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Genetic differentiation in the yellow fever virus vector, *Aedes simpsoni* complex, in Africa: Sequence variation in the ribosomal DNA internal transcribed spacers of anthropophilic and non-anthropophilic populations

L. G. Mukwaya,[1] J. K. Kayondo,[1] M. B. Crabtree,[2] H. M. Savage,[2] B. J. Biggerstaff[2] and B. R. Miller[2]

[1]*Department of Entomology, Uganda Virus Research Institute, Entebbe, and* [2]*Division of Vector-Borne Infectious Diseases, National Center for Infectious Diseases, Centers for Disease Control and Prevention, Public Health Service, US Department of Health and Human Services, Fort Collins, CO, USA*

Abstract

Mosquitoes of the *Aedes simpsoni* complex are important vectors of yellow fever virus in Africa. We examined the ribosomal DNA sequence divergence in the internal transcribed spacer regions (ITS-1 and ITS-2) for populations of mosquitoes that were determined to be anthropophilic or non-anthropophilic in their bloodmeal host preference. A neighbour-joining tree produced two clades: one contained all of the individual mosquitoes from anthropophilic populations and the other contained all of the individual mosquitoes from non-anthropophilic populations. There was no segregation of the taxa within each of the two clades based on geographical origin. The data suggest the exisťtence of two distinct species of *Ae. simpsoni* s.l. in Uganda that correlates with their host blood-feeding preference. The current taxonomic status of the complex is discussed in relation to these findings.

Keywords: *Aedes simpsoni*, *Aedes bromeliae*, *Aedes lilii*, ribosomal DNA, yellow fever, anthropophily.

Introduction

The *Aedes (Stg.) simpsoni* complex currently comprises three described species. *Aedes simpsoni* (*sensu stricto*) occurs only in South Africa and Zimbabwe and is not involved in the transmission of yellow fever virus. The anthropophilic species, *Ae. bromeliae,* originally described as *Ae. simpsoni* (Haddow, 1945, 1968), is common throughout the Afrotropical region and is considered the principal vector of yellow fever virus in Central and East Africa. *Aedes lilii* occurs throughout Africa but is less common than *Ae. bromeliae* and does not blood-feed on humans (Huang, 1979, 1986). Because of the morphological similarity of members of the group and the considerable range of anthropophily, the taxonomic status within the complex has been controversial (Huang, 1986; Lutwama & Mukwaya, 1994; Jupp & Kemp, 1999). A diagnostic, illustrated key has been published that differentiates the three species within the *Ae. simpsoni* complex, based on pale scale ornamentation of the fore and mid tarsomeres and the presence or absence of a tooth on the mid-tarsal claws (Huang & Ward, 1981). Recently, workers in Uganda and South Africa have called into question the absolute diagnostic quality of these morphological characters (Lutwama & Mukwaya, 1994; Jupp & Kemp, 1999).

The correct species determination of members of the *Ae. simpsoni* complex is critical because of the importance of anthropophilic members (*Ae. bromeliae*) in the transmission of yellow fever virus to non-human primates and humans. This species was incriminated as the principal vector in the Ethiopian yellow fever epidemic of 1960–62, where an estimated 30,000 deaths occurred (Haddow, 1968).

The purpose of the study reported here was to examine the sequence diversity in the internal transcribed spacers (ITS-1 and ITS-2) of the ribosomal DNA (rDNA) array from geographical populations of anthropophilic (*Ae. bromeliae*) and non-anthropophilic (*Ae. lilii*) members of the *Ae. simpsoni* complex from Uganda, Nigeria and Kenya. These non-coding, nuclear sequences have proved useful in differentiating closely related members of species complexes within the mosquito family, Culicidae (Porter & Collins, 1991; Wesson *et al*., 1992; Paskewitz *et al*., 1993; Fritz *et al*., 1994; Crabtree *et al*., 1995; Miller *et al*., 1996).

Received 3 August 1999; accepted 7 September 1999. Correspondence: Barry R. Miller, Division of Vector-Borne Infectious Diseases, Centers for Disease Control, PO Box 2087, Fort Collins, CO, 80522, USA. Tel.: (970) 221–6413; fax: (970) 221–6476; e-mail: brm4@cdc.gov

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Table 1. Mosquitoes used in this study.

