

Swimming out of Africa: mitochondrial DNA evidence for late Pliocene dispersal of a cichlid from Central Africa to the Levant

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The rich Levantine fauna and flora were shaped by millions of years of migration across the region, from Africa to Eurasia and vice versa. Most large-scale processes that led to this diversity have been relatively well studied. However, small-scale processes, and details such as the area of origin of particular groups, and the route and time of dispersal are often not as clear. This is the case with the endemic Levantine representatives of the fish family Cichlidae. In this work we combine genetic, palaeontological and geological data in an attempt to understand the dispersal of the cichlid fish *Astatotilapia flavijosephi* (Lortet, 1883) from sub-Saharan Africa to the Levant. *A. flavijosephi* is unique among the Levantine cichlids in being the only non-tilapiine. It is also the only haplochromine cichlid to be found out of Africa. A partial sequence of the control region of the mitochondrial DNA was used to determine *A. flavijosephi*'s phylogenetic relationships with other African haplochromines, and to estimate its time of divergence from this group. Combining our findings with palaeontological and geological data, we suggest that *A. flavijosephi* separated from the other haplochromines during the middle to late Pliocene (2.5–3.3 Mya) and probably dispersed from Africa to the Levant via the Nile. © 2004 The Linnean Society of London, *Biological Journal of the Linnean Society*, 2004, 82, 103–109.

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INTRODUCTION

The Levant has long been considered a bridge between three continents as it connects the Eurasian and African plates (Frankenberg, 1999). Millions of years of migration across this bridge have created relatively varied flora and fauna in the region. For example, Israel, which is situated in the southern Levant, harbours richer flora and fauna than other areas with a similar climate, despite its small area (Frankenberg, 1999). Whilst the large-scale processes that led to this variety have received considerable attention (e.g. Tchernov, 1979), important details such as the region of origin or the time and route of dispersal of the differ-

ent organisms, are often not well understood (Tchernov, 1992: 172). One such case, for which conflicting interpretations exist, is that of the cichlid fish.

The inland-water ichthyofauna of Israel comprises 32 indigenous species that represent families originating from Africa, Central Asia, Europe, the Levant, the Mediterranean and the Red Sea (Goren & Ortal, 1999). The family Cichlidae is represented in the Levant by six indigenous species, all of which are of African descent. Researchers agree that the arrival of these species represents at least two different migrations from Africa into the Levant (Tchernov, 1988; Por, 1989; Goren & Ortal, 1999). *Astatotilapia flavijosephi*, together with *Tristramella simonis* and *Tristramella sacra* that comprise a Levantine endemic genus, probably arrived in an earlier migration or migrations.

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Tilapia zilli, *Sarotherodon galileus* and *Oreochromis aureus*, all representing widely distributed species from the Nile, North and sub-Saharan Africa, are probably relatively recent immigrants (Tchernov, 1988; Por, 1989; Goren & Ortal, 1999). While researchers agree that the latter migrated through the Nile into the Israeli coastal rivers system (Tchernov, 1988; Por, 1989; Goren & Ortal, 1999), the route by which the earlier immigrants arrived and the time of their arrival are not as clear.

Based on palaeontological remains of several taxa from Levantine Tertiary sites, Tchernov (1988) concluded that an archaic Ethiopian stock had colonized the Jordan–Dead Sea Rift Valley during that era. Cichlids are known from the Levant from as early as the lower Miocene (Wadi Araba; Weiler, 1970). Later they appear in the upper Pliocene site of Erq-el-Ahmar and the early Pleistocene site of Ubeidiya (Tchernov, 1979, 1988). During this time span, Ethiopian elements could have migrated into the Levant when Africa and Arabia were still one geological unit (early Miocene), connected by land bridges (middle and late Miocene) or through hydrographic connections with Africa (Pliocene) (see also Murray, 2001). Later, during the early Pleistocene, Tchernov (1979, 1988) suggests, this archaic stock was cut off by the development of the Saharo-Arabian desert belt, often causing autochthonous speