Molecular Phylogenetics and Evolution 52 (2009) 588–601

Contents lists available at ScienceDirect

Molecular Phylogenetics and Evolution

journal homepage: www.elsevier.com/locate/ympev

Molecular phylogenetics and biogeography of the Neocellia Series of *Anopheles* mosquitoes in the Oriental Region

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ARTICLE INFO

Article history:
Received 29 July 2008
Revised 28 January 2009
Accepted 29 January 2009
Available online 6 February 2009

Keywords:
Anopheles
Southeast Asia
Phylogeography
Biogeography
Pleistocene climatic change
Allopatric speciation
Ecological speciation

ABSTRACT

Molecular studies of population divergence and speciation across the Oriental Region are sparse, despite the region's high biodiversity and extensive Pliocene and Pleistocene environmental change. A molecular phylogenetic study of the Neocellia Series of *Anopheles* mosquitoes was undertaken to identify patterns of diversification across the Oriental Region and to infer the role of Pleistocene and Pliocene climatic change. A robust phylogeny was constructed using CO2 and ND5 mitochondrial genes and ITS2 and D3 nuclear ribosomal markers. Bayesian analysis of mitochondrial genes was used to date divergence events. The repeated contraction and expansion of forest habitat resulting from Pleistocene climatic fluctuations appears to have had a substantial impact on intraspecific diversification, but has not driven speciation within this group. Primarily early to mid Pliocene speciation was detected within the Annularis Group, whereas speciation within the Maculatus and Jamesii Groups occurred during the mid and late Pliocene. Both allopatric divergence driven by late Pliocene environmental changes and ecological adaptation, involving altitudinal replacement and seasonality, are likely to have influenced speciation in the Maculatus Group.

1. Introduction

Southeast Asia is an important region in terms of global biodiversity, containing four of the 25 global biodiversity hotspots described by Myers et al. (2000). These are the island regions of Sundaland, Wallacea and the Philippines, and the mainland region of Indo-Burma. Together these regions contain an estimated 9.7% of the world's known endemic plant species and 8.3% of the known endemic vertebrate species, the majority of which are concentrated within tropical forest habitat (Taylor et al., 1999; Myers et al., 2000; Brook et al., 2003). Sri Lanka and the Western Ghats of India represent an additional biodiversity hotspot in the Oriental Region, which shares similar flora and fauna to the Southeast Asian hotspots. Research on Oriental biodiversity has been neglected relative to that of other regions, and consequently little is understood of the processes underlying the generation of diversity across the region (Sodhi et al., 2004). The mosquito genus *Anopheles* is species rich within the Oriental Region, containing multiple sibling species complexes that occupy a wide variety of ecological niches (Reid, 1968; Collins and Paskewitz, 1996; Foley et al., 2007a). The genus *Anopheles* is therefore a good model for studying the distribution of biodiversity and the factors influencing population divergence and speciation across the region.

Numerous hypotheses have been put forward to explain the species richness of the tropics, a phenomenon that has been demonstrated in mosquitoes (Foley et al., 2007a), as well as numerous other taxa (for reviews see Gaston, 2000; Hill and Hill, 2005; Mittlebach et al., 2007). One of the oldest and most debated of these hypotheses is the refuge model, originally proposed by Haffer

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doi:10.1016/j.ympev.2009.01.022

(1969). This states that the repeated expansion and contraction of tropical forest in response to climatic fluctuation resulted in allopatric divergence and eventual speciation. The Pleistocene, with its alternating cool, arid glacial periods and warm, moist interglacial periods, might therefore be expected to generate biodiversity according to the refuge hypothesis. However, since the majority of speciation events in a wide range of tropical vertebrate and invertebrate taxa pre-date the Pleistocene (Moritz et al., 2000; Burns and Naoki, 2004; Bush, 2005; Hill and Hill, 2005; Perez-Eman, 2005), the importance of Pleistocene climatic change in generating tropical diversity remains unclear. Rather than allopatric fragmentation, the importance of environmentally driven adaptation in generating tropical biodiversity has been highlighted in several recent studies across South America, Africa and Australia (Smith et al., 1997, 2005; Schneider et al., 1999; Ogden and Thorpe, 2002). Models of ecological speciation that have been proposed include the gradient (reviewed in Moritz et al., 2000) and altitudinal replacement models (Norman et al., 2007).

The role of Pleistocene climatic change in driving speciation in South America and Africa continues to be debated (Moritz et al., 2000; Pennington et al., 2004). It has been suggested that the Pleistocene refuge model is a particularly implausible explanation for the generation of Amazonian biodiversity due to accumulating palaeoecological evidence indicating that this region remained largely forested during glacial periods (Mayle et al., 2004). In contrast, Pleistocene climatic change may have had a particularly strong environmental influence in Southeast Asia. Southeast Asia is unique among tropical regions with regard to the dramatic effect of changes in Pleistocene sea level, which rose and fell during interglacial and glacial periods, respectively (Voris, 2000). This caused the repeated destruction and formation of land bridges between the mainland and island regions, with the large landmass of the Sunda Shelf being exposed during periods of lowered sea level (see Fig. 1), leading to increased inland aridity (Heaney, 1991). Consequently, whereas Pleistocene tropical forest cover across South America and Africa was primarily affected by temperature and precipitation, sea level fluctuations are thought to have had an equal or even greater impact on tropical forest cover across Southeast Asia (Heaney, 1991). Palaeoenvironmental reconstructions of the last glacial maximum indicate that the dominant tropical forest habitat of interglacial periods was largely replaced by grassland and savannah habitat during glacial periods, particularly in mainland Southeast Asia (Heaney, 1991; Hope et al., 2004; White et al., 2004).

Molecular studies indicate that forest fragmentation in the Sunda Shelf region, encompassing insular and mainland Southeast Asia, led to population fragmentation and divergence in both rodent (Gorog et al., 2004) and plant taxa (Cannon and Manos, 2003). Within the insular Sundaic region, speciation within the *Macaca silensis* Group of macaques (Ziegler et al., 2007) and the *Pteruthius* genus of shrike babblers (Reddy, 2008) is thought to have been triggered by the allopatric fragmentation of populations on different islands, as rising sea levels submerged land bridges between them. On the mainland, the interglacial expansion of tropical forest from refugial regions has been suggested as the cause of population expansions in *Anopheles* mosquito species (Walton et al., 2001; Chen et al., 2004; O'Loughlin et al., 2008) and in *Simulium* blackfly species (Pramual et al., 2005). However, the question of whether allopatric divergence associated with habitat fragmentation has resulted in speciation within forest taxa within the mainland Oriental Region is unanswered and is a question we address here.

The Neocellia Series (Christophers, 1924) of the *Anopheles* subgenus *Cellia* comprises some 31 currently recognised species (Harbach, 2004; Gad et al., 2006). The majority of species in the Neocellia Series are found within the Oriental Region; however