Population origin	Latitude/longitude	A/NA[a]	Individual designation	Clone number	GenBank accession no.
Bwamba, Uganda	0.8N, 30.13E	A	Bwamba 1	59–1	AF158194
			Bwamba 2	60–4	AF158193
			Bwamba 3	63–1	AF158192
			Bwamba 4	68–1	AF158191
			Bwamba 5	74–2	AF158190
			Bwamba 6	79–1	AF158189
			Bwamba 7	89–1	AF158188
			Bwamba 8	99–1	AF158187
			Bwamba 9	101–1	AF158232
Nkokonjeru, Uganda	0.233N, 32.917E	A	Nkokonjeru 1	210–3	AF158223
			Nkokonjeru 2	212–3	AF158222
			Nkokonjeru 3	215–4	AF158221
			Nkokonjeru 4	216–1	AF158220
			Nkokonjeru 5	217–2	AF158219
			Nkokonjeru 6	220–1	AF158218
Bussi Island, Uganda	0.03N, 32.4E	A	Bussi Island 1	436–2	AF158199
			Bussi Island 2	437–3	AF158198
			Bussi Island 3	438–3	AF158197
			Bussi Island 4	440–3	AF158196
			Bussi Island 5	441–3	AF158195
Taveta, Kenya	3.39S, 37.664E	A	Taveta 1	349–4	AF158211
			Taveta 2	350–3	AF158210
			Taveta 3	351–3	AF158209
			Taveta 4	352–2	AF158208
			Taveta 5	353–2	AF158207
			Taveta 6	355–2	AF158206
Entebbe, Uganda	0.067N, 32.467E	NA	Entebbe 1	149–4	AF158231
			Entebbe 2	154–2	AF158230
			Entebbe 3	157–1	AF158229
			Entebbe 4	162–1	AF158228
			Entebbe 5	164–1	AF158227
			Entebbe 6	169–1	AF158226
			Entebbe 7	176–1	AF158225
			Entebbe 8	180–4	AF158224
Mbale, Uganda	1.079N, 34.171E	NA	Mbale 1	398–3	AF158205
			Mbale 2	399–4	AF158204
			Mbale 3	400–2	AF158203
			Mbale 4	401–2	AF158202
			Mbale 5	402–2	AF158201
			Mbale 6	404–1	AF158200
Enugu, Nigeria	6.45N, 7.45E	NA	Nigeria 1	287–2	AF158217
			Nigeria 2	300–2	AF158216
			Nigeria 3	301–2	AF158215
			Nigeria 4	302–3	AF158214
			Nigeria 5	303–1	AF158213
			Nigeria 6	307–3	AF158212

[a]A, anthropophilic; NA, non-anthropophilic.

Results

Table 1 lists the population origin, anthropophily, clone number, and GenBank accession number of the ITS sequences of the forty-six individual mosquitoes examined in this study. Table 2 lists the guanine–cytosine (GC) content (%) and the length in nucleotides of the ITS-1 and ITS-2 regions. The ITS-1 was GC rich for all populations and the length of the region varied from 353 to 414 nucleotides due primarily to deletion/insertion events. The length in nucleotides of the ITS-2 varied from 201 to 212; interestingly, the GC content of the anthropophilic populations was slightly less (48.1–49%) than that of the non-anthropophilic populations (50.0–50.5%) (Table 2).

An edited nucleotide alignment for the combined ITS-1 and ITS-2 rDNA sequences was constructed that contained 642 sites; forty-four sites (6.8%) were variable and eighteen sites were phylogenetically informative. A neighbour-joining (NJ) tree produced from estimates of Kimura's two-parameter distances of the forty-six individual mosquitoes examined is given in Fig. 1. This NJ tree was generated without outgroup designation and shows

Table 2. Guanine–cytosine (GC) content and length of internal transcribed spacer (ITS) sequences analysed; *n* = number of individuals sequenced.

