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MOLECULAR SYSTEMATICS AND DIVERGENCE ESTIMATES OF PAKISTANI MOSQUITOES IN GENUS *ANOPHELES*

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ABSTRACT

The present study explored the phylogenetic utility of mt-ND5 gene sequences for mosquitoes in genus *Anopheles*. Phylogenetic tree employing the Maximum Parsimony methods in PAUP strongly supported the traditional taxonomy of genus. Subgenus *Anopheles* represented by a single species i.e. *Anopheles nigerrimus* initially separated out from the rest with a strong bootstrap value of 100. Subgenus *Cellia* representing bulk of Pakistani mosquitoes showed a monophyletic lineage (bootstrap value: 97). Using two fossil calibration points (both at 34 Mya) mtDNA divergence times were estimated within a sympatric group of Anophelines of Pakistan. The divergence time between Subgenera *Anopheles* and *Cellia* was estimated at approximately 6 million years ago i.e. between mid to late Pliocene. Series Neocellia (*Anopheles splendidus, Anopheles stephensi, Anopheles annularis, Anopheles pulcherrimus*) diverged some 5.7 million years ago from Myzomyia (*Anopheles culicifacies* and *Anopheles fluviatilis*), while speciation within the respective series took place during the Pleistocene about 0.7 million years ago. The present study, shows Pliocene as the starting point for mosquito evolution while Pleistocene as the point of diversification. The ongoing climate change, uplifting of Himalaya, evolution of a monsoon system, rising and falling of the sea level are perhaps those features of Pleistocene that might have led to the process of speciation in genus *Anopheles* of Pakistan.

Keywords: malaria, Pakistan, *Anopheles*, Pleistocene.

INTRODUCTION

Anopheline systematics has reached its zenith in classical morphology some years ago and is now dominated by molecular genetics research (Harbach, 2004). DNA based studies of this genus have mainly focused on the intra/interspecific identification, phylogenies and divergence dates for various species and complexes (Collins and Paskewitz, 1996; Foley *et al*., 1998; Krzywinski *et al*., 2001 a and b; Chen *et al*., 2004; Donnelly *et al*., 2004; Foley *et al*., 2007; Morgan *et al*., 2009). Despite the global advances in molecular systematics of the group; such studies are still lagging behind in Pakistan.

Pakistan (mainly Khyber Pakhtunkhwa and Baluchistan) has hosted millions of Afghans during the past few decades. The admittance and repatriation of these refugees has been linked to the changing disease patterns, especially malaria (Kazmi and Pandit, 2001). It is a disease that flourishes in conditions of crisis and population displacement for many reasons: the breakdown of health services and control pro-grammes; the displacement or concentration of non-immune refugees in malaria-risk areas; malnourishment within displaced populations; the siting of camps on marginal land prone to vector breeding; problems in gaining access or supplying medicine to displaced populations; and the lack of protective shelter and other man± vector barriers (Rowland, 2001). Malaria is regionally listed as a high priority health problem by the World Health Organization. In Pakistan two species are recognized as vectors i.e. *Anopheles stephensi* and *Anopheles culicifacies* (Mahmood *et al*., 1984) while many others are suspected vectors (Suleman, 1993).

In this perspective, genetic studies of Anopheline mosquitoes greatly deserve attention in Pakistan. Here we present, for the first time, baseline data on molecular systematics of seven Anopheline mosquitoes from Pakistan. The objectives are to determine (1) whether significant sequence heterogeneity exists within the mt-ND5, mtDNA sequences for biological species identification (2) to construct an Anopheline phylogeny for Pakistani mosquitoes (3) and to assess rate of speciation and divergence of a sympatric group of species from Pakistan. Fossil constraints are used to estimate divergence of taxa based on molecular data.

MATERIALS AND METHODS

Sample species: The present study analyzed mitochondrial sequences of seven Anopheline species from Pakistan. The species were exclusively eusynanthropic and came from yearlong collections (2007) in three villages of district Charssada (34-03' and 34º-28'N and 71º-28' and 71º-53'E), Khyber Pakhtunkhwa, Pakistan. The study sites within the district were selected for their high prevalence of malaria. The

specimens, all adults and preserved in silica gel, were kept at the department of Zoology (University of Peshawar, Pakistan). About 22 mosquito specimens belonging to seven species were selected and sequenced for the present study.