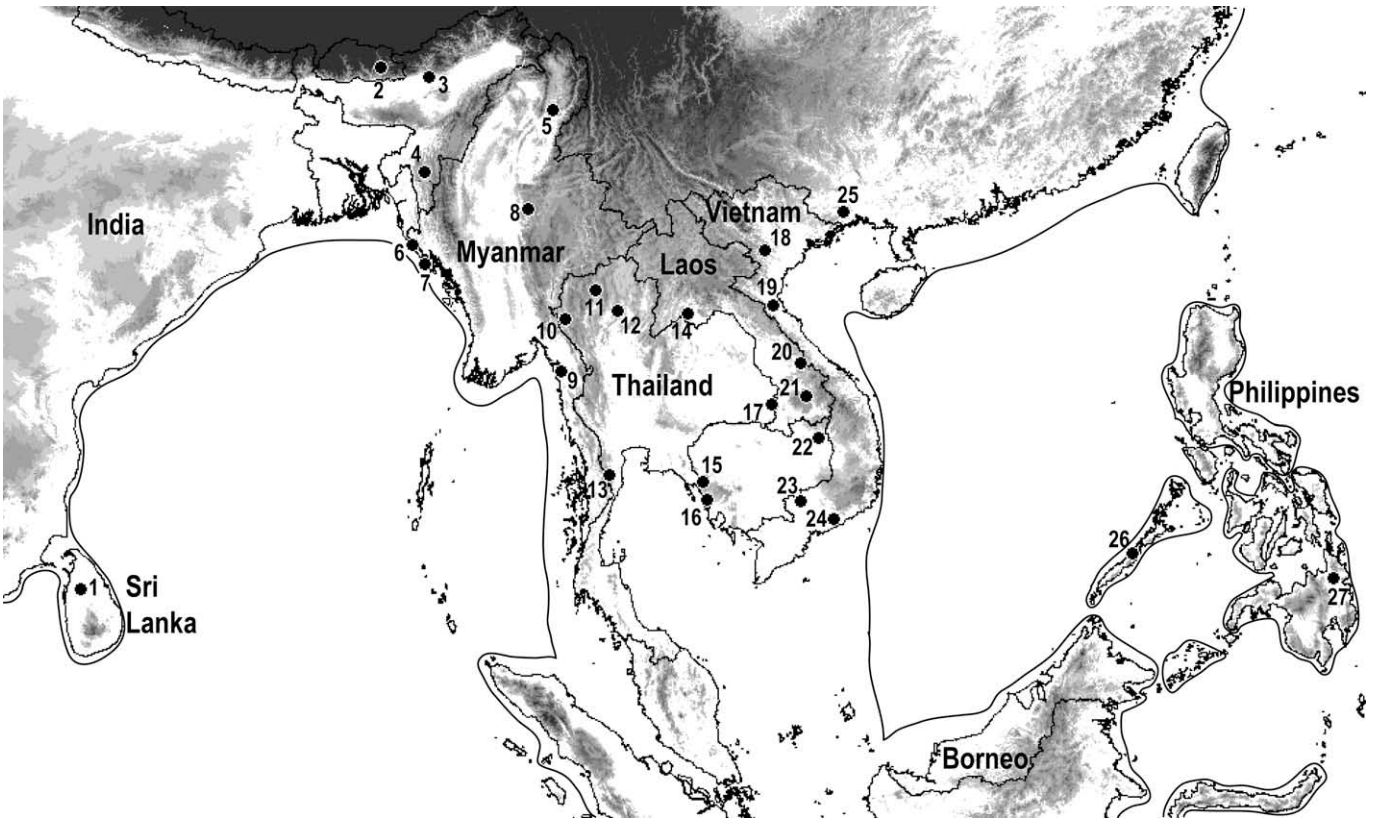

Fig. 1. Relief map of Southeast Asia indicating the locations of sample sites (see Table 1). The smooth black line indicates the approximate landmass configuration when sea levels were 120 m below their current level, the lowest levels reached during the Pleistocene (data taken from Voris, 2000).

the Series also includes African, Middle Eastern and European taxa. Within the Series, three groups have been assigned, based predominantly on morphology. These include the Annularis Group (*An. annularis*, *An. nivipes*, *An. pallidus*, *An. philippinensis, An. schueffneri*), the Jamesii Group (*An. jamesii, An. pseudojamesi, An. splendidus*) and the Maculatus Group of eight species. The Maculatus Group has recently been subdivided into two further groupings: the Maculatus Subgroup (*An. dravidicus* and *An. maculatus*) and the Sawadwongporni Subgroup (*An. sawadwongporni* and *An. notanandai*) in addition to four unassigned species (*An. greeni, An. dispar, An. willmori* and *An. pseudowillmori*). Several additional species remain unplaced within the Series (Table 1) and, with the exception of the internal systematics of the Maculatus Group (Ma et al., 2006), the molecular support for this classification has been largely untested. A thorough and comprehensive study of the molecular phylogeny of the Neocellia Series is therefore needed.

The three taxonomic groups within the Neocellia Series differ in their ecology as well as their morphology. Whereas members of the Maculatus Group are mainly associated with tropical forest habitat and hilly or mountainous areas, the Annularis Group are much less dependent on forest, being found in a wide range of habitats including heavily cleared areas such as rice paddies, and at generally lower altitudes (Horsfall, 1955; Reid, 1968; Shrestha et al., 1988; Upatham et al., 1988; Darsie and Pradhan, 1990; Rahman et al., 1993; Bangs et al., 2002). Within the Jamesii Group, both *An. jamesii* and *An. pseudojamesi* have similar habitats to the Annularis Group species, whereas those of *An. splendidus* are more similar to the Maculatus Group (Reid, 1968; Darsie and Pradhan, 1990; Rattanarithikul et al., 2006). All mosquito species are dependent on water for larval development. These species will therefore have been influenced by fluctuating levels of aridity, making the Series a suitable model system for determining the effect of historical climatic change on diversification. However, we would expect changes in tropical forest cover associated with climatic perturbation to have had an especially strong impact on the Maculatus Group taxa due to their close association with tropical forest habitat.

Here we use a molecular phylogenetics approach, using two mitochondrial genes and two ribosomal DNA (rDNA) regions, to investigate the pattern and timing of diversification of species within the Neocellia Series. In addition, a larger range of specimens from one species within the Neocellia Series, *An. annularis*, was sequenced for the CO2 mitochondrial gene to examine intraspecific population structure. The following questions are addressed: What are the processes of interspecific and intraspecific diversification within the Neocellia Series? Has the Pleistocene been important for speciation events within the Neocellia Series, or do speciation events pre-date this period? Have the Pleistocene climatic fluctuations had a greater impact on diversification within the forest-dependent Maculatus Group relative to other members of the Series? These questions are aimed at increasing our understanding of the processes influencing species richness within Southeast Asia, and of the biogeography of the region in general. An additional taxonomic question asked is whether the morphologically based classification of the Neocellia Series (Harbach, 2004) is supported by the molecular phylogeny. Several species within the Series are important vectors of malaria within Southeast Asia and knowledge of the taxonomic relationships of species may help to explain differences in vectorial capacity between them.

2. Materials and methods

2.1. Sampling techniques

A total of 130 mosquitoes representing 21 of the 31 species in the Neocellia Series were used in the study (Table 1 and Fig. 1). Despite our efforts to obtain specimens for the remaining 10 species this was ultimately not possible and the implications for this are discussed later. Where possible each species was sampled across its distribution range, however in some cases availability was limited. Two outgroup species were selected from within the genus *Anopheles* (subgenus *Cellia*); *Anopheles gambiae* (Pyretophorus Series) and *Anopheles dirus* (Neomyzomyia Series) (in Harbach, 2004). For an in-depth analysis of intraspecific population structure, 115 *An. annularis* individuals were sampled from 14 locations across the species' range. (See Supplementary material for *An. annularis* specimen information.) The majority of specimens were collected as adults. All immature specimens collected in larval surveys were reared to adulthood prior to identification. Specimens were first identified to species level using the keys of Harrison and Scanlon (1975) and Rattanarithikul and Panthusiri (1994) and then preserved by desiccation. Identifications were confirmed within the Maculatus and Annularis Groups by comparison of the ITS2 sequences obtained here to those of reference specimens (Walton et al., 2007a,b) and those in GenBank (e.g. from Ma et al., 2006). Several specimens, which are indicated in Table 1, were obtained as morphologically identified, pinned mosquitoes from the collection of the Natural History Museum, London.

2.2. DNA extraction and amplification

DNA extractions from whole adult mosquitoes were carried out using the phenol–chloroform method (Sambrook et al., 1989). A 621 bp fragment of the mitochondrial CO2 gene, 500 bp of the ND5 mitochondrial gene, 343 bp of the D3 region of the 28S ribosomal gene and approximately 620 bp of the ITS2 spacer region of the rDNA were PCR-amplified using the primer pairs leu/lys (Sharpe et al., 2000), ND5/Phe (Krzywinski et al., 2001), D3a/D3b (Sharpe et al., 1999) and 5.8f/28s (Paskewitz and Collins, 1990), respectively. All PCRs were carried out using a GeneAmp 9700 thermocycler (Applied Biosystems, USA). Each PCR had a total volume of 50 μl, and contained 200 μM dNTP, 1.5 mM $MgCl_2$, and primers at concentrations of 0.24 μM, 0.8 μM, 0.6 μM and 1.0 μM for the CO2, ND5, D3 and ITS2 primers, respectively. For the amplification of all regions, initial denaturation was carried out for 5 min at 94 °C. For the amplification of CO2, ND5 and ITS2, 39 cycles consisting of 1 min at 94 °C for denaturation, 1 min at 51 °C for annealing and 2 min at 72 °C for extension were performed, followed by 3 min at 72 °C for final extension. For the amplification of the D3 region, 39 cycles consisting of 30 s at 94 °C, 30 s at 55 °C and 1 min at 72 °C were carried out, followed by 4 min at 72 °C for final extension. Purification of PCR products using Millipore® Montage columns (Billerica, USA) was carried out prior to sequencing (Macrogen Inc., South Korea), which was performed in both directions using the original PCR amplification primers specified above. Sequences were assembled and manually edited in the program SEQUENCHER 4.6 (Gene codes, Ann Arbor, USA). The sequences used in this study were deposited in GenBank with Accession Nos. FJ526394–FJ526741 and EU882061.

2.3. Phylogenetic analysis

The mitochondrial DNA (mtDNA) sequences were translated with DnaSP 4.50.3 (Rozas and Rozas, 1999) using the invertebrate mitochondrial genetic code to test for the amplification of pseudogenes. No stop codons were found indicating the sequences were mitochondrial. Nucleotide sequences were aligned using the program ClustalX 1.81 (Thompson et al., 1997) and the resulting alignments verified by eye. After experimenting with several combinations of gap opening and extension penalties, the ribosomal dataset was aligned using penalties of 20 and 2, respectively, prior to making appropriate manual adjustments. Due to high interspecific variation at both ribosomal loci, reliable alignment

Table 1
Specimens sampled for this study, with their associated sampling localities (see Fig. 1).

