An. cruzii* is broadly distributed and occurs in the states of Pernambuco, Sergipe, Bahia, Espírito Santo, Rio de Janeiro, São Paulo, Paraná, Santa Catarina, and Rio Grande do Sul (Forattini, 1962; Wilkerson and Peyton, 1991). *An. homunculus* occurs only in São Paulo, Paraná, and Santa Catarina (Calado and Navarro-Silva, 2005). The two species co-occur in São Paulo Atlantic Forest, an important spot for autochthonous malaria in Brazil (Marrelli et al., 2007; Pina-Costa et al., 2014). According to

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the Brazilian Ministry of Health (SINAN, 2004), between 1994 and 2004, around 8000 cases of malaria were reported in southeastern Brazil, with the highest number of cases reported in the state of São Paulo (Marrelli et al., 2007). However, despite the epidemiological importance of *Anopheles*, only a few studies have examined the populations of these two species in this area.

Both species belong to the subgenus *Kerteszia* and exploit bromeliad phytotelmata as larval habitats (Downs and Pittendrigh, 1946), a distinctive feature among Anophelinae. The larval colouration (Fig. 1) is diagnostic for these two species (Lima, 1952), but adult females are very similar. Because congeneric females show interspecific similarity, some occurrence records of *An. cruzii* may not be accurate, and some observations may have confused the species with other species belonging to *Kerteszia* (Calado and Navarro-Silva, 2005). In addition to their morphological similarities and use of similar habitat, the two species are closely related phylogenetically. The monophyly of *Kerteszia* is well documented in the literature (Besansky and Fahey, 1997; Sallum et al., 2000, 2002). Some authors believe that *An. cruzii* and *An. homunculus* belong to a group of phylogenetically related species (Rosa-Freitas et al., 1998), but the proposition has never been tested empirically. Although some molecular studies on the phylogenetic positions of *Kerteszia* species have been conducted (Krzywinski et al., 2001; Sallum et al., 2002), no research based on both mitochondrial and nuclear genes has been reported; species divergence times among *An. cruzii*, *An. homunculus*, and other anopheline mosquitoes remain unknown; and an appraisal of their population dynamics is lacking.

Other studies have suggested that *An. cruzii* constitutes a species complex (Ramirez and Dessen, 2000; Rona et al., 2009, 2013), and therefore, different populations could co-exist within the samples analysed. This is not the case for the population examined here (Carvalho-Pinto and Lourenço-de-Oliveira, 2004; Malafronte et al., 2007; Zavortink, 1973). Although there are behavioural differences between the two populations, including preferences for types of bromeliads that grow at different heights in the forest (Veloso et al., 1956) and vectorial status (Rachou et al., 1958; Forattini, 1962, 2002), to date almost no evident morphological or genetic differences have been observed between females. In the present study, we compared a population of *An. cruzii* with one of *An. homunculus* at this important malaria spot. Our goal was to test whether there were measurable differences between the species at the morphological and molecular levels that might contribute to further control measures, as well as to better understand important evolutionary parameters associated with those populations. To achieve this, we: (1) attempted to improve species identification, (2) investigated differences in their genetic composition using COI, a typical molecular marker employed in genetic comparisons, (3) examined differences in morphology using wing geometry, an accepted low-cost method used for studying Culicidae and other insects, (4) estimated their phylogenetic position within other Anophelinae using five genes (three nuclear and two mtDNA), and (5) dated the origin of each species.

2. Material and methods

2.1. Mosquito collection and identification

All specimens were collected in the municipality of Cananeia, Sao Paulo, Brazil (24°53′06 "S/47°51′01" W), situated in the Atlantic Forest biome. This region was selected because it is one of the main autochthonous malaria spots in Brazil (Pina-Costa et al., 2014) and the species *An. cruzii* and *An. homunculus* are sympatric there (Marrelli et al., 2007). The eggs and larvae of *An. cruzii* and *An. homunculus* were obtained from terrestrial, epiphytic, and saxicolous bromeliads using a manual suction pump (Lozovei and Silva, 1999). The great majority of the immature specimens collected were in the early stages of development (eggs or L1). The specimens were taken to the laboratory and raised to adulthood, under standard conditions of temperature and humidity (25 ± 1 °C; 80% ± 10%) (Rúa et al., 2005). Morphological identification was conducted according to Forattini (2002). The adults were sacrificed and stored in a freezer at −80 °C in order to preserve their DNA.

2.2. Morphometric analysis

Geometric morphometrics of the wing were analysed according to the procedure described by Lorenz et al. (2012). We analysed the right wings of 133 *An. cruzii* (66 females and 67 males) and 105 *An. homunculus* (56 females and 49 males). All the specimens were collected in July 2011 and January 2012. Because of their sexual dimorphism, the males and females were analysed separately. Eighteen wing landmarks (Fig. 2) from each individual were digitised using TpsDig v.1.40 (Rohlf, 2006). All specimens were scored by a single experimenter (C.L.). The coordinates were analysed using TpsRelw 1.36 (Rohlf, 2003a) and relative warps analyses (Principal Components) were conducted.