Sequences generated DE novo: DNA extractions from whole adult mosquitoes were carried out using the phenol chloroform method. In a 1.5ml eppendorf tube each mosquito specimen was ground with a pestle following the addition of 300µl of TENS buffer and 6µl of proteinase- K. The samples were incubated at 37^{O}C overnight. Afterwards, 400µl of phenol/chloroform/isoamyl alcohol (in ratio 25:24:1) were mixed in each sample and centrifugation was performed at 13000 rpm for 10 minutes. The upper phase was pipetted out. About 30µl of chloroform/isoamyl alcohol was added again to the sample which was then centrifuged (13000 rpm for 2 minutes). The upper aqueous phase was removed and 1ml of 100% ethanol was added in the aqueous solution. Sample was stored at -20ºC for 1-hr and then centrifuged (13000 rpm for 15 minutes) resulting in precipitation of DNA in a pellet form at the bottom of the tube. The supernatant was removed and the precipitate was washed. The pellet was suspended in molecular biology grade water/D2H2O. Neat stocked DNA were stored at 4ºC for short term use and at -20ºC for intermediate periods (Sambrook *et al.*, 1989). Primers reported previously i.e. ND5, 5'-GGHYTAACTGT WWGTTATTCATTTC-3' (Kyrzwensky *et al*., 2001b) were utilized in order to amplify the mitochondrially encoded NADH dehydrogenase 5 (mt-ND5) gene segment. Thermal cycling was performed in Mastercycler (Eppendorf). Each PCR amplicon had a total volume of 50µl. For amplification, 39 cycles consisting of 1 minute at 94ºC for denaturation, 1 minute at 51ºC for annealing and 2 minutes at 72 ºC for extension were performed, followed by 3 minutes at 72 ºC for final extension. Sequencing was performed using external services (Macrogen Inc., South Korea). Sequences generated during the present study were deposited in the Genbank with Accession Nos. JX985561-JX985583 as listed in Table 2.

Chromas Pro 34-Version 1.33 was used for manual edition of sequences while Clustal X was used for alignment and comparison of sequences.

Phylogenetic Analysis: Each mt-ND5 sequence constituted 500 nucleotide base pairs. Gaps were treated as missing data in all analysis. Parsimony analyses (MP) were implemented in PAUP4.0b10 (Swofford 2002) using the heuristic search option with Tree Bisection-Reconnection (TBR) branch-swapping. MP topologies were evaluated by bootstrap method with 1000 replicates (TBR) of 100 random stepwise addition replicates each. Parsimony-uninformative characters were excluded. A Majority rule consensus tree was thus obtained.

The software BEAST 1.4 (Drummond and Rambaut, 2007) was used to calculate a time line for Anopheline evolution. Modeltestv 3.7 (Posada and Crandall, 1998) suggested the general time reversible (GTR) + I + Γ model of evolution. *Culex pipiens* was used as outgroup. The root age prior between Genus *Anopheles* and outgroup *C. pipiens* was set according to fossil-based minimum ages: *Culex winchesteri*, 34 Ma; *Anopheles dominicanus*, 34 Ma (Reidenbach *et al*., 2009) respectively. One tree was saved every 100 cycle for a total of 1.1×10^6 cycles. The first 1000 cycles were discarded as burn-in. The final trees are displayed in results.

RESULTS

Molecular systematics of Pakistani Anophelines

Species identification: Mitochondrially encoded mt-ND5 gene segment was used during the present study. We also amplified COI and COII for some species, though were unsuccessful in the case of others. Thus for the phylogenetic analysis of *Anopheles* species only mt-ND5 was used. For this purpose two year old specimens preserved in silica gel were successfully amplified and sequenced. Sequence variation accommodated identification of Pakistani taxa (Figure 1).

Phylogenetic relationships: The 50% majority rule consensus tree employing the Maximum Parsimony methods in PAUP strongly supported the traditional taxonomy of genus *Anopheles* (Figure 1).