Taxonomical group	Species	Sample location (country, province/town)	Sample size	Georeference (latitude, longitude)	Collection site (Fig. 1)
Pyretophorus Series					
Gambiae Complex	*Anopheles gambiae*	Uganda	1	Unavailable	
Neomyzomyia Series					
Leucosphyrus Group	*Anopheles dirus*	Laos, Sekong	3	15.37, 106.67	21
Neocellia Series	*Anopheles karwari*	India, Assam	3	26.94, 92.99	3
		Cambodia, Ratanakiri	1	13.86, 107.10	22
		Cambodia, Koh Kong	3	11.60, 103.05	16
		Myanmar, Rakhine	2	20.82, 92.40	6
		Vietnam, Tay Ninh	2	11.56, 106.44	23
		Sri Lanka	1	8.35, 80.38	1
	Anopheles paltrinierii	United Arab Emirates (UAE)[a]	2	Unavailable	
	Anopheles maculipalpis	Zambia[a]	1	Unavailable	
	Anopheles pulcherrimus	Pakistan[a]	2	Unavailable	
	Anopheles stephensi	Laboratory colony, original material from Dubai	2	Unavailable	
	Anopheles superpictus	Greece, Ioannina	1	39.67, 20.85	
	Anopheles ainshamsi	Egypt, Râs Shukeir[a,b]	1	Unavailable	
Annularis Group	*Anopheles annularis*	Sri Lanka	3	8.35, 80.38	1
		Myanmar, Rakhine	2	20.16, 92.85	7
		Myanmar, Myitkyina	3	25.72, 97.48	5
		Myanmar	2	16.25, 97.75	9
		Thailand, Mae Hong Son	1	18.16, 97.93	10
		Thailand, Phetchaburi	1	12.51, 99.52	13
		Cambodia, Ratanakiri	1	13.86, 107.10	22
		Laos, Vientiane	1	18.35, 102.37	14
		Vietnam, Hoa Binh	1	20.64, 105.17	18
		Philippines, Palawan Island	1	9.65, 118.46	26
	Anopheles pallidus	Sri Lanka	1	8.35, 80.38	1
	Anopheles philippinensis	India, Assam	2	26.94, 92.99	3
		Thailand, Chiang Mai	2	19.20, 99.00	11
		Cambodia, Ratanakiri	2	13.86, 107.10	22
		Laos, Vientiane	1	18.35, 102.37	14
	Anopheles nivipes	China, Guangxi	1	22.04, 108.00	25
		Myanmar, Myitkyina	2	25.72, 97.48	5
		Thailand, Chiang Mai	2	19.20, 99.00	11
		Cambodia, Ratanakiri	1	13.86, 107.10	22
		Laos, Vientiane	1	18.35, 102.37	14
Jamesii Group	*Anopheles jamesii*	India, Assam	1	22.92, 92.47	4
		Sri Lanka	2	8.35, 80.38	1
		Myanmar, Rakhine	1	20.86, 92.46	6
		Thailand, Phetchaburi	1	12.51, 99.52	13
		Vietnam, Tay Ninh	2	11.56, 106.44	23
	Anopheles pseudojamesi	Thailand, Chiang Mai	3	19.20, 99.00	11
	Anopheles splendidus	Myanmar, Myitkyina	1	25.72, 97.48	5
		Thailand, Chiang Mai	2	19.20, 99.00	11
		Thailand, Lampang	1	18.45, 99.79	12
		Cambodia, Ratanakiri	3	13.86, 107.10	22
		Vietnam, Binh Thuan	2	10.93, 107.67	24
Maculatus Group	*Anopheles dispar*	Philippines, Mindanao Island	1	8.76, 125.77	27
	Anopheles greeni	Philippines, Mindanao Island	1	8.76, 125.77	27
	Anopheles willmori	Bhutan	1	27.26, 91.26	2
		Thailand, Chiang Mai	1	Unavailable	11
		Vietnam	1	Unavailable	
	Anopheles pseudowillmori	India, Assam	4	26.93, 92.99	3
		Myanmar, Myitkyina	2	25.72, 97.48	5
		Thailand, Chiang Mai	2	19.20, 99.00	11
		Laos, Vientiane	1	18.35, 102.37	14
	Anopheles maculatus species K	Thailand, Ubon Ratchathani	2	Exact location unknown	17
		Cambodia, Ratanakiri	2	13.86, 107.10	22
		Vietnam, Quang Binh	1	17.90, 105.90	19
Maculatus Subgroup	*Anopheles dravidicus*	Thailand, Chiang Mai	2	19.20, 99.00	11
	Anopheles maculatus	India, Assam	3	22.92, 92.47	4
		Sri Lanka	1	8.35, 80.38	1
		Myanmar, Myitkyina	2	25.72, 97.48	5
		Myanmar, Pyin Oo Lwin	1	22.11, 96.60	6
		Thailand, Chiang Mai	3	19.20, 99.00	11
		Thailand, Phetchaburi	1	12.50, 99.53	13
		Cambodia, Ratanakiri	3	13.86, 107.10	22

(*continued on next page*)

Table 1 (*continued*)

Taxonomical group	Species	Sample location (country, province/town)	Sample size	Georeference (latitude, longitude)	Collection site (Fig. 1)
		Savannakhet, Laos	1	16.57, 106.44	20
		Sekong, Laos	1	15.37, 106.67	21
		Vietnam, Nghe An	1	18.64, 105.48	19
Sawadwongporni Subgroup	*Anopheles sawadwongporni*	India, Assam	1	22.92, 92.47	4
		Myanmar, Myitkyina	1	25.72, 97.48	5
		Thailand, Chiang Mai	1	19.20, 99.00	11
		Thailand, Phetchaburi	1	12.50, 99.53	13
		Cambodia, Pursat	2	12.26, 102.92	15

[a] Morphologically identified specimens from the collections of the Natural History Museum, London, collected in 1983 (*An. paltrinierii* and *An. ainshamsi*), 1936 (*An. maculipalpis*) and 1984 (*An. pulcherrimus*).
[b] Specimen from Gad et al. (2006).

of the entire dataset was not possible. All taxa that could not be aligned were omitted from the rDNA analysis, leaving a subset of taxa (*An. maculatus, An. dravidicus, An. willmori, An. greeni, An. dispar, An. sawadwongporni, An. maculatus* species K, *An. annularis, An. philippinensis, An. nivipes, An. pallidus, An. pulcherrimus, An. jamesii, An. pseudojamesi* and *An. splendidus*). Two concatenated datasets were created, one containing the mitochondrial CO2 and ND5 gene fragments for the entire dataset, and the other containing the ITS2 and D3 fragments for the subset of taxa. Partition homogeneity tests (Farris et al., 1995) were conducted in PAUP version 4b10 (Swofford, 2002) to test for incongruence between the mtDNA gene regions and between the rDNA regions and none was detected. Subsequent analyses were carried out separately on both the concatenated mitochondrial and the concatenated nuclear datasets. The most appropriate model of evolution was determined for each dataset using MODELTEST (Posada and Crandall, 1998), and the parameters specified by this program were implemented in further analyses. Gaps in the ribosomal dataset were treated as missing data. Prior to phylogenetic analyses, plots of the number of transitions and transversions against the genetic distance were examined for each gene region to detect any substitution saturation. Statistical tests for substitution saturation were also conducted using the method of Xia et al. (2003) and none was detected.

The Neighbour Joining (NJ) and Maximum Likelihood (ML) methods of phylogeny inference were conducted in PAUP version 4b10 (Swofford, 2002), and MCMC Bayesian analysis was carried out using MrBayes v3.0b4 (Huelsenbeck and Ronquist, 2001). Due to the large size of the dataset it was necessary to use the heuristic search for the ML analysis, with 20 replicate random addition sequences and using tree bisection and reconnection (TBR). To test the reliability of the ML analysis, 100 bootstrap replicates were performed. MrBayes was run for 4 million generations, using one cold and 5 incrementally heated chains and sampling every 100 generations. Two independent runs were performed to confirm convergence. The number of generations taken to reach the stationary phase was determined by plotting the likelihood scores of trees against generation time, and all trees produced before the stationary phase was reached were discarded with a burnin of 100,000 generations. A 50% majority rule consensus tree was created from the remaining trees. Bayesian support values of 95% and Maximum Likelihood Bootstrap values of 70% were taken as being significant support for a node (Hillis and Bull, 1993).

The Shimodaira–Hasegawa (SH) test (Shimodaira and Hasegawa, 1999), was used to test whether the data supports the internal taxonomic relationships proposed by Christophers (1924), Rattanarithikul and Green (1987), Rattanarithikul et al. (2006) and Ma et al. (2006). The SH test compares the likelihood scores of the optimal ML tree to those in which phylogenetic relationships are constrained to conform to *a priori* hypotheses. ML trees were constructed under the following constraints: (1) *An. pseudowillmori* was included within a monophyletic Maculatus Group; (2) *An. splendidus, An. pseudojamesi* and *An. jamesii* were constrained within a monophyletic Jamesii Group. The likelihood scores of the constrained trees and the optimal tree were determined using the resampling estimated log-likelihood (RELL) method with 1000 bootstrap replications. The SH tests were performed using PAUP version 4b10 (Swofford, 2002).