	ITS-1		ITS-2	
Population (*n*)	GC content (%)	Length (nt)	GC content (%)	Length (nt)
Bwamba, Uganda (9)	57.4–58.7	365–380	48.6–49.0	204–208
Nkokonjeru, Uganda (6)	56.1–57.6	353–372	48.1–49.0	204–212
Bussi Island, Uganda (5)	57.1–58.4	364–375	48.6–49.0	208
Taveta, Kenya (6)	57.2–57.8	363–372	48.1–48.6	207–208
Entebbe, Uganda (8)	57.0–58.2	397–405	50.3–50.5	201–206
Mbale, Uganda (6)	57.6–58.1	394–414	50.3–50.5	201–206
Nigeria (6)	56.6–58.4	394–410	50.0–50.3	201–206

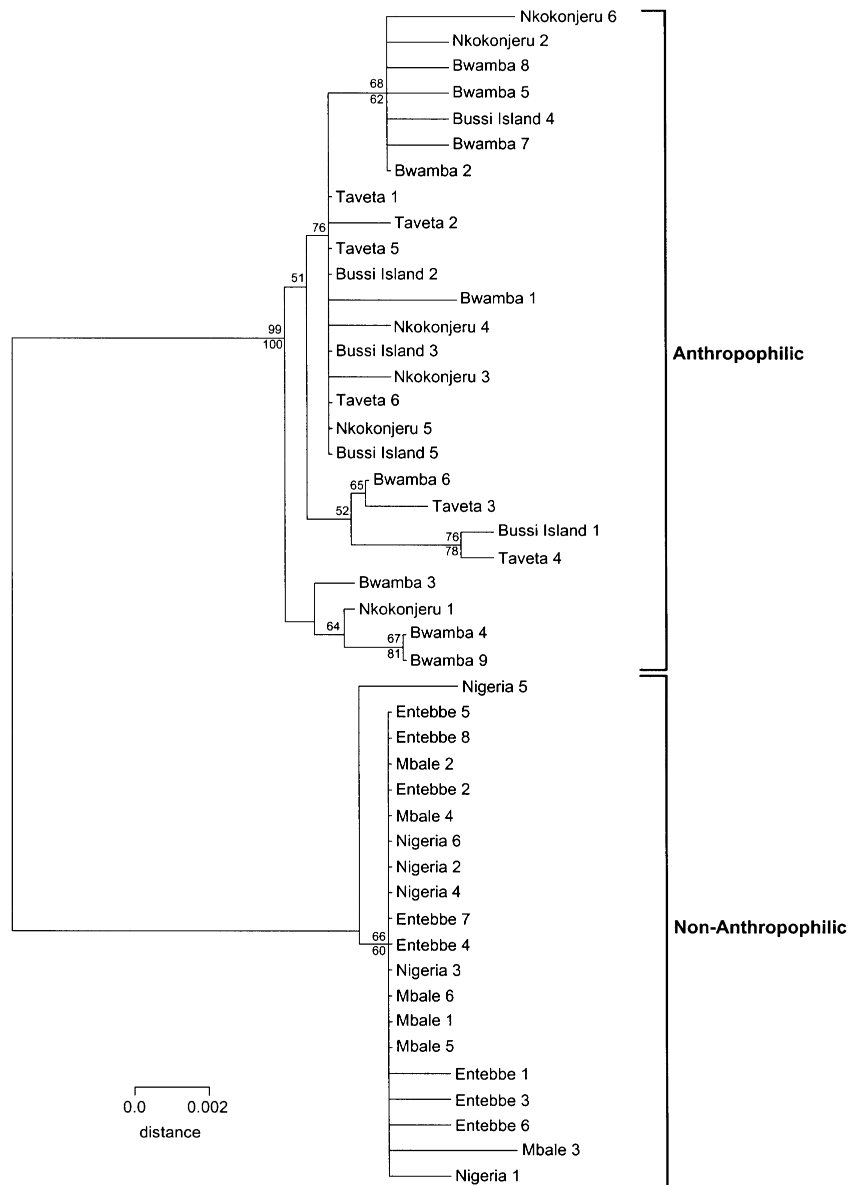

Figure 1. Phylogenetic tree based on rDNA ITS-1 and ITS-2 sequence data (642 sites) for forty-six individual mosquitoes of the *Aedes simpsoni* complex. Host blood-feeding preferences are indicated on the right side of the figure. The unrooted tree was constructed using neighbouring-joining with the Kimura two-parameter distance calculation (scale bar). The confidence probability value is given above the branch tested and the bootstrap confidence level (500 replications) is shown below the branch for values above 50%.