These data were used to calculate the canonical variables and the Mahalanobis distance using TpsUtil 1.26 (Rohlf, 2004), TpsRelw 1.36 (Rohlf, 2003a), TpsRegr 1.28 (Rohlf, 2003b), Statistica 7.0 (StatSoft, 2004), and MorphoJ 1.02 (Klingenberg, 2011) software programs. The allometric effect was removed in all analyses of shape through the regression between the components of shape and size of the centroid. Discriminant analysis and reclassification tests were performed using the Mahalanobis distances as estimators of the metric distance. Morphological diversity based on principal component analysis was estimated according to Petersen et al. (2015). Thin-plate splines were obtained by regression of the canonical scores versus the shape components using TpsRegr 1.28 (Rohlf, 2003b). In order to calculate the most influential landmarks between species, we used the TET and COV programs (MOME, 2010). In order to compare the wing shapes of *An. cruzii* and *An. homunculus* with those of other species, we used wing data from *Anopheles* (*Kerteszia*) *bellator*, *Anopheles* (*Kerteszia*) *laneanus*, *Anopheles* (*Nyssorhynchus*) *darlingi*, and *Anopheles* (*Nyssorhynchus*) *aquasalis* obtained from the WingBank (http://wingbank.com.br/) database.

2.3. DNA extraction and mtDNA sequencing

Whole mosquito tissue, except wings, was homogenised according to the procedure described by Jowett (1986) in order to extract genomic DNA. A total of 50 specimens of *An. cruzii* and 31 specimens of *An. homunculus* were used for the genetic analyses. Polymerase chain reaction (PCR) analysis was used to amplify the fragment of the mitochondrial gene COI. The reaction mixture contained 1× buffer stock solution (20 mM Tris–HCl, pH = 8.4), 0.4 mM dNTP, 2.5 mM of $MgCl_2$, 0.5 mM each primer (forward and reverse), 0.5 μl of *Taq* polymerase, 2.5 μl of genomic DNA, and sufficient sterile water to produce a final volume of 20 μl in each 0.2-ml reaction tube. The primers used in the amplification of the mitochondrial cytochrome oxidase subunit I (COI) gene have been published by Zhang and Hewitt (1997) and the sequences have been deposited in GenBank: UEA-7 [5′-TACAGTTGGAATAGA CGTTGATAC-3′] for the forward primer and UEA-10 [5′ TCCAATG CACTAATCTGCCATATTA-3′] for the reverse primer. The anchoring of the primers occurs in the final portion of the COI gene. The PCR program consisted of an initial temperature of 95 °C for 3 min, followed by 40 cycles of 94 °C for 40 s, annealing for 40 s (52 °C for *An. cruzii*, 47 °C for *An. homunculus*), and 72 °C for 1 min, with a final extension step of 72 °C for 10 min. The size of

Fig. 1. Immature forms. Larvae of *An. cruzii* (left) and *An. homunculus* (right).

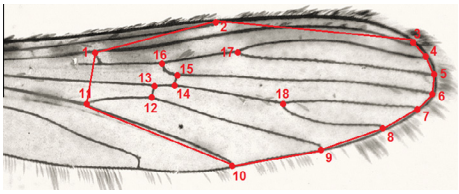

Fig. 2. Wing of *Anopheles*. Location of the 18 wing landmarks used in the morphometric analysis of *Anopheles cruzii* and *An. homunculus*. In red: imaginary geometric diagram representing the portion of wing considered in this study. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

the final amplified fragment was approximately 390 bp for *An. cruzii* and 513 bp for *An. homunculus*, and it did not include the barcode region of mitochondrial DNA that is used in species identification (De Azeredo-Espin, 2011; Ruiz-Lopez et al., 2012). The GenBank accession numbers for the sequences obtained are KC992738-KC992770 for *An. cruzii* and KC992783-KC992791 for *An. homunculus*. The PCR product was purified using a commercial kit (PureLinkTM PCR Purification kit; Invitrogen Corporation, Melle, Germany) and following the manufacturer's instructions. The fragment was sequenced in the forward and reverse directions using the commercial kit ABI PRISM dGTP BigDye ® Terminator v.3 (Applied Biosystems, Foster City, CA, USA). Samples were precipitated, stored at −20 °C, and sequenced using the Applied Biosystems model 3130xl sequencer.

2.4. Genetic analyses and population dynamics

The DNA sequences were analysed using the Lasergene program suite ® Core (DNAstar) and aligned and edited using the MEGA 6.0 program (Tamura et al., 2013). The minimum spanning network of the *An. cruzii* and *An. homunculus* haplotypes was created using TCS 1.21 (Clement et al., 2000) with the *median-joining* algorithm. To assess the genetic differences between species, the analysis of haplotype diversity and the number of polymorphic sites in the sequences were calculated with MEGA 6.0, using the Kimura-2-Parameter model. Population dynamics was inferred using D statistics (Tajima, 1989), Fs (Fu, 1997), and mismatch distributions from pairwise differences of COI sequences, all calculated in DnaSP v.5 (Rozas et al., 2003).