2.4. Divergence time estimation

Due to the lack of reliable and recent calibration points, molecular dating methods assuming a relaxed molecular clock (Drummond et al., 2006) were not suitable for this dataset. To estimate the timing of divergence events it was therefore necessary to assume a strict molecular clock, and to specify a rate of molecular evolution. The estimation of divergence times was carried out using the concatenated ND5 and CO2 sequences. The relative rates test (Tajima, 1993) was first applied to the sequence data in MEGA3.1 (Kumar et al., 2004) to test the hypothesis of mutation rate homogeneity across lineages. In addition, ML phylogenies were inferred both in the presence and absence of the assumption of a strict molecular clock, and their likelihood scores compared using the SH test, conducted in PAUP version 4b10 (Swofford, 2002). As no significant difference in likelihood scores was detected, and as the relative rates test did not detect any evidence of rate heterogeneity across lineages, a molecular clock-like model of evolution was assumed.

Divergence times and their confidence intervals were estimated using a Bayesian MCMC coalescent method, implemented in BEAST 1.4 (Drummond and Rambaut, 2007). Initial MCMC chains were run for 1,000,000 generations and the scale factors adjusted as suggested by the operator analysis. This was repeated until the effective sample size of all parameters exceeded 100 (Drummond et al., 2002). The uncorrelated log normal rate variation model was specified with the Yule tree prior distribution. Finally, two independent MCMC chains, each of 10,000,000 generations, were performed with a discarded burnin of 1,000,000 generations. The chains were sampled every 1000 generations and, after checking for convergence, the results of the independent chains were combined. Tracer v1.4 (Rambaut and Drummond, 2007) was used to obtain the posterior probability density distribution and 95% confidence intervals of divergence times, and to examine the effective sample size of all parameters.

For the dating with BEAST, a standard arthropod mtDNA sequence divergence rate of 2.3% per million years was used, based on concordant estimates of the rate of mtDNA evolution from a variety of taxa across five arthropod genera (Brower, 1994). This estimate of the molecular clock is based on the apparently linear relationship between uncorrected sequence divergence and divergence time with an implicit simple underlying model of evolution. To estimate divergence in BEAST we therefore applied a GTR model (with the associated parameters specified by MODELTEST as the pri-

ors) without a gamma distribution or proportion of invariant sites. Our estimates should therefore be reliable for the time period for which the Brower (1994) molecular clock was determined (at least the last 3.25 million years), but since mutation saturation starts to occur after this time (DeSalle et al., 1987), divergences substantially older than this may be underestimated. The 2.3% divergence pmy rate of mitochondrial evolution has been used to estimate the dates of events in the histories of a wide range of insect taxa (Salvato et al., 2002; Hundsdoerfer et al., 2005; Garrick et al., 2007; Albre et al., 2008), including *Anopheles* mosquitoes (Foley et al., 2007b). However, there is likely to be a degree of error associated with assuming this general rate, so some caution should be exercised in the interpretation of the estimated speciation dates.

2.5. Phylogeography of An. annularis

Firstly, mitochondrial CO2 sequence data were used to create a median joining haplotype network using NETWORK 4.500 (Bandelt et al., 1999). Secondly, CO2 sequence data were analysed using Nested Clade Phylogeographic Analysis (NCPA), which is designed to distinguish between historical and contemporary processes, such as range expansion, allopatric fragmentation and restricted gene flow, that have shaped the phylogeographic structure of species or populations (Templeton et al., 1995). NCPA was implemented in ANeCA (Panchal, 2007) which includes the software TCS v1.18 (Clement et al., 2000) and GeoDis v2.2 (Posada et al., 2000) and provides a fully automated implementation of NCPA. A haplotype network was constructed from sequence data using the statistical parsimony algorithm (Templeton et al., 1992) and haplotypes were clustered hierarchically into a set of nested clades (Templeton, 1998). Using this nested design, genetic and geographic distances were compared within and between clades to detect deviations from the null hypothesis of no association between haplotype variation and geography (Posada et al., 2000). Finally, the automated published inference key (available at http://darwin.uvigo.es/software/geodis.html), was used to infer the historical processes that have shaped the phylogeographic structure of each of the nested clades. The efficacy of NCPA as a method for inferring gene flow regime and demographic history has been questioned, based on a lack of statistical hypothesis testing and high proportions of false inferences recovered in simulation studies (e.g. Knowles and Maddison, 2002; Panchal and Beaumont, 2007; Petit, 2008). It has, however, been argued that much of this criticism is based on misrepresentations and invalid implementation of NCPA, simulation artefacts and unrealistic sampling assumptions (Templeton, 2004, 2008). Taking both sides of the argument into consideration, we consider NCPA to be a credible and useful tool when the results are assessed in conjunction with those from other methods.

3. Results

3.1. Sequence variation

It was not possible to amplify the mitochondrial DNA or ITS2 sequences for either *An. paltrinieri* or *An. maculipalpis.* It is likely that their DNA was degraded since both were pinned museum specimens that were collected several decades ago (Table 1).

Both mitochondrial gene regions were AT rich, with an overall AT content of 87%. MODELTEST identified the GTR+I+G model as the most appropriate for both the mitochondrial and ribosomal datasets. For the rDNA dataset, a total of 198 bp were removed due to ambiguities in the alignment, leaving a concatenated rDNA dataset of 644 bp, of which 57% were variable. The concatenated mtDNA dataset consisted of 1131 bp, 49% of which were variable. The mtDNA alignment was unambiguous, with no indels.

3.2. Phylogenetic analysis

3.2.1. Mitochondrial DNA data

Fig. 2 shows the 50% majority rule consensus tree obtained using Bayesian analysis, the topology of which is in agreement with the phylogenetic trees obtained using both ML and NJ analyses. These show that the Neocellia Series does not form a monophyletic group. One main monophyletic clade was recovered (Bayesian Posterior Probability value, PP = 100, ML Bootstrap value, BS = 99), however both Oriental *An. karwari* and African *An. ainshamsi* fell outside of this clade. This main clade is composed of six lineages, including two major clades, in an unresolved polytomy. The first of these clades contains all of the currently classified Maculatus Group species (Rattanarithikul and Green, 1987; Harbach, 2004) with the exception of *An. pseudowillmori*, which forms a separate lineage in the unresolved polytomy. This well-supported clade (PP = 100, BS = 99) is therefore referred to here as the Maculatus Group clade (Fig. 2). The SH test reveals that the likelihood score of the ML tree in which *An. pseudowillmori* was constrained within a monophyletic Maculatus Group clade was significantly lower than that of the optimal ML tree ($p = 0.014$). The Maculatus Group clade is divided into two well-supported subclades (PP = 99, BS = 79 and PP = 77, BS = 91), supporting the division of the Maculatus Group into the Maculatus and Sawadwongporni Subgroups in the current classification (Rattanarithikul et al., 2006).

The second major clade recovered contains species currently classified within the Annularis and Jamesii Groups, with the exception of *An. splendidus* that is currently placed in the Jamesii Group but, like *An. pseudowillmori*, forms a separate lineage in the unresolved polytomy. The SH test reveals that the likelihood score of the ML tree in which *An. splendidus* was constrained within a monophyletic Jamesii Group clade was significantly lower than that of the optimal ML tree ($p = 0.021$). This second major clade is itself poorly supported (PP = 63, BS < 50), and is split into the poorly supported Annularis Group clade (PP = 85, BS < 50) and the more strongly supported Jamesii Group clade (PP = 97, BS < 50). The Annularis Group clade (Fig. 2) includes all currently classified Annularis Group species as well as, in a basal position, *An. pulcherrimus*, which according to the current taxonomy is unplaced within the Neocellia Series (Harbach, 2004). The two members of the Jamesii Group clade, *An. jamesii* and *An. pseudojamesi*, appear to be sister taxa. *Anopheles superpictus* and *An. stephensi*, both of which are currently unplaced within the Neocellia Series (Harbach, 2004), form the two final lineages within the unresolved polytomy.

3.2.2. Ribosomal DNA data

Anopheles maculipalpis, An. paltrinierii and *An. ainshamsi* are all currently classified within the Neocellia Series (Harbach, 2004) but have distributions outside of the Oriental Region, in either Africa or the Middle East. The D3 rDNA region sequence data from these species is so divergent from Oriental members of the Series that it is impossible to align them with any confidence, suggesting that these non-Asian species should not be included within a monophyletic Neocellia Series. In the cases of *An. maculipalpis* and *An. paltrinierii*, an alternative possibility is sample contamination due to the problem of DNA degradation in these old specimens. However, the fact that unique haplotypes were obtained for both of these species suggests contamination is unlikely. The concordance of rDNA and mtDNA in *An. ainshamsi* supports its position outside of the Neocellia Series.