Subgenus *Anopheles* represented by a single species i.e. *Anopheles nigerrimus* initially separated out from the rest with a strong bootstrap value of 100. Subgenus *Cellia* representing bulk of Pakistani mosquitoes showed a monophyletic lineage (bootstrap value: 97).

Within Subgenus *Cellia* the series Myzomyia showed paraphyly by branching *A. culicifacies* along with series Neocellia (bootstrap value: 78.7) though the clade *culicifacies* branched distinctly from those taxa representing Neocellia (89.7).

At a bootstrap value of 89.6, the Pakistani Neocellia formed a monophyletic clade showing four distinct lineages i.e. *An. Pulcherrimus An. Annularis An. Stephensi and An. Splendidus*.

Divergence estimates and speciation: Using two fossil calibration points (both at 34 Mya) we estimated mtDNA divergence times within a sympatric group of Anophelines from Pakistan. The divergence time between Subgenera *Anopheles* and *Cellia* was estimated at approximately 6 million years ago i.e. between mid to late Pliocene. Series Neocellia (*An splendidus, An stephensi, An annularis, An pulcherrimus*) diverged from Myzomyia (*An. Culicifacies* and *An fluviatilis*) some 5.7

million years ago, while Species diversification took place during the Pleistocene about 0.7 million years ago for almost all species (Figure 2). The complex *annularis* diverged about 4 million years ago.

Table 1. Brief taxonomy of species investigated analyzed during the present study.

Subgenus Anopheles	***Subgenus Cellia***	
Series Myzorhynchus (Edwards 1932)	*Series Myzomyia*	*Series Neocellia* (Christophers 1924)
Anopheles nigerrimus Giles 1900	*Anopheles culicifacies* Giles 1901 *Anopheles fluviatilis* James 1902	*Anopheles annularis* van der Wulp 1884 *Anopheles pulcherrimus* Theobald 1902 *Anopheles splendidus* Koidzumi 1920 *Anopheles stephensi* Liston 1901

Table 2. Genebank accession numbers for the ND5 Anopheline sequences reported from Pakistan.

Species	Accession Numbers
Anopheles annularis 1	JX985561
*Anopheles culicifacies*1	JX985562
*Anopheles culicifacies*2	JX985563
*Anopheles fluviatilis*1	JX985564
*Anopheles fluviatilis*2	JX985565
*Anopheles fluviatilis*3	JX985566
*Anopheles nigerrimus*1	JX985567
*Anopheles nigerrimus*2	JX985568
*Anopheles nigerrimus*3	JX985569
*Anopheles nigerrimus*4	JX985570
Anopheles pulcherrimus 1	JX985571
Anopheles pulcherrimus 2	JX985573
Anopheles pulcherrimus 3	JX985574
*Anopheles splendidus*1	JX985575
*Anopheles splendidus*2	JX985576
*Anopheles splendidus*3	JX985577
*Anopheles stephensi*1	JX985578
*Anopheles stephensi*2	JX985579
*Anopheles stephensi*3	JX985580
*Anopheles stephensi*4	JX985581
*Anopheles stephensi*5	JX985582
*Anopheles stephensi*6	JX985583

Source locality: Charssada District, Pakistan 34◦-03'N & 71◦-53'E; Area: 996 km2 /385 sq mi.