Estimates of population dynamics from our samples of *An. cruzii* and *An. homunculus* were calculated using BEAST v.1.8.0 (Drummond et al., 2012). We tested four models of population dynamics (constant, expansion, exponential, and logistic) using the stepping-stone method of marginal likelihood estimation (Xie et al., 2010) to infer the model that best described the evolution of such populations. A total of 20 steps were used in the bridge between posterior and prior, and each run was performed twice to check for repeatability.

2.5. Phylogenetic analysis and estimation of divergence time

In addition to analysing the COI sequences of *An. cruzii* and *An. homunculus* obtained in this study, we analysed the COI sequences of all *Kerteszia* species available in GenBank (http://www.ncbi.nlm.nih.gov/), as well as those of four other genes (Table 1). *An. darlingi* and *An. aquasalis* formed the outgroup, because those species belong to the subgenus *Nyssorhynchus* (Sallum et al., 2002). We used all available sequences from different individuals of the same species for each gene (i.e., all those sequenced by us or retrieved from GenBank) by maximum parsimony, to test whether different species form reciprocally monophyletic clades. If monophyly is detected for all the genes tested, then intraspecific variability cannot interfere with phylogenetic estimation of the species relationships. Therefore, whenever reciprocally monophyletic clades for a gene were found, we selected the largest sequence for that gene to be representative of the species, or if more than one sequence was equally large, we chose one of them haphazardly. The alignment for each gene was obtained using the Muscle algorithm (Edgar, 2004) within the MEGA 6.0 interface, with default parameters. Concatenation of all gene alignments was done in SequenceMatrix v.1.7.8 (Vaidya et al., 2001). The best data partitioning strategy (i.e., whether it is best to assume a single partition, to partition by codon position, or to partition by gene) was inferred by PartitionFinder v.1.1.1 (Lanfear et al., 2012).

Phylogenetic analyses were performed in two ways: (1) using the concatenated alignment described (supermatrix analysis, SM) by maximum parsimony (MP), maximum likelihood (ML), and Bayesian analysis (BA); and (2) estimating a species tree that maximised the probability of encompassing the five different gene trees (species tree analysis, ST) under a Bayesian framework. SM was done by MP with 1000 bootstraps in MEGA and by ML with 1000 bootstraps in IQTree v0.9.6 (Minh et al., 2013), using the best partitioning strategy and respective reversible models selected by PartitionFinder. SM was also performed by BA using BEAST v.1.8.0. In the latter case, we used the same ML partitioning and models, assuming a lognormal distribution of rates among branches with the mean arbitrarily fixed to 1.0 (ucld.mean = 1.0). For ST, a new PartitionFinder run was carried out in which each non-linked genic region was separated to prevent nuclear genes and mtDNA from being present in the same partition. After partitioning and models were selected, ST was conducted in *BEAST v.1.8.0 with the appropriate settings, assuming a Birth–Death

Table 1
Anopheles species used in phylogenetic analyses. The number refers to the sequence obtained from GenBank. Species in grey were used as outgroup.

Species	COI	ITS2	CYTB	28S	*White*
An. cruzii	*KC992738 – KC992770	AF027165	AF311248	AF417810	AF318203
An. homunculus	*KC992783 – KC992791	JQ291248			
An. bellator	AF417704	DQ364652	AF311249	AF311243	AF318201
An. laneanus		DQ364656			
An. neivai			AF311260	AF311244	AF318205
An. darlingi	JX435797	AF051274	NC014275	AF417805	GQ121273
An. aquasalis	AF548902	U92324		AF417804	

*Sequences obtained in this study.

process prior with default parameters, and ucld.mean = 1.0 as above. All BEAST and *BEAST runs were carried out for 100,000,000 generations twice to check for repeatability, with convergence and effective sample size of parameters checked by Tracer v.1.6 (Drummond and Rambaut, 2007). The final ST tree was the topology that showed the maximum *a posteriori* product of clade credibilities, annotated with median branch relative times and posterior node probabilities, obtained by TreeAnnotator v.1.8.0 (Drummond and Rambaut, 2007).

We estimated the age of divergence between the *Kerteszia* and *Nyssorhynchus* lineages by implementing another ST under BEAST, this time using a node calibrated with fossil information, together with an inclusive prior for the lognormal distribution of absolute rates. The absence of well-preserved fossils in Culicidae makes it difficult to estimate the emergence of groups precisely (Besansky and Fahey, 1997); we used the fossil described in Zavortink and Poinar (2000) as a hard minimum bound for the divergence between *Kerteszia* and *Nyssorhynchus*, as it is the oldest known fossil attributed to the latter lineage, with a minimum of 15 MY. A conservative soft upper maximum of 260 MY was used, as it marks the separation of *Drosophila* and *Anopheles* according to Gaunt and Miles (2002) and marks the origin of the Diptera stem group in Wiegmann et al. (2011). The conservative interval 15–260 MY spans recent published estimates (Reidenbach et al., 2009) without using methodological extrapolations that are typical of secondary calibrations (we do not use their new inferred bounds here, in order to avoid such potential biases). As a prior for the rates for each genic partition identified by PartitionFinder, we used an Exponential [0.04] (=0.04 substitution/site/branch/million years), spanning a 95% range of 0.001–0.148 s/s/b/MY, which conservatively encompasses published rates for insect mitochondrial and nuclear genes (Brower, 1994; Papadopoulou et al., 2010). Convergence, repeatability, effective sample sizes, and annotation of the final tree were calculated as described above.