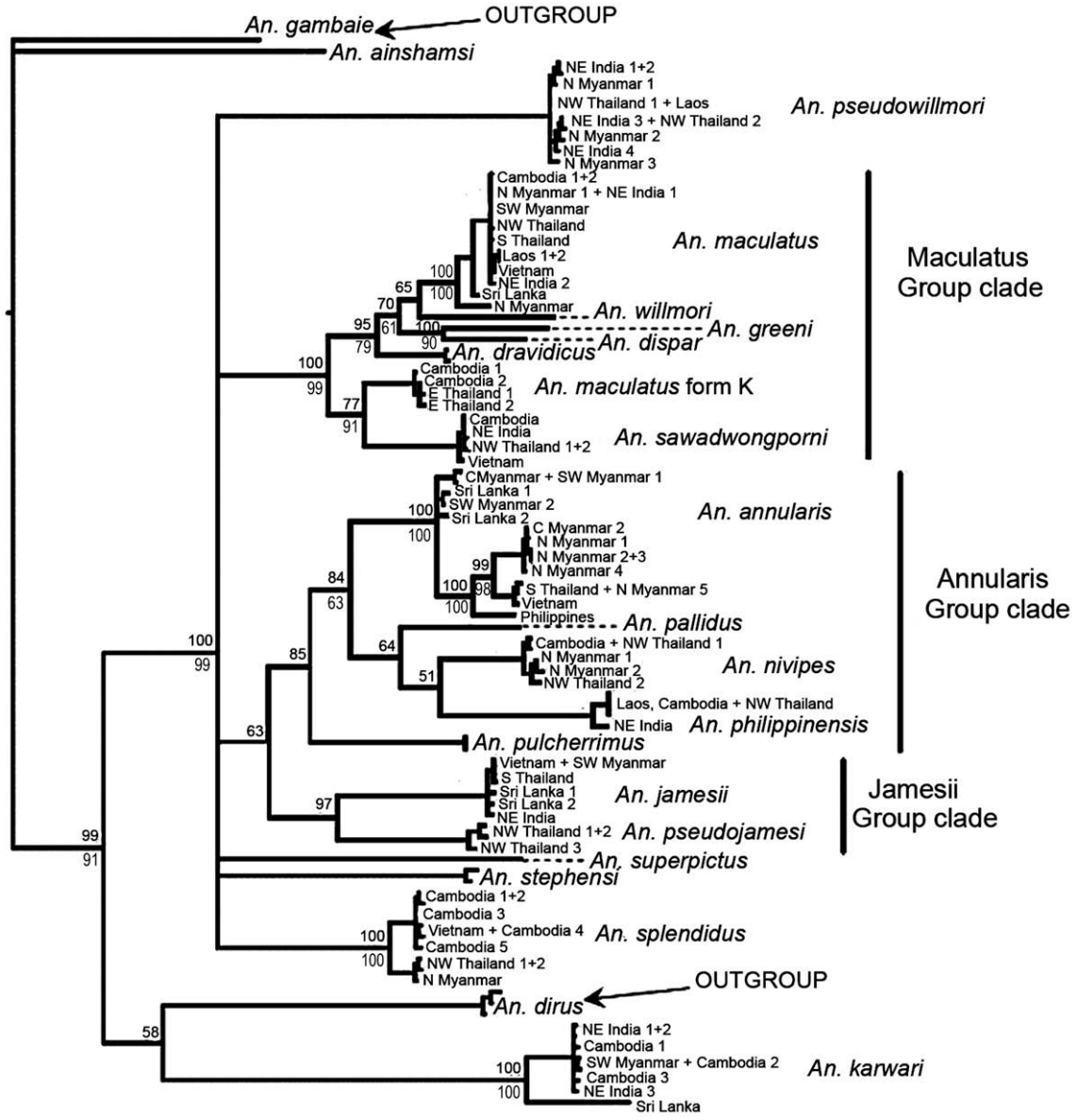

Fig. 2. Bayesian 50% majority rule consensus tree obtained through analysis of the concatenated mtDNA dataset. Bayesian support values and ML Bootstrap values >50% are shown above and below each node, respectively. The support indices for the majority of intraspecific nodes are not given due to a lack of space. *An. dirus* and *An. gambiae* are indicated as outgroup species.

Fig. 3 shows the 50% majority rule consensus tree obtained using Bayesian analysis of the restricted rDNA dataset, the topology of which is in agreement with the phylogenetic trees obtained using both ML and NJ analyses. The Maculatus Group clade, as defined by the mtDNA data analysis, was strongly supported (PP = 100, BS = 100). The exclusion of *An. pseudowillmori* from this clade was also supported, as the rDNA sequence of this species could not be aligned reliably with those from the other Maculatus Group species. The Maculatus Group clade was split into two well-supported subclades, as in the mtDNA analysis. The first of these contains *An. maculatus, An. dravidicus, An. greeni, An. dispar* and *An. willmori* (PP = 100, BS = 79); the second contains *An. sawadwongporni* and *An. maculatus* species K (PP = 100, BS = 100).

The clade containing the Annularis Group species and *An. pulcherrimus*, *An. jamesii* and *An. pseudojamesi* was well supported (PP = 100, BS = 88). *Anopheles jamesii, An. pseudojamesi* and *An. splendidus* did not group together, in disagreement with the current classification of the Jamesii Group (Rattanarithikul et al., 2006). As in the mtDNA analysis, the Annularis Group clade included all species currently classified within the Annularis Group (Harbach, 2004), as well as the currently unplaced *An. pulcherrimus*. The Annularis Group clade, although poorly supported in the mtDNA analysis, received strong support from the rDNA (PP = 100, BS = 98). As in the mtDNA analysis, the majority of nodes within the Annularis Group were poorly supported.

Although rDNA evolves by concerted evolution (Elder and Turner, 1995), and so is expected to homogenize rapidly within species, some intraspecific variation was noted (Fig. 3). The highest amount of intraspecific ribosomal divergence was seen within *An. karwari*, with both the ITS2 and D3 sequences sampled from Sri Lanka differing from the mainland haplotype, by 15 bp and 4 bp, respectively. The single Sri Lankan *An. maculatus* specimen has ITS2 and D3 sequences that differ from the mainland sequences by one substitution and one indel, and by two substitutions, respectively. In *An. annularis*, the ITS2 locus had five sequence types involving a total of six polymorphic sites as reported in Walton et al. (2007a). Some association of rDNA with geography can be detected, however there is no association with the mitochondrial haplotype.

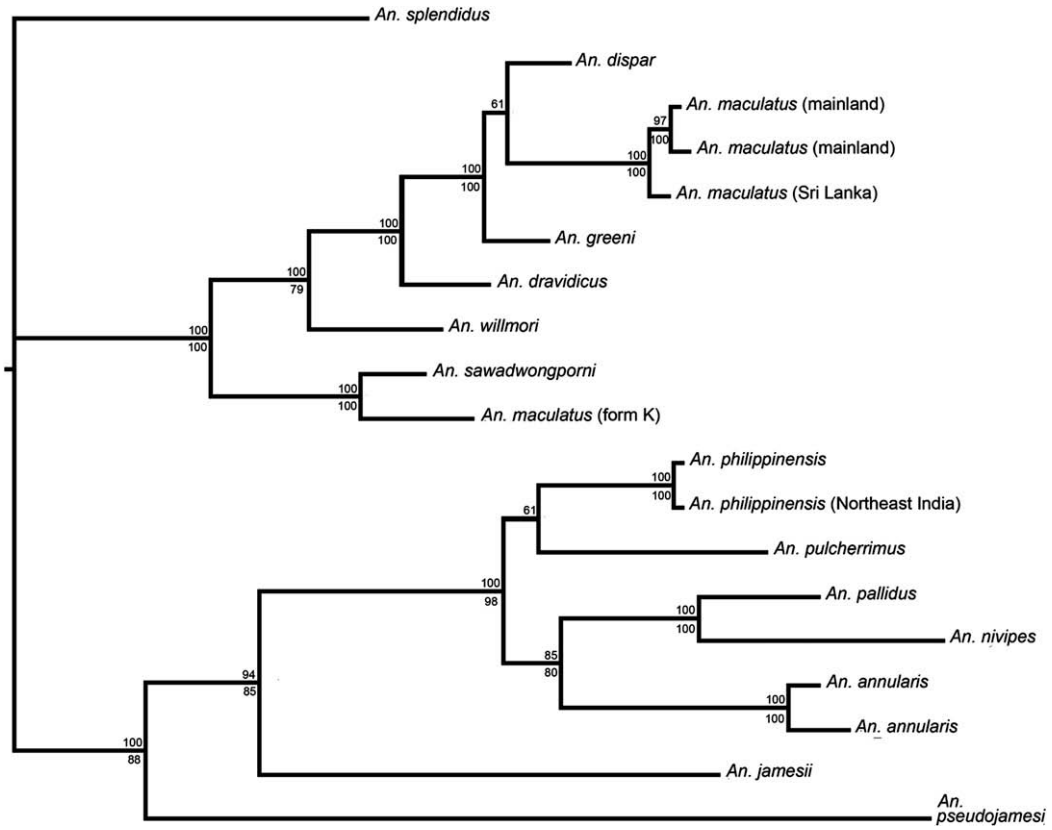

Fig. 3. Bayesian 50% majority rule consensus tree obtained through analysis of the concatenated rDNA dataset, using the restricted set of taxa. Bayesian support values and ML Bootstrap values >50% are shown above and below each node, respectively.