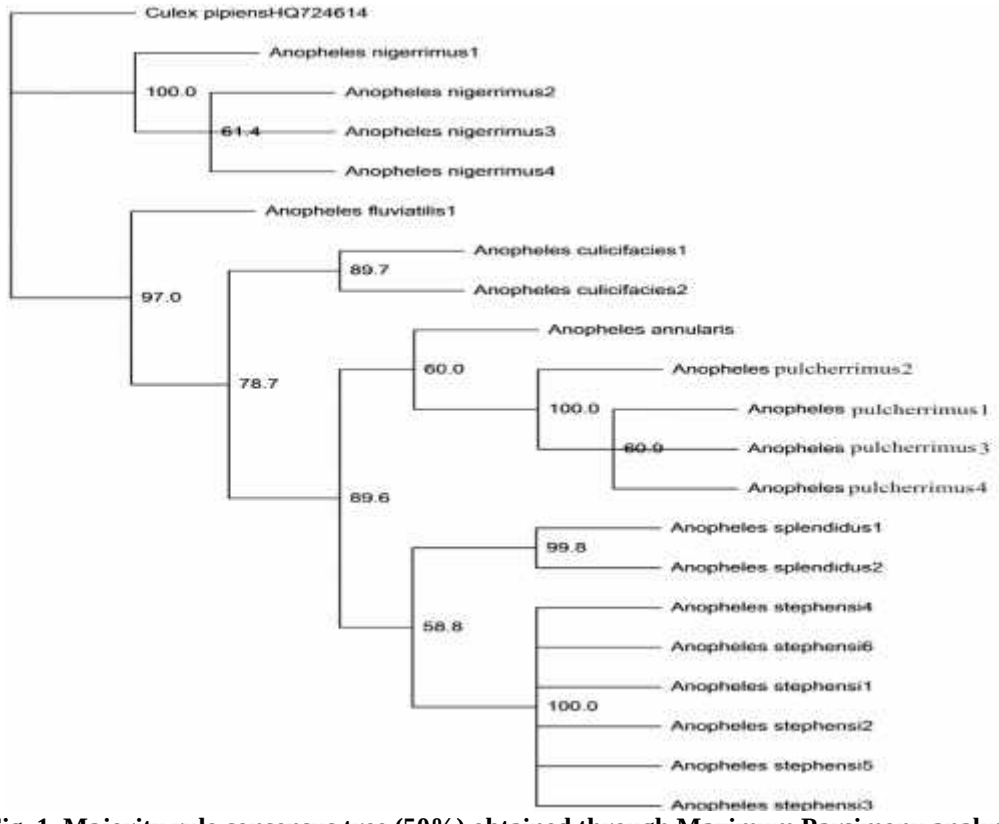

Fig. 1. Majority rule consensus tree (50%) obtained through Maximum Parsimony analysis