3. Results

3.1. Wing morphology

Discriminant analysis revealed the species are significantly distinct regarding the wing shape (Fig. 3). The multivariate Mahalanobis distance between *An. cruzii* and *An. homunculus* was more pronounced in females ($d = 3.97$) than in males ($d = 3.05$). The accuracy scores after a cross-validated reclassification test were 89.7% for female *An. cruzii* and 94.6% for female *An. homunculus*, compared with only 74.6% for male *An. cruzii* and 90.0% for male *An. homunculus*. Morphological diversity was higher in *An. cruzii* (md = 8.74) than in *An. homunculus* (md = 5.58). Thin-plate splines with pairwise comparison between species showed greater displacement of landmarks #2 and #16 for females and #1 and #2 for males (Fig. 3), calculated by the software program COV.

3.2. Genetic data and population dynamics

For *An. cruzii*, we obtained a 390-bp sequence located at the 3′ end of the COI gene. A total of 33 haplotypes were observed in this population and the degree of similarity among them is depicted in Fig. 4. About 92% of the substitutions (mostly transitions) were located at the third codon position (synonymous).

For *An. homunculus*, a 513-bp sequence of the COI gene was obtained; 87% of the mutations occurred in the third codon position and the majority of them were transition mutations. Among the 31 specimens sampled, there were nine different haplotypes (Fig. 4). Scores of the main genetic parameters (haplotypic and nucleotide diversity), as well as the results of Tajima and Fs tests, are listed in Table 2. In order to compare the haplotypes of species, we used 390-bp sequences for both species. Our analysis revealed that 23 mutational steps separate *An. cruzii* from *An. homunculus* (Fig. 4).

The Fs statistics is largely negative for *An. cruzii*, whilst for *An. homunculus* the signal for expansion is not significant. The stepping-stone tests showed that a model of logistic growth for the *An. cruzii* population is more likely than other models (including constant population size through time), whereas for *An. homunculus* the assumption of a constant population through time cannot be rejected (data not shown). The graphs of mismatch distribution (Fig. 5) also confirm these findings ($p < 0.001$).

3.3. Phylogenetic analysis

Phylogenetic analyses for each gene showed that individuals from the same species clustered together. We selected the largest sequence of each gene for each species (or chose one haphazardly among the largest, when more than one sequence was equally large). The best partitioning schemes for SM and ST, and the evolutionary model chosen for each partition, are shown in Supplementary File 1. For ST, a separate model was used for each individual gene. MP, ML, and BA converged to the same tree, in which [*An. bellator* + *An. homunculus*] and [*An. cruzii* + *An. laneanus*] are sister clades (Fig. 6). Furthermore, the clade [*An. bellator* + *An. homunculus*] had significant support in the MP and ML analyses (see Supplementary File 2). Regarding ST, except for the [*An. cruzii* + *An. laneanus*] clade, the ingroup was unresolved. The separation between *Nyssorhynchus* and *Kerteszia* lineages was inferred to be between 15.0 and 40.5 MYA, with all posterior divergences being <15 MY (Fig. 6).

The molecular species tree topology was plotted on the morphospace of Principal Components of female wing shape to relate the two analyses (Fig. 7). The wing shapes of the *Anopheles* in the *Kerteszia* group are more similar to one another than to those belonging to the subgenus *Nyssorhynchus* (*An. darlingi* and *An. aquasalis*).