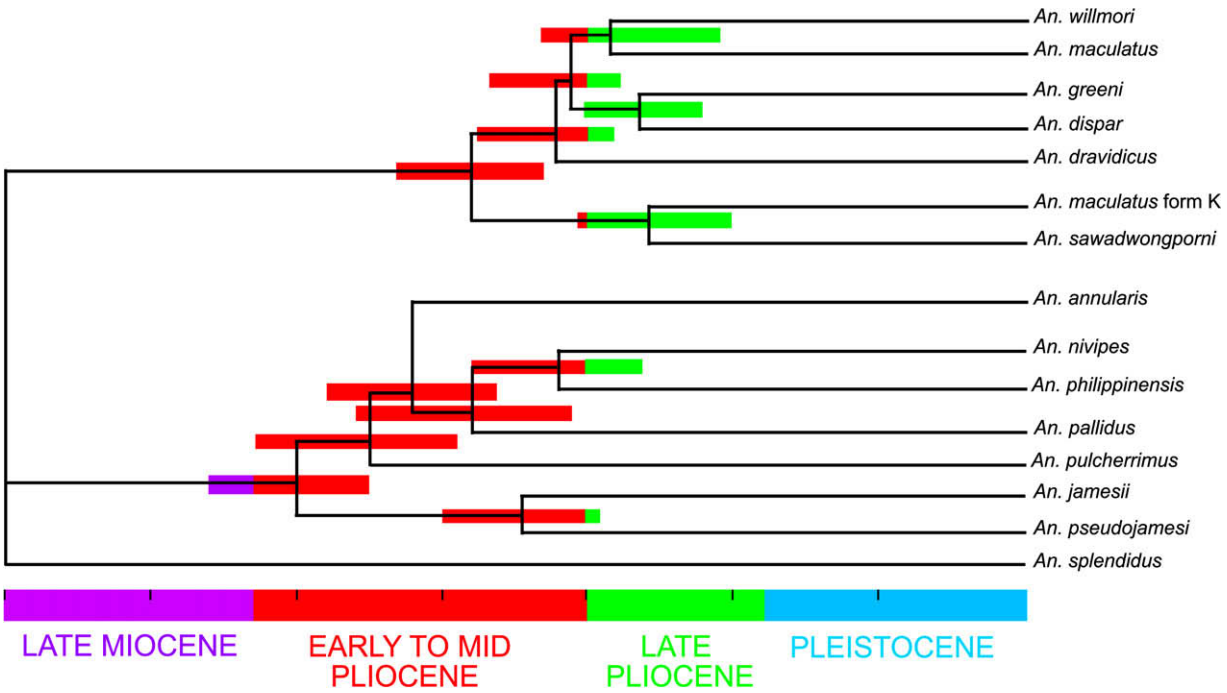

Fig. 4. Chronogram for the Neocellia Series. Divergence times estimated using the BEAST software (Drummond and Rambaut, 2007) are shown on the Bayesian consensus topology obtained from the analysis of the mtDNA dataset. Node positions indicate mean estimated divergence times, and node bars indicate the 95% confidence intervals.

Within *An. philippinensis*, the ITS2 sequence from northeastern India differed by one substitution and an indel from the mainland sequence. None of the other species where there were specimens from two or more geographical locations exhibited intraspecific variation.

3.3. Divergence times

The estimated divergence times and their associated 95% confidence intervals are shown in Fig. 4. Whereas speciation within the Annularis Group dates mainly to the early to mid Pliocene (3.2–4.5 mya), speciation within the Maculatus Group is inferred to have occurred more recently, during the late Pliocene, with most speciation dated to between 2.6 and 3.2 mya. The divergence of the Jamesii Group (*An. jamesii* and *An. pseudojamesi*) is estimated to have occurred slightly earlier than the Maculatus Group, during the mid to late Pliocene (3.4 ± 0.5 mya).

The divergence of the Sri Lankan and mainland *An. karwari* haplotypes occurred during the late Pliocene (2.4 mya, 95% CI: 1.9–2.9 mya). All other intraspecific divergence dates to the Pleistocene. The two deep mtDNA clades within *An. splendidus*, composed of eastern individuals from Vietnam and Cambodia, and western individuals from northwestern Thailand and Myanmar, differ by 23 fixed differences corresponding to an estimated divergence time of 1.16 mya (95% CI: 0.79–1.55 mya). Within *An. maculatus*, the northern Myanmar and Sri Lankan haplotypes were basal to the mainland haplotypes and diverged 1.27 mya and 0.6 mya, respectively (95% CI: 0.92–1.67 mya and 0.39–0.87 mya, respectively). The most divergent and basal haplotype within *An. philippinensis* also came from the west, from northeastern India, with an estimated divergence time of 0.62 mya (95% CI: 0.36–0.89 mya). Within *An. annularis* the divergence between the Philippines and mainland haplotypes occurred 1.8 mya (95% CI: 1.49–2.26 mya), whereas eastern and western lineages (see below) diverged slightly later, 1.12 mya (95% CI: 0.78–1.75 mya).

3.4. Altitudinal replacement

Members of the Annularis Group are commonly found in lowland regions, whereas species within the Maculatus Group tend to be found at a wider range of altitudes, from the foothills to mountain peaks (Covell, 1927; Reid, 1968; Rattanarithikul et al., 2006). Within the Maculatus Subgroup, the relationship between phylogenetic position and approximate altitudinal distribution (Fig. 5) indicates speciation by altitudinal replacement, in which the distribution of one species replaces that of the sister species along an altitudinal gradient (Moritz et al., 2000; Norman et al., 2007). Whereas the majority of species within the Maculatus Group are found in the foothills, both *An. dravidicus* and *An. willmori* replace *An. maculatus* at submontane and montane altitudes, respectively. The maximum altitudinal distribution of *An. dravidicus* has been reported as 1820 m, in the submontane tracts of the Himalayas (Covell, 1927) but it has also been collected at lower altitudes; at 950 m in Myanmar, Pyin Oo Lwin District, and 450–500 m in Thailand, Mae Hong Son Province (K. Morgan, P. Somboon and C. Walton, unpublished data). *Anopheles willmori* generally has a higher altitudinal distribution having been recorded at 990–1475 m in northern Thailand (Rattanarithikul et al., 2006), 2500 m in the Himalayas (Covell, 1927), 3170 m in Nepal (Darsie and Pradhan, 1990) and 1800 m in Bhutan (P. Somboon, unpublished data).

3.5. Phylogeography of An. annularis

The mtDNA phylogeny (Fig. 2) shows that intraspecific divergence in *An. annularis* was high relative to other taxa. A separate phylogeographic study of this species was therefore carried out

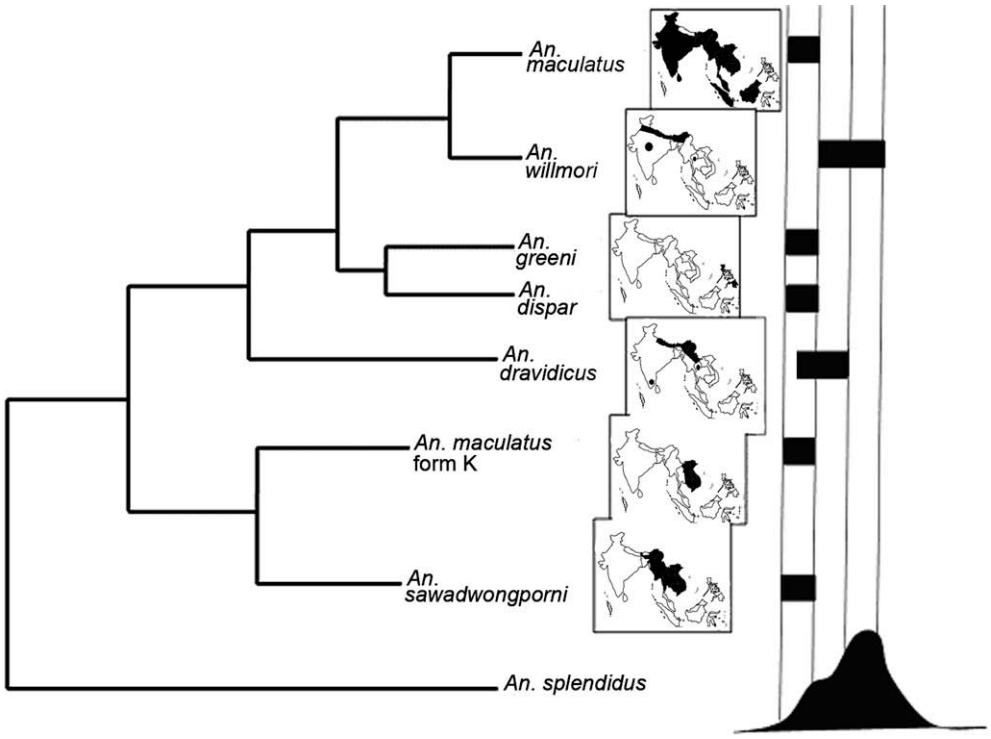

Fig. 5. The distribution of each species within the Maculatus Group clade plotted against phylogenetic position. The altitudinal range across which each species can be found (foothill, submontane or montane) is also shown next to each species. Distributions and altitudinal ranges are approximate.