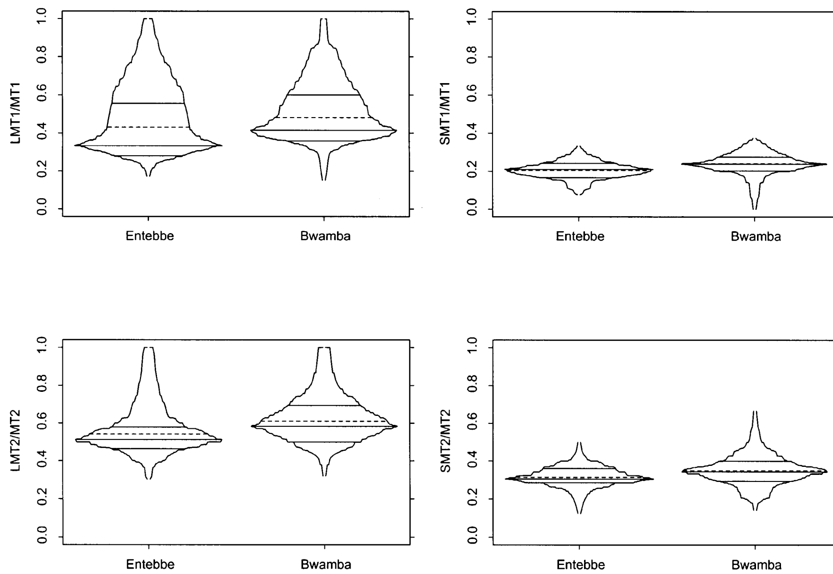

Figure 2. Box-percentile plots comparing mid-tarsi pale band ratios for adult female mosquitoes from Bwamba, an anthropophilic population, with females from Entebbe, a non-anthropophilic population. The solid line located at maximum width corresponds to the median, while the solid lines located below and above the median line represent the 25th and 75th percentiles, respectively. The dashed line indicates the sample mean. The length of each pale band was expressed as a ratio of the respective tarsus length and abbreviated as follows: SMT1/MT1, shortest length of pale band on MT1/length of MT1; LMT1/MT1, longest length of pale band on MT1/length of MT1; SMT2, shortest length of pale band on MT2/length of MT2; and LMT2/MT2, longest length of pale band on MT2/length of MT2.

two major clades. There is strict segregation of populations into each clade; for instance, all of the individuals from Taveta, Kenya, are in the upper clade, while all the individuals from Entebbe, Uganda, are in the lower clade. Importantly, the two major clades also segregate based on their members blood-feeding preference for humans. However, within each major clade there is absolutely no segregation of individuals from a geographical population. For example, in the anthropophilic clade, individuals from different geographical populations form minor clades (Bussi Island, Uganda, and Taveta, Kenya) while in the non-anthropophilic clade, individual Nigeria 5 forms a sister relationship to all the remaining individuals from Nigeria, Entebbe, Uganda, and Mbale, Uganda.

Morphological examination of 103 individuals from a population representing each major clade (anthropophilic Bwamba and non-anthropophilic Entebbe) showed that the observed ratios for all four pale bands overlapped widely and displayed similar distributions (Fig. 2). Although clear distinctions between the populations were not evident, the means and medians for each of the four ratios were greater in the Bwamba population than the Entebbe population. Thus, the portion of the tarsi covered by pale scales on MT1 and MT2 was longer among females of the Bwamba population. The observed population medians for each of the four pale band ratios were significantly different (for each test Kruskal–Wallis $\chi^2 > 10.70$, $P < 0.01$).

Discussion

The taxonomy and identification of many mosquito taxa are problematic because of the high degree of morphological similarity and/or the variance in certain diagnostic characters; the *Ae. simpsoni* complex is no exception. Theobald (1905, 1910, 1915) originally described three species, and these were subsequently synonymized by Edwards (1912), who later (1941) recognized two forms or variants (see Lutwama & Mukwaya, 1994, for a complete taxonomic synopsis). Finally, Huang (1979, 1986) resurrected three species in the complex, and Huang & Ward (1981) provided a group of key characters for separating these species.

In this study, comparison of the ratios of the length of pale bands to the length of mid-tarsi 1 and 2 for two populations of *Ae. simpsoni s.l.* from Uganda demonstrated different host preferences and ITS sequences consistent with a comprehensive study that compared the length of pale bands in a larger number of anthropophilic and non-anthropophilic populations from East Africa (Lutwama & Mukwaya, 1994). The range of observed ratio values overlapped extensively, thus excluding the use of these characters as diagnostic tools to separate anthropophilic (*Ae. bromeliae*) and non-anthropophilic (*Ae. lilii*) individuals and populations. However, the medians for each of the four ratios were significantly larger in the Bwamba or anthropophilic population than in the non-anthropophilic population from Entebbe (Fig. 2). Lutwama & Mukwaya (1994) analysed twelve populations and observed that means for pale band ratios were typically longer among anthropophilic populations, although one anthropophilic population had mean values typical of non-anthropophilic populations. The data suggest that the loci controlling host preference and leg banding pattern are weakly associated. Nonetheless, our current inability to correctly diagnose individual mosquitoes as *Ae. bromeliae*, an important vector of yellow fever virus, or *Ae. lilii*, a mosquito that does not

blood-feed on humans, is disturbing, as it compromises our ability to assess the risk of yellow fever virus transmission in East Africa.