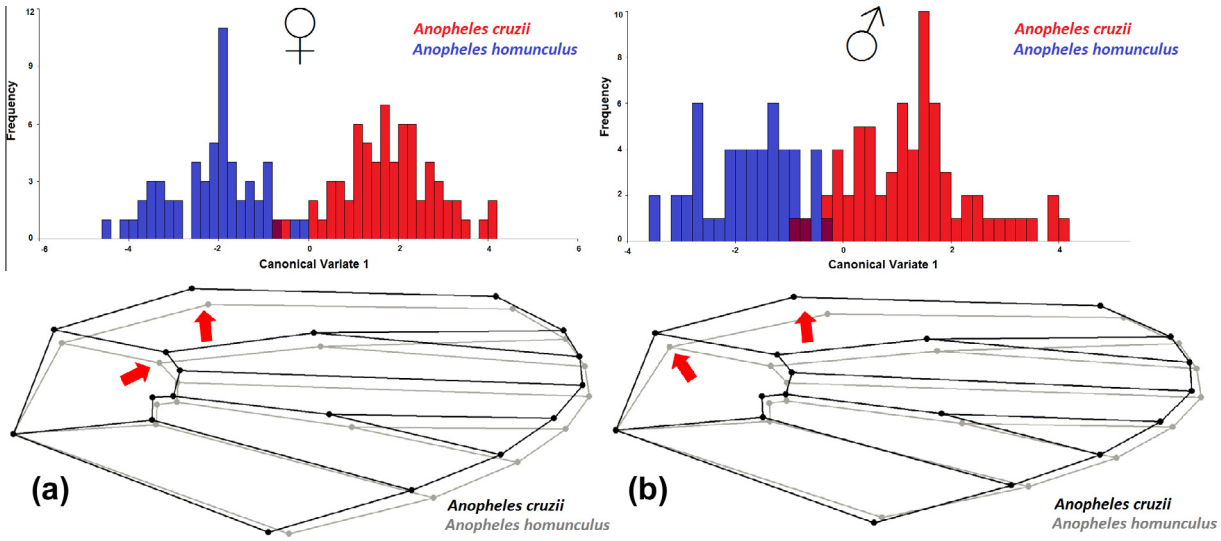

Fig. 3. Geometric morphometrics of *Anopheles cruzii* and *An. homunculus*. Above: Canonical Variate Analysis – comparisons between two species. Below: Thin-plate spline between *An. cruzii* and *An. homunculus* (a) females and (b) males. Arrows indicate the most influential landmarks.

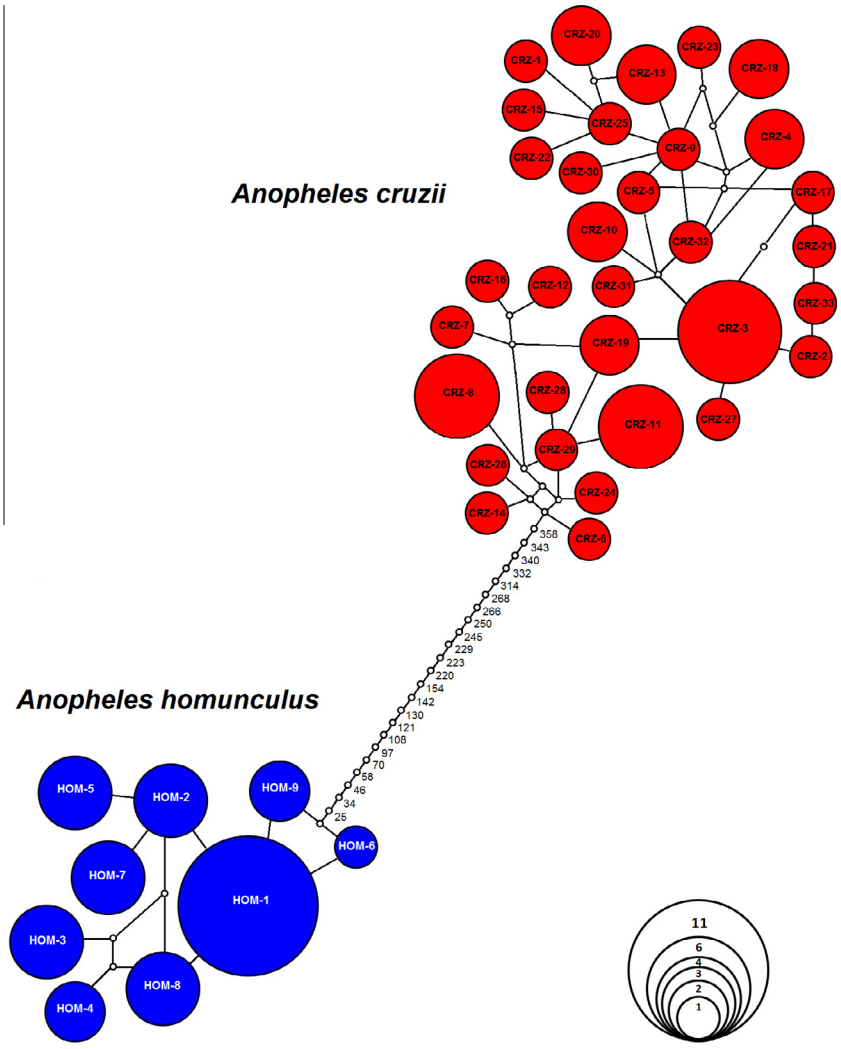

Fig. 4. Haplotype networks of COI gene for *Anopheles cruzii* and *An. homunculus*. Circles sizes correspond to haplotype frequency. Each step represents a single mutational event and the white circles are hypothetical single substitutions in the position indicated by numbers. Size of the circles is proportional to the number of the occurring haplotypes. The number of individuals can be derived from the scale shown.

Table 2
Summary statistics for CO-I data of *Anopheles cruzii* and *An. homunculus*.

Species	*H*/*n*	Number of polymorphic sites	*A*–*T* content	*h*	π	*DT*	*Fs*
An. cruzii	33/50	29/390	72%	0.97	0.012	−1.06	−26.65*
An. homunculus	9/31	14/513	71.1%	0.84	0.005	−0.76	−0.987

H = number of haplotypes; *n* = individuals sampled; *h* = haplotype diversity; π = nucleotide diversity; *DT* = Tajima's *D* test; *Fs* = Fu's *F* test;
* Shows a significant *p*-value.