to determine if there has been allopatric fragmentation, and to infer the likelihood of speciation. A total of 115 individuals across the species' distributional range were sequenced for the mitochondrial CO2 gene, and several individuals within each mitochondrial haplogroup were sequenced for the ribosomal ITS2 region. In both the median joining haplotype network and the statistical parsimony nested cladogram, haplotypes within *An. annularis* cluster into two highly divergent lineages separated by 14 mutations (Fig. 6). Both of these lineages (1 and 2) are further divided into two divergent clades (a and b). Clade 1a is made up primarily of haplotypes derived from the eastern mainland of Southeast Asia, specifically Vietnam, Cambodia and Laos, but also includes haplotypes from northwestern Thailand, and the north and southwest of Myanmar. Clade 1b comprises mainly haplotypes from northern Myanmar, as well as some from northwestern Thailand and central and westerly Myanmar. Within the second more western distributed lineage, clade 2a contains haplotypes derived from northeastern India, Sri Lanka and western and central Myanmar, whereas clade 2b contains only specimens from northeastern India and Sri Lanka. The *An. annularis* individual from the Philippines was highly divergent from all other haplotypes in the network, differing from the most closely related haplotype by 17 mutations (Fig. 6). The NCPA inferred a pattern of contiguous range expansion in the eastern clade 1a, with restricted gene flow and some long distance dispersal in the western clade 2b. Allopatric fragmentation was inferred to have influenced the population history of the western lineage (clade 2). Due to the high divergence between the eastern and western lineages a conclusive outcome could not be reached for the total cladogram. However, the considerable divergence between all four clades and their distinctive geographical composition clearly indicates allopatric fragmentation, with the deepest, and therefore probably oldest or most persistent, separation being on the east and west of the mainland. Since individuals sampled from the same site frequently have haplotypes from several different clades it indicates that, on the mainland, populations have remixed following allopatric fragmentation.

4. Discussion

4.1. Systematics

The ribosomal DNA dataset had limited use for resolving deeper phylogenetic relationships due to the difficulty of aligning sequences from more distantly related taxa, and was much more useful for resolving relationships within clades. Overall, the tree topologies obtained using a variety of phylogenetic methods and from both mitochondrial and nuclear markers were in good agreement, indicating that the gene trees presented here are a reliable indication of species relationships.

There are several differences between the implied taxonomic relationships of the morphological-based classification (Harbach, 2004), and the molecular phylogenies presented here. Notably, the Neocellia Series is not monophyletic. Indeed, four of the 21 species sampled in this study, *An. karwari, An. ainshamsi, An. paltrinierii* and *An. maculipalpis*, were not placed within the ingroup of the Neocellia Series clade based on rDNA and (where available) mtDNA data. Our results support the suggestion of Foley et al. (1998) that the Neocellia Series is paraphyletic, which was based on their

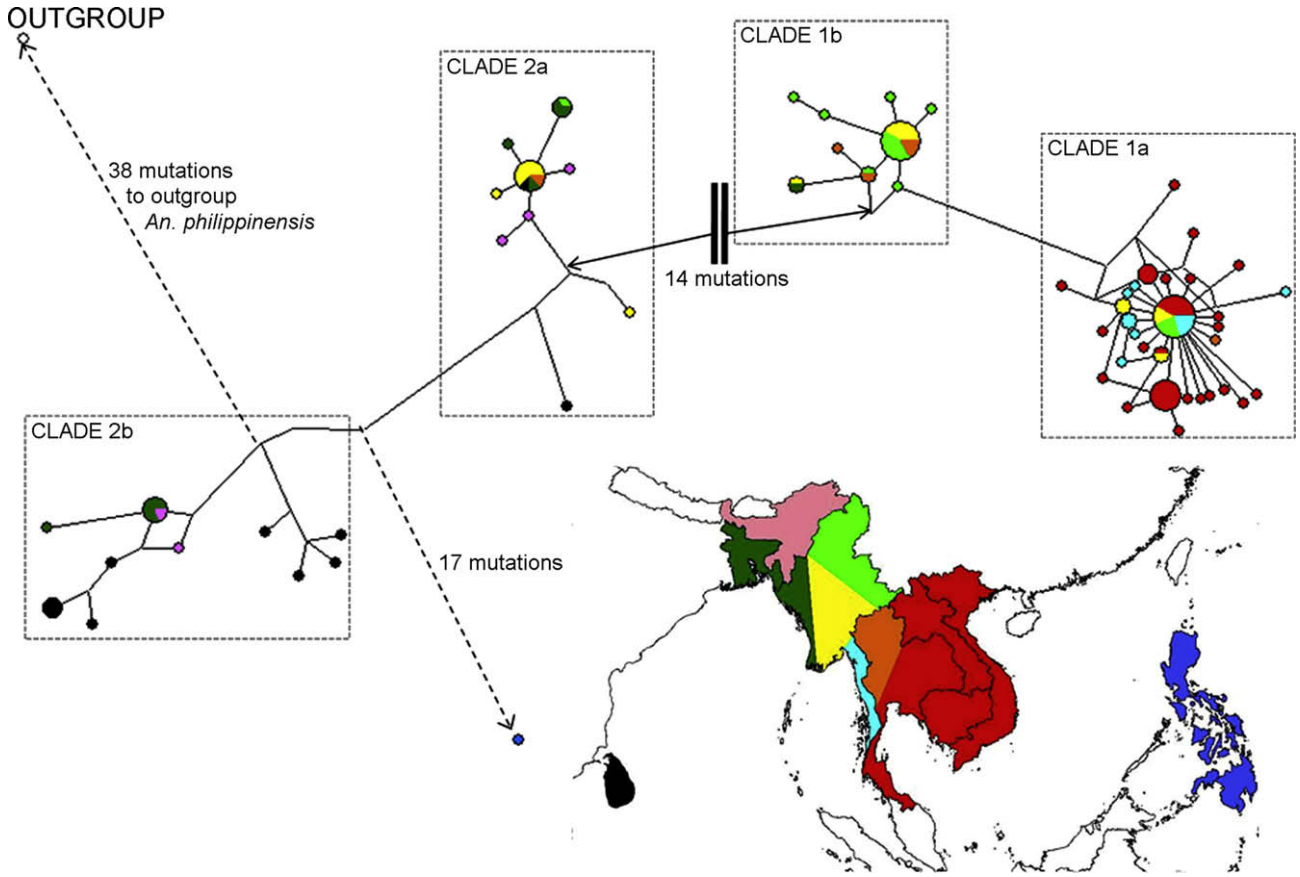

Fig. 6. Median joining haplotype network for *An. annularis*. Circles represent unique haplotypes with the diameter proportional to haplotype frequency. Unless otherwise indicated, branch lengths are proportional to the number of mutations separating haplotypes. Unsampled intermediate haplotypes are not shown. The colour of each haplotype represents the sampling location, as indicated on the map.

mtDNA molecular phylogeny of Australasian *Anopheles* species in which two species of the Neocellia Series, *An. karwari* and *An. annularis*, fell into different clades. The apparent grouping of *An. karwari* with the outgroup species, *An. dirus*, is likely to be an artifact due to long-branch attraction (Bergsten, 2005). This relationship may therefore be removed by wider taxon sampling including the putative members of the Neocellia Series we were unable to obtain. Even if increased taxon sampling resulted in the inclusion of *An. karwari* in the monophyletic main clade (Fig. 2) the conclusion that the Series is paraphyletic would be unchanged as *An. ainshamsi* and the other African taxa sampled, *An. paltrinierii* and *An. maculipalpis*, are highly divergent. These results indicate that further molecular phylogenetic analyses are required to fully test morphological classifications within the genus *Anopheles*.

The Maculatus, Annularis and Jamesii Groups listed in Harbach (2004) receive mixed support from the molecular data. All classified Maculatus Group species fell into a monophyletic clade, with the exception of *An. pseudowillmori*, which appears to be distantly related to the rest of the Maculatus Group. The grouping of Maculatus Group species into two distinct subgroup clades was supported, with the Maculatus Subgroup containing the currently unplaced *An. willmori, An. greeni* and *An. dispar* as well as *An. maculatus* and *An. dravidicus*, and the Sawadwongporni Subgroup containing *An. maculatus* species K in addition to *An. sawadwongporni*. The findings of Ma et al. (2006), based on phylogenetic analysis of ITS2 and D3 sequences of five Maculatus Group species, agree largely with our grouping structure but differ in not placing *An. willmori* within either subgroup. The grouping of *An. sawadwongporni* with *An. maculatus* species K concurs with their morphological similarity and the results of cross mating experiments (Thongwat et al., 2008). Although the Annularis Group was supported by the molecular phylogeny, the clade also included the currently unplaced *An. pulcherrimus*, as has also been suggested by Ma et al. (2006). The molecular phylogeny did not support the Jamesii Group; the grouping of *An. jamesii* and *An. pseudojamesi* in a monophyletic clade received moderate support but the inclusion of *An. splendidus* within this clade was not supported. The unsampled putative Neocellia Series taxa, particularly *An. schueffneri* and *An. notanandai*, which are currently classified in the Annularis and Maculatus Groups, respectively, could affect the extent of support for distinct Annularis and Maculatus and Group clades. However, the unsampled taxa will not have any major effect on the dating and locations of speciation events within these groups, so the biogeographical inferences (see below) are expected to be robust.