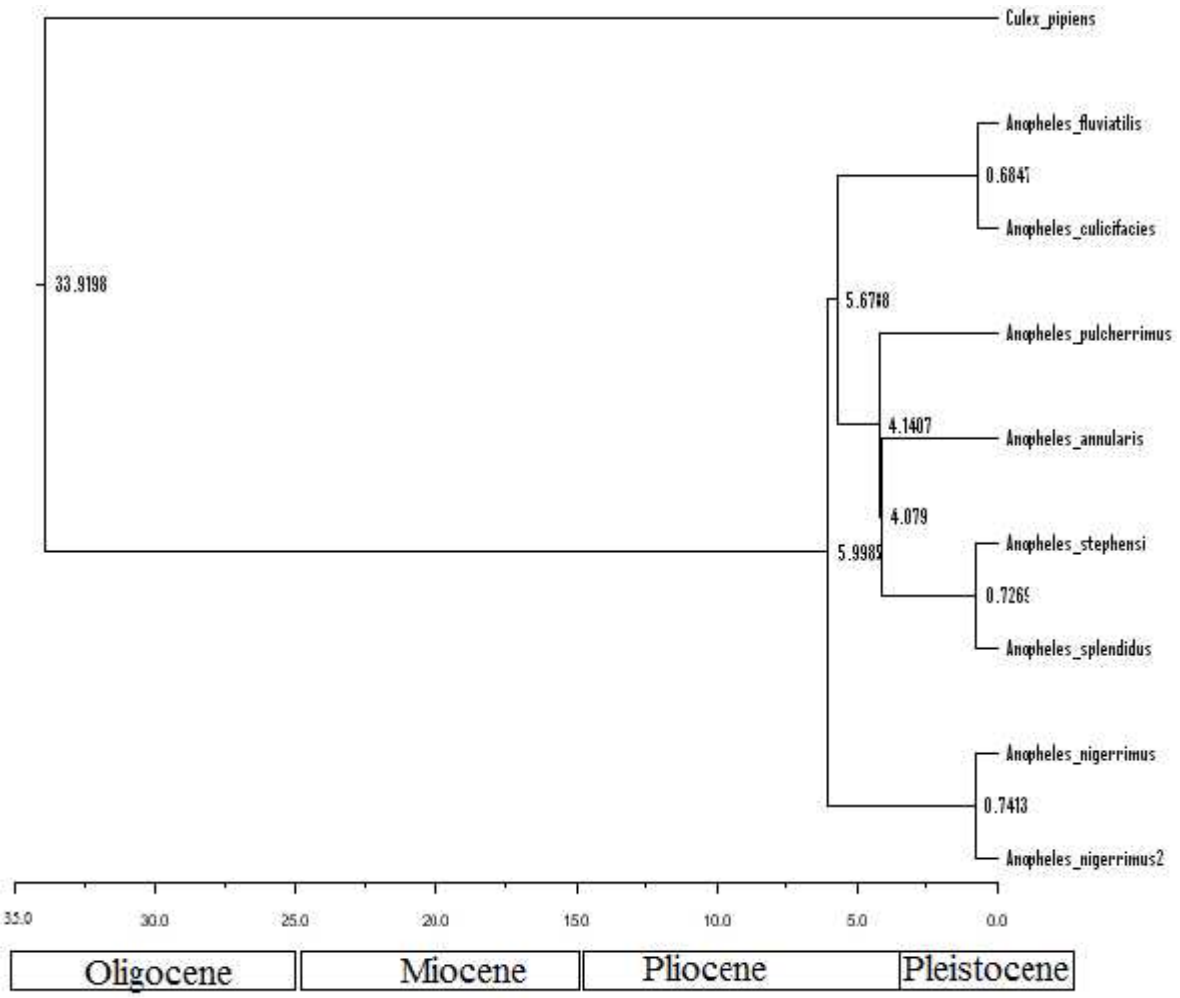

Fig. 2. Chronogram for Anopheline species of Pakistan.

DISCUSSIONS

Anopheline systematic: Anopheline mosquitoes (Culicidae, Anophelinae) are of prime medical importance as human malaria vectors, yet their phylogeny is poorly known (Krzywinski *et al*., 2001b). *Anopheles* with 437 species worldwide is classified into six subgenera: the cosmopolitan *Anopheles*, the Old World *Cellia*, and the Neotropical *Kerteszia*, *Nyssorhynchus*, *Lophopodornyia* and *Stethornyia* (Krzywinski *et al*., 2001b, Harbach 2004). In Pakistan there are 24 anophelines species (Mehmood *et al*., 1984) with 11 in Khyber Pakhtunkhwa province i.e., *A. stephensi* and *A. culicifacies, A. splendidus, A. pulcherrimus, A. annularis, A. maculatus, A. nigerrimus, A. subpictus, A. superpictus, A. fluviatilis* and *A. tarkhudi* (Suleman, 1993). Most of the species belong to subgenus *Cellia*. The present study investigated molecular systematics of seven of these species (Table 1). Mt-ND5 gene segment was employed for this purpose. Mt-ND5 has previously been successfully utilized for resolving taxonomic problems within genus *Anopheles* as it provides adequate variation for identification and phylogenetic reconstruction (Besansky *et al*., 1997; Lehman *et al*., 1997; Krzywinski *et al*., 2001a; Donnelly *et al*., 2004; Morgan *et al*., 2009).

Subgenus *Cellia* representing bulk of Pakistani mosquitoes showed a monophyletic lineage (bootstrap value: 97). The results are in accordance to current phylogenetic scheme for Anopheline relationships as presented by Harbach, 2004 and other studies (Sallum *et al*., 2000).

Series Myzomyia showed paraphyly for our results according to which *A. culicifacies* (Myzomyia; funestus group) originated from the same ancestral stock as rest of Neocellia (Figure 1). In classical morphological taxonomy, phenotypic overlap is known to exist between members of the funestus group of Myzomyia and the annularis group of the Neocellia series making it difficult to identify them as groups (Swain *et al*., 2010). Our results however showed that despite the paraphyly of Myzomyia series; *A. culicifacies* (funestus) was itself a monophyletic lineage and mt-ND5 facilitated the

identification of funestus and annularis groups (Figure 1). As for Series Neocellia monophyly was obvious in the results which are similar to a study carried out in neighboring India (Swain *et al*., 2010). On contrary the phenomenon of paraphyly has been well documented in Neocellia throughout the globe (Pape, 1992; Foley *et al*., 1998; Ma *et al*., 2011).