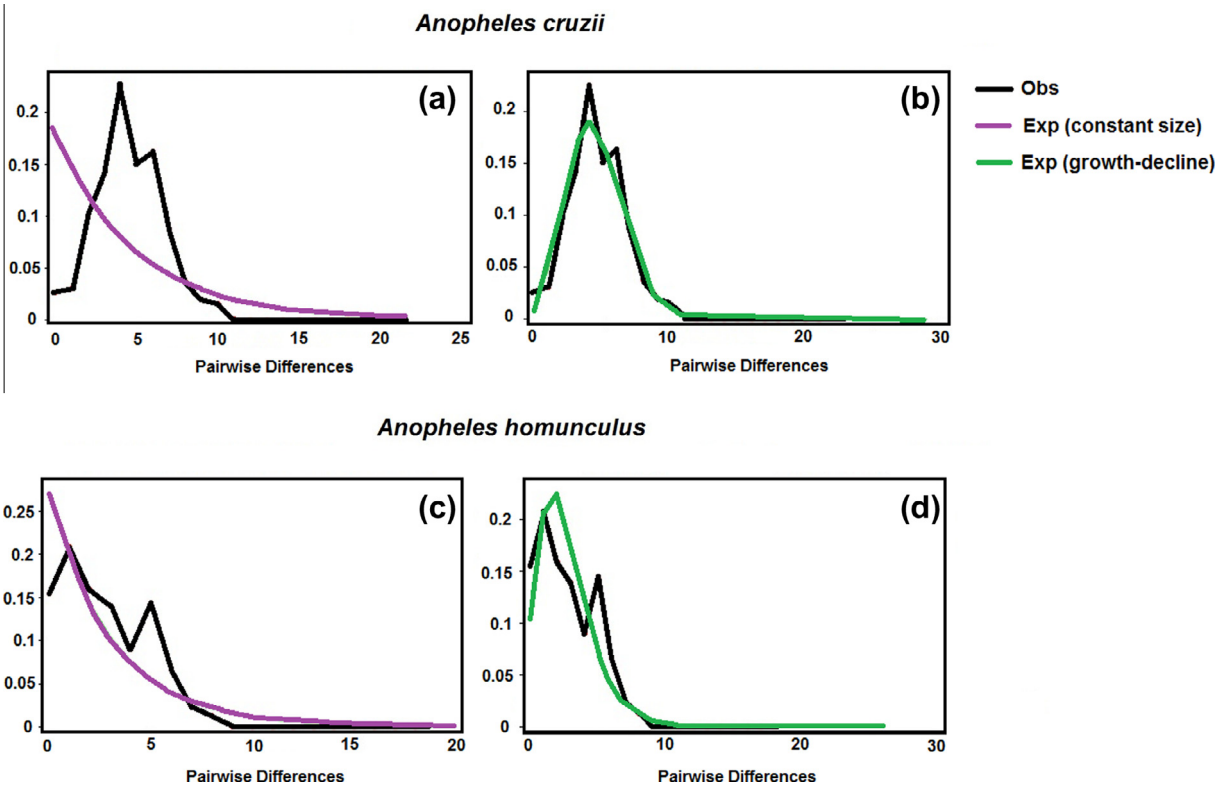

Fig. 5. Population dynamics. Observed mismatch distributions among haplotypes in *An. cruzii* (a and b) and *An. homunculus* (c and d). The population of *An. cruzii* is consistent with a growth-decline model, as shown in b. The green line represents the expected results, which are closest to the observed results (black line) in this case. In *An. homunculus* the graphs are not sufficient for differentiating between the hypotheses of constant population and expansion. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

4. Discussion and conclusions

Information about the genetic and morphological variability of *An. cruzii* and *An. homunculus* are of particular interest because they are sympatric vectors of etiological agents of malaria in south-eastern Brazil, one of the main Brazilian regions of concern regarding malaria (Pina-Costa et al., 2014). We begin our discussion with their inferred phylogenetic relationship with other species in the *Kerteszia* group and divergence times, and then, we examine the morphological and genetic differences between them.

It can be concluded that *An. cruzii* and *An. homunculus* are not sister-groups as evidenced by phylogenetic analysis based on five genes and by the (at least) 23 mutational steps that separate the mtDNA haplotypes of the two taxa. All topologies constructed by MP, ML, and BA converged and indicated that *An. bellator* was a sister to *An. homunculus*, while *An. laneanus* was a sister to *An. cruzii*. The subgenus *Kerteszia* is known to be monophyletic within *Anopheles* (Krzywinski et al., 2001; Sallum et al., 2002), but few studies have examined the relationships between the species within this group. In the present study, analyses of five genes of different origin (mitochondrial, nuclear non-coding and nuclear coding) were conducted to improve the accuracy of the topology. Analyses indicate that *Anopheles* emerged during the Cenozoic Era (50 MYA). Estimated divergence time between the subgenus *Nyssorhynchus* and *Kerteszia* was 15–40.5 MYA, which is in reasonable agreement with the results of Reidenbach et al. (2009). Diversification within *Kerteszia* was relatively recent (2–14.2 MYA) and may be related to the spread of bromeliads in South America. Givnish and Barfuss (2011) showed that a major radiation of bromeliads in Brazil occurred in Atlantic Forest and adjacent regions roughly 9.1 MYA. During the last three million years (i.e., during the Pleistocene), major climatic oscillations caused various speciation events, including insect speciation (Hewitt, 2000), and could explain the recent large radiation of *Kerteszia* species. According to Rona et al. (2010), the divergence of species within the *An. cruzii* complex occurred between 1.1 and 3.6 MYA, which agrees with our dating of the appearance of *An. cruzii* being at most 4.8 MYA.