The genetic basis of the host blood-feeding preference of *Ae. simpsoni* complex has been described in laboratory-based studies (Mukwaya, 1977) and the difference in behavioural host-feeding preferences has been noted by workers conducting studies in the field (Gibbons, 1942; Gillett, 1951, 1955; Mattingly, 1952; Mukwaya, 1974). Further, work by Mukwaya (1974) in Uganda has shown that the non-anthropophilic form feeds preferentially on rodents. Differences in blood-feeding preferences have been noted in other mosquito taxa that have been investigated, particularly vectors of human malaria including *Anopheles gambiae* complex in the Gambia (Bryan *et al.*, 1987), *An. arabiensis* in Madagascar (Ralisoa Randrianasolo & Coluzzi, 1987), *An. vestitipennis* in Mexico (Arredonodo-Jimenez *et al.*, 1996) and *An. funestus* in Senegal (Lochouarn *et al.*, 1998). Although anthropophily in mosquitoes undoubtedly has a genetic basis, the underlying molecular mechanisms that direct this important behaviour are unknown.

In summary, analyses of ITS sequence data indicate the presence of two distinct taxa of the *Ae. simpsoni* complex in Uganda. Examination of single-copy loci from these taxa as well as *Ae. simpsoni sensu stricto*, would help to clarify their specific status. Based on morphological analysis and host-seeking behaviour, these are without doubt the entities previously described as *Ae. bromeliae* and *Ae. lilii*. Unfortunately, at present, no constellation of explicit morphological characters or molecular procedures exists to identify these species definitively.

Experimental procedures

Determination of blood-feeding behaviour

Table 1 lists the populations used in this study, their geographical location and human biting preference. Anthropophily was determined as described by Haddow *et al.* (1947). Essentially, we canvassed an area with abundant *Ae. simpsoni* complex immatures and adults, as measured by examination of larval habitats and adult collections by sweeping with nets. Yellow fever virus-immunized human bait was stationed in these areas, and biting mosquitoes were collected. If no females could be attracted to human bait, despite their abundance, the population was considered to be non-anthropophilic.

Field collections

Aedes simpsoni complex mosquito eggs were collected in Nigeria by using ovitraps provisioned with strips of cotton cloth that served as oviposition substrates. The traps were left in the field for 3–4 days, and the cotton cloth with attached eggs was dried and sent to Entebbe, Uganda, by courier. The eggs were induced to hatch, and the resulting larvae reared to adults.

Aedes simpsoni complex mosquito females were collected in Taveta, Kenya, from yellow fever virus-immunized human bait as they landed and were placed in liquid nitrogen.

In Uganda, populations of non-anthropophilic mosquitoes were collected as larvae and reared in the laboratory into adults and then frozen. On other occasions, resting, gravid females were captured with sweep nets and allowed to lay eggs in the laboratory and reared to adults. Anthropophilic populations were collected on human bait as above.

Morphological measurements

The length of basal pale bands on mid-tarsus 1 (MT1) and mid-tarsus 2 (MT2) of 103 adult female specimens from each of two populations, Bwamba (anthropophilic) and Entebbe (non-anthropophilic), were measured. A mid-leg was removed from each specimen examined and measured at 50× magnification with a dissecting microscope. Three measurements were made along the length of each tarsal segment: the shortest length of pale band – the length from the base of the tarsal segment to the shortest termination of the pale band; the longest length of pale band – the length from the base of the tarsal segment to the longest termination of the pale band; and the tarsal segment length – the length from the base to apex of the tarsal segment. The termination of the pale band was defined as the position at which pale scales were not present or interrupted by dark scales. Values from our study cannot easily be compared with those of previous studies in which the bands were measured either along the anterior or posterior surface. To avoid errors associated with orientation of the leg, we simply measured the pale band at both its longest and shortest extension. The length of each pale band was expressed as a ratio of the respective tarsus length and abbreviated as follows: SMT1/MT1, shortest length of pale band on MT1/length of MT1; LMT1/MT1, longest length of pale band on MT1/length of MT1; SMT2, shortest length of pale band on MT2/length of MT2; and LMT2/MT2, longest length of pale band on MT2/length of MT2.