The refuge model of Anopheline speciation: The abounding biodiversity of Orient has been attributed to habitat fragmentation and creation of refuges over time (Haffer, 1969). The refuge model thus states that the repeated expansion and contraction of tropical forests in response to climatic fluctuation resulted in allopatric divergence and eventual speciation within many taxa including mosquitoes (Morgan *et al*., 2009). The question is when did it happen? Many studies have indicated Pliocene to be the time when major Anopheline speciation events took place in the world (Donnelly *et al*., 2004; Morgan *et al*., 2009). The present study, however, shows Pliocene as the starting point while Pleistocene as the point of diversification for the genus *Anopheles.*

It has previously been asserted that Pleistocene with its changing climate might have favored radiation of species yet evolutionary role of Pleistocene has remained doubtful (Morgan *et al*., 2009). Quite the opposite Pleistocene in Pakistan was a suitable candidate for mosquito evolution. Following factors might have played an important role in this regard.

Changing Climate: The changing climate of Pleistocene favored mosquito evolution (Morgan *et al*., 2009). Alternation of cool glacial periods with the warm interglacial periods (Hills and Hills, 2005) perhaps gave way to refuges that acted as hotspot zones where speciation ran rampant.

Rising and Falling Sea levels: Pleistocene was the time when sea levels were continuously rising and falling in South East Asia (Heaney, 1991). The phenomenon perhaps further assisted in the creation of refuges on the Indian Subcontinent.

Evolution of Monsoon and phased uplift of Himalaya: Since antiquity the climates of Asia are affected significantly by the extent and height of the Himalaya (Kutzbach *et al*., 1993; Kutzbach *et al*., 1997). Uplift of this region began about 50Myr ago. Significant increases in altitude occurred in the mid Pliocene i.e., some 10-8 million years ago (Molnar *et al.*, 1993, Harrison *et al*., 1992). The formation of Himalaya is correlated with evolution of the Monsoon system. Three stages are recognized in this perspective; the enhanced aridity and onset of the Indian and east Asian monsoons in Pliocene; continued intensification of Summer and Winter Monsoons during early Pleistocene; and increased variability and possible weakening of the Indian and east Asian summer monsoons and continued strengthening of the east Asian winter monsoon since about 2.6Myr ago (Zhisheng *et al*., 2001).

Today Monsoons are known to have a profound impact on natality/mortality of Anopheline species. In the arid Punjab for example rainfall facilitates increased breeding and lifespan of the malarial vectors (Bouma and Kaay, 1996).

The historical development of a Monsoon system since the late Pliocene through the Pleistocene supports the refuge model of *Anopheles* speciation in Pakistan.

Changing Habitats: Reconstruction of Pleistocene environment in the last glacial maximum suggests major habitat replacements in South Asia (Morgan *et al*., 2009). Thus "moist" thick forests continuously interchanged with "arid" grasslands and Savannahs (Heaney, 1991; Hope *et al*., 2004; White *et al*., 2004; Morgan *et al*., 2009). Similar changes were occurring in vegetation i.e. from C3 (forests) to C4 (grasses) in Pakistan beginning about 8Myr ago (from mid Pliocene onwards) (Cerling *et al*., 1997). As mosquitoes are very much sensitive to habitat nature, such events might have inevitably led to spatial and temporal isolation of mosquito populations which ultimately gave rise to new species.

Conclusions: The present study demonstrated the utility of mitochondrial mt-ND5 sequences in phylogenetic inferences of Anopheline species. It also showed a recent speciation for Pakistani mosquitoes, about 0.7 million years ago, which might be due to the major climatic and geological changes that occurred in Pakistan during the Plesitocene. These events such as development of monsoon system, uplifting of himalaya, glaciation etc might had played an important role in evolution of various taxa including mosquitoes.

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