The interspecific similarity between female *An. cruzii*, *An. homunculus*, and other *Kerteszia* species may be attributed to the relatively young evolutionary age of the group compared with other Anophelinae groups. However, the wing shape of each subgenus seems to have followed the evolution of groups, because one pattern of clustering was observed among *Kerteszia* species

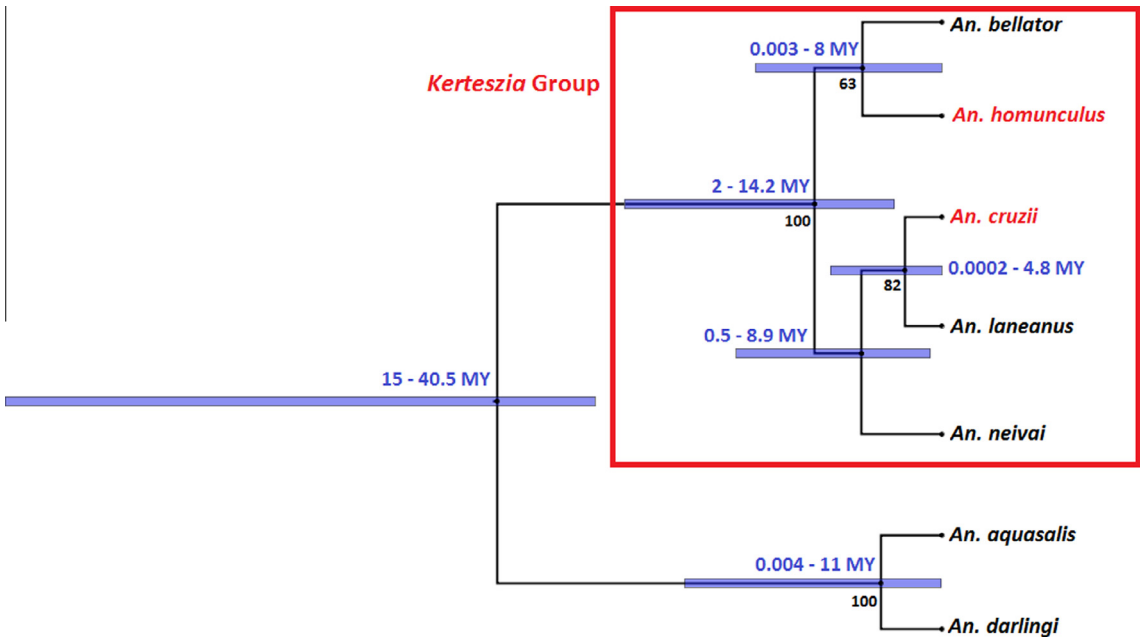

Fig. 6. Species tree and divergence times for *Anopheles*. Species tree of combined mtDNA (CO-I and CYT-B), ribosomal DNA (ITS2 and 28S) and nuclear gene (*white*) data. Node positions indicate divergence times in the maximum clade credibility Bayesian tree, and node bars indicate associated 95% confidence intervals. Number below each branch indicates the posterior probability according to the bayesian species tree analysis.