Statistical and graphical analyses of morphological data

Ratio data for the Bwamba and Entebbe populations were compared graphically by using box-percentile plots (Fig. 2). The width of the irregular box-percentile plot is proportional to the percentage of observations that are more extreme in that direction. The solid line located at maximum width corresponds to the median, while the solid lines located below and above the median line represent the 25th and 75th percentiles, respectively. The dashed line indicates the sample mean. The non-parametric Kruskal–Wallis rank sum test was used to test separately for each ratio the null hypothesis, H_O = the observed population medians are equal.

DNA extraction, amplification, cloning and sequencing

Ribosomal DNA amplification and sequencing were carried out on selected individuals from each population listed in Table 1. Nine mosquitoes were selected from the Entebbe and Bwamba populations. Three individuals were selected that conformed most closely to Huang & Ward's diagnostic leg banding patterns (Huang & Ward, 1981) along with three individuals that did not conform and three individuals selected at random. For all other populations, five to six individuals were selected at random.

Individual mosquitoes were ground in 180 µl sterile phosphate-buffered saline (PBS) with microfuge pellet pestle grinders (Kontes,

Vineland, NJ). Genomic DNA was extracted with the use of the QIAamp Tissue Kit (QIAGEN, Inc., Chatsworth, CA) by using protocol B for insects, followed by the blood and body fluid protocol according to manufacturer's directions. The desired region of the rDNA gene group was amplified by PCR by using a modification of the procedure previously described by Crabtree *et al*. (1995). Each 100-µl reaction contained 15 ng genomic DNA, 50 mM KCl, 10 mM Tris–HCl, pH 8.3, 1.5 mg $MgCl_2$, 0.001% gelatin, 0.2 mM each dATP, dCTP, dGTP, dTTP (Roche Molecular Biochemicals, Indianapolis, IN), 0.16 µM each of the 18SFHIN and CP16 primers, and 2.5 units of Taq DNA polymerase (PE Applied Biosystems, Foster City, CA). The amplification program consisted of one cycle at 95°C, 4 min; thirty-five cycles of 95°C, 30s; 48°C, 30 s; 72°C, 2 min; and one cycle of 72°C, 4 min; 21°C, 1 min.

PCR products were either purified by using the QIAquick PCR Purification kit (QIAGEN) or by agarose gel electrophoresis, followed by extraction from the agarose by using the QIAquick Gel Extraction kit (QIAGEN). Purified DNA fragments were then cloned into either the pGEM-T or pGEM-T Easy vector (Promega, Madison, WI) as recommended by the manufacturer. One clone per individual was selected and sequenced as previously described (Miller *et al*., 1996).

Sequences have been submitted to GenBank (accession numbers listed in Table 1). With the use of PILEUP (Wisconsin Package, v. 9.0, Devereux *et al*., 1984), aligned sequences with gap creation and gap extension penalties were set at 3.0 and 0.1, respectively. The alignment was edited to remove ambiguously aligned regions. The combined ITS-1 and ITS-2 alignment used to produce the phylogram in Fig. 1 is available at the European Bioinformatics Institute Alignment Database and can be accessed by anonymous FTP from FTP@EBI.AC.UK in the directory/pub/databases/embl/align, or via the EBI Web page at: ftp://ftp.ebi.ac.uk/pub/databases/embl/align/.

Phylogenetic analysis of the edited alignment was accomplished by using the program MEGA (Molecular Evolutionary Genetics Analysis, v. 1.01, Kumar *et al*., 1993). NJ trees were generated by using the Jukes–Cantor and Kimura two-parameter distance-calculating algorithms, with sites containing alignment gaps excluded from the analysis. Confidence probabilities of the NJ trees produced in MEGA were calculated by using the standard error test for the interior branch lengths (Rzhetsky & Nei, 1992, 1993); statistical confidence was also evaluated by calculating the bootstrap confidence level, which gives the percentage of bootstrap trees in which the same interior branch as that of the original tree appears.

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