Anopheles karwari from Sri Lanka is very likely to be a distinct species from that present on the mainland, based on the high level of divergence observed in both mtDNA and rDNA, which dates to a similar time (the late Pliocene) as the most closely related species of the Maculatus Group. It has been suggested that *An. annularis* is a complex of at least two sibling species (Rattanarithikul et al., 2006) and a considerable level of east–west divergence was noted in *An. annularis*, and also in *An. splendidus*. However, the lack of corresponding divergence in their rDNA indicates that allopatric fragmentation in these species has most likely not resulted in speciation as the rDNA appears to have largely homogenised, suggesting ongoing gene flow.

The Mosquito Barcoding Initiative has selected the mitochondrial gene CO1 as the marker for species identification (Y. Linton, personal communication). All species within this study could be differentiated from one another based on their CO2 and ND5 sequences indicating that mtDNA has the resolving power to distinguish species. However, large levels of intraspecific divergence were detected within several species, including *An. annularis* and *An. splendidus*. The possibility of such intraspecific divergence should be taken into consideration when using mtDNA as a barcoding marker, as it could lead to the erroneous identification of individuals from divergent intraspecific lineages as separate species.

4.2. Biogeography

The majority of speciation events within the Annularis Group occurred during the early and mid Pliocene, predating the onset of the Pleistocene glaciations by more than a million years. The climate during this period was warm and humid (Chandler et al., 1994), so tropical forest is likely to have been widespread across Southeast Asia and larval habitats abundant. This may have increased niche availability and facilitated the spread of the Annularis Group, the distribution of which extends from Southwest to Southeast Asia. With the exception of *An. pulcherrimus* in Southwest Asia and India (Glick, 1992), most species in the Annularis Group are found across the Oriental Region and do not exhibit allopatric distributions that might indicate allopatric speciation (Walton et al., 2007a). However, this signal often erodes over time due to dispersal (Barraclough and Nee, 2001). This could well be the case in the Annularis Group, as suitable habitat is available across Southeast Asia, the dispersal capacity of the mosquitoes is high (Reid, 1968; Scanlon et al., 1968) and there has been substantial time since speciation. In the Annularis Group we are therefore unable to distinguish between the alternative hypotheses of allopatric speciation and sympatric ecological speciation, particularly as we have very little data on the ecology of each species within the group.

Speciation of *An. jamesii* and *An. pseudojamesi*, *An. nivipes* and *An. philippinensis*, and within the Maculatus Group, is estimated to have occurred during the mid to late Pliocene. This period also appears to have been an important time for diversification in other forest-dependent taxa across mainland and insular Southeast Asia; within the forest-dependent *Lophura* gallopheasants (Randi et al., 2001), and in the Southeast Asian Black-browed Barbets (Feinstein et al., 2007). The driving force for this diversification was suggested to have been increasing forest fragmentation, which is likely to have been associated with Pliocene climatic change (Ravelo et al., 2004). The late Pliocene was characterised by a large global climatic transition that began 2.8 mya, before which global temperatures were an estimated 3 °C higher than those today, precipitation levels were high and consequently tropical forest cover across Southeast Asia would have been extensive (Chandler et al., 1994; Cronin et al., 1994). The climatic transition was followed by a period of rapidly decreasing temperatures and increasing aridity, during which tropical forest is likely to have been increasingly replaced with grassland and savannah (Willis et al., 1999; Ravelo et al., 2004). The dating of speciation to this period in the Maculatus Group, and the tendency of the species to be distributed somewhat allopatrically (Fig. 5), is consistent with allopatric speciation triggered by isolation in fragmented forest refugia. In particular, the species of the Sawadwongporni Subgroup have more easterly distributions whereas the basal taxon in the Maculatus Subgroup, *An. dravidicus*, has a more westerly distribution (Fig. 5) indicating an early east–west split. The clearer signal of allopatric distribution in the Maculatus Group than the Annularis Group may indicate a greater importance of allopatric speciation in these more forest-dependent species. Alternatively, there may simply have been less opportunity for the signal of allopatric speciation to be eroded in the Maculatus Group.

The molecular phylogeny indicates that *An. greeni* and *An. dispar* speciated during the late Pliocene. The unique biodiversity of the Philippines is attributed partly to the fact that this is the only major island region in Southeast Asia that remained entirely disconnected from the mainland throughout the Pleistocene (Sodhi

et al., 2004). Although there are currently sea barriers between the Philippines and the mainland, dispersal of an ancestral form may have been facilitated during the late Pliocene if sea levels were lower, as during the Pleistocene glacial maxima, decreasing the distance between the Philippines and Borneo (Voris, 2000). This is in accord with the suggestion that another *Anopheles* species, *An. flavirostris*, dispersed from Borneo to the Philippines at periods of lower sea level (Foley and Torres, 2006).

There is also evidence that ecological adaptation has played a role in driving diversification of the Maculatus Group. Whereas the majority of Neocellia species are found at relatively broad altitudinal ranges and lay their eggs in stagnant water, all species within the Maculatus Group are found in forested foothill or mountainous regions and lay their eggs primarily near to running water such as along the margins of streams, or in the rocky pools associated with streams (Reid, 1968; Rattanarithikul et al., 2006). The radiation of the Maculatus Group may therefore have been in response to the opening up of a new ecological niche, as has been observed in other taxa (Schluter, 1996; Rainey and Travisano, 1998). Since ecological conditions, particularly larval habitat, are likely to vary with altitude, the altitudinal replacement in the Maculatus Group may be the result of ecological speciation, with populations at differing altitudes becoming adapted to the ecological conditions predominant at each altitudinal zone (Moritz et al., 2000). There is also some suggestion of altitudinal replacement in the Philippines species with *An. dispar* being more common at higher altitudes than *An. greeni* (Rattanarithikul and Harbach, 1990). Finally, it seems very likely that seasonality has pressured ecological adaptation and influenced divergence within the Maculatus Group; mosquito collections made in the Mae Hong Song Province of northwestern Thailand throughout 2001 and 2002 revealed a higher prevalence of *An. maculatus* during the dry season and of *An. sawadwongporni* during the wet season ($\chi^2 = 44.35$, $p < 0.0001$; total number of mosquitoes = 176 (P. Somboon, C. Walton, unpublished data)).

All Pleistocene-dated divergence events occurred within species indicating that, although this time period has not been important for speciation in the Neocellia Series, the Pleistocene glaciations have shaped genetic diversity within species. Although (with the exception of *An. annularis*) sample sizes within species are limited, some general patterns of geographical divergence emerged. Firstly, divergent, and sometimes basal, haplotypes were found in Sri Lanka (*An. maculatus* and *An. karwari*), northeastern India (*An. philippinensis*) and northern Myanmar (*An. maculatus*), suggesting that these western regions may have contained forest refugia during Pleistocene glacial periods. This concurs with previous suggestions based on zoogeographic and systematic evidence in the case of Sri Lanka (Brandon-Jones, 1996). Furthermore, both northeastern India and northern Myanmar are mountainous (Fig. 1), and therefore likely to have retained pockets of forest habitat at intermediate elevations throughout the Pleistocene (Brandon-Jones, 1996; Gathorne-Hardy et al., 2002; O'Loughlin et al., 2008). The second pattern of intraspecific divergence detected (in *An. annularis* and *An. splendidus*) was that of east–west divergence centred approximately on the Thai–Myanmar border. This suggests that at least one refugium existed in the east of Southeast Asia, as well as the proposed refugial regions in the west. These processes of allopatric fragmentation within species inferred from both the phylogenetic and phylogeographic data may well reflect those that have contributed to allopatric speciation in earlier time periods.

5. Conclusions

There are several discrepancies between the molecular phylogeny and the current classification of the Neocellia Series, indicating that the classification may require revision. Although Pleistocene environmental fluctuation appears to have influenced intraspecific population history, driving allopatric diversification during periods of habitat fragmentation, it has not triggered speciation in the Neocellia Series. Instead, all speciation dates to the Pliocene but could have been influenced by climatic shifts that occurred at this earlier time. Even if allopatric fragmentation has played some role in speciation in the Maculatus Group, ecological adaptation, following a shift into a new ecological niche and involving altitudinal replacement and seasonality, appears to have played an important role in driving divergence. The importance of both pre-Pleistocene allopatric divergence and ecological adaptation in driving speciation within *Anopheles* mosquitoes, and of forest fragmentation driven by Pleistocene climatic change in driving intraspecific divergence, is likely to be mirrored in other forest-dependent taxa across the region. Studies of such taxa are needed to determine which of the above processes have played major roles in the generation of species richness and intraspecific diversity across the region.

Acknowledgments

We thank the staff at the Office of Disease Prevention and Control in Chiang Mai, Thailand, the Centers of Malariology, Parasitology and Entomology in Vientiane, Laos (under the direction of Dr. Samlane Phompida), and Phnom Penh, Cambodia (under the direction of Dr. Duong Socheat), the Department of Medical Research in Myanmar, Gauhati University, and the Malaria Research Centers in Assam and Bangladesh, for all their invaluable help in mosquito collection. David Robertson and James Eales, University of Manchester, provided much appreciated help and advice regarding the phylogenetic analysis. We thank the anonymous reviewers for their helpful comments and suggestions. This work was funded by a NERC studentship to Katy Morgan with the Natural History Museum as a CASE partner.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ympev.2009.01.022.

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