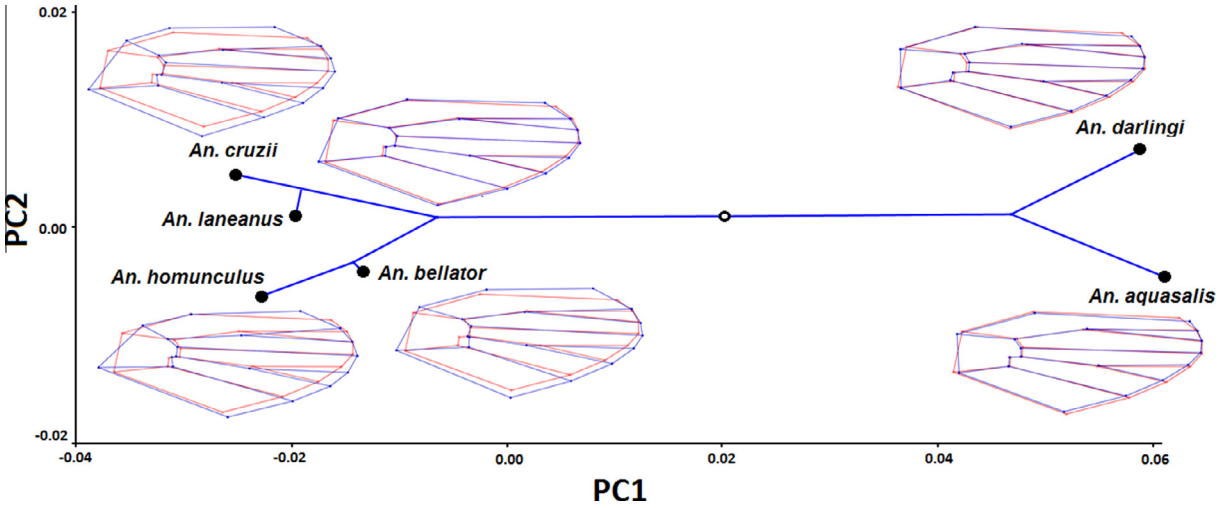

Fig. 7. Reconstruction of evolutionary changes in wing shape of *Anopheles*. The phylogenetic tree has been superimposed onto a plot of the first two principal components of the wing shape. The wings diagrams show the shape change from the reconstructed common ancestor (red contour and empty circle) to the mean shape for the respective species (blue contour and solid dots). *An. neivai* was omitted because there was no wing available. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

and another among *Nyssorhynchus* species. The wing is typically used as a marker of microevolutionary processes (Dujardin, 2008; Gómez et al., 2014; Henry et al., 2010); within *Anopheles*, it also appears to be a good indicator of macroevolution. In this case, the landmark data revealed a phylogenetic signal in wing shape, according to the genetic topology found in our study. Other studies in sea urchins and simians also found good agreement between morphometric data and phylogenies that had been estimated from other information (David and Laurin, 1996; Lockwood et al., 2004).

Despite that they share the same habitat and are believed to be morphologically similar, *An. cruzii* and *An. homunculus* showed marked differences, both morphologically and genetically. The wing shape analyses revealed species-specific wing venation patterns that could be attributed to their different evolutionary histories. Both females and males consistently differed in wing shape, also supporting the conclusion that *An. cruzii* and *An. homunculus* are morphologically different. Some of the landmarks in the wings were more variable than others and were consequently more useful in species identification. Lorenz et al. (2012) observed that female *An. cruzii* and *An. homunculus* collected in 2009 could be distinguished by their wing shapes, and that wing shape was a diagnostic characteristic helpful in the taxonomic classification of these species. Here, we extended this previous study and also

demonstrated that the diagnosis based on wing shape was still valid after some years (samples analysed here were collected in 2011–2012).

On analysing the polymorphism of the COI gene we concluded that both species differed significantly, in spite of their overall morphological resemblance. The higher haplotype diversity of *An. cruzii* indicates that the COI gene for this species is less conserved than that of *An. homunculus*. Accordingly, *An. cruzii* was morphologically more distinct (dm = 8.74). Regarding evolutionary population dynamics, *An. cruzii* showed population growth according to the stepping-stone model, whereas the population of *An. homunculus* apparently remained constant over time, agreeing with D and Fs statistics, and also with the mismatch distribution analysis (though the latter analysis was not sufficient to infer constant population for *An. homunculus*).

Overall, these morphological, genetic and population differences may be due to different evolutionary histories or to differences in their ability to disperse. Our findings are consistent with those of Veloso et al. (1956), who studied *An. cruzii* in several habitats across a broad altitude range (0–600 m). They reported that the species breeds in bromeliads with different water capacities, ranging from 5 to 2000 ml, located either on the ground or in the canopy, including in trees up to 25 m tall. *An. cruzii* has the advantage for dispersal by being acrodendrophilic (Ueno et al., 2007); it may fly to the canopy of the forest in search of breeding sites and blood supply (from monkeys and birds). In contrast, *An. homunculus* prefers more humid, lower forest habitats and breeds in small bromeliads located in biotopes that are less than 5 m in height, which are more affected by anthropogenic effects and therefore more susceptible to the impacts of human activities. In the 1940s, malaria control measures were implemented in the Atlantic Forest in Brazil by targeting anopheline *Kerteszia* species associated with bromeliads – to curb 'bromeliad-malaria' (Marrelli et al., 2007). These measures included manual removal of bromeliads and use of chemical insecticides. They resulted in a significant decrease in the mosquito population and, consequently, a reduction in the number of cases of malaria (Deane, 1988). The malaria control efforts could have altered the population structure of *An. homunculus*, causing the population to go through a bottleneck effect that reduced its haplotype diversity.

Furthermore, it is known that the two species also exhibit various behavioural differences (e.g., vector status) which may be correlated with their particular vector-competence and adaptation (Veloso et al., 1956). It is possible that the great variability found in the mtDNA of *An. cruzii* is correlated with intraspecific polymorphism in other genes that can influence biting times, feeding and resting sites, and anthropophily (Lounibos and Conn, 2000). Further genetic studies with this scope would be welcome.

This study focused on two Cananeia sympatric populations and we do not know if all the resulting interpretations can be extended to populations from elsewhere. Nonetheless, it was an important step towards understanding relevant parameters related to the evolution and sympatry of these two species in a known autochthonous region of malaria in Brazil.

Competing interests

The authors declare that they do not have competing interests.

Authors' contributions

C.L., J.S.L.P., and L.S. conceived the study, carried out data analysis, interpreted the results, and wrote the manuscript. C.L. collected data in the field. All authors approved the final version of the manuscript.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.meegid.2015.08.011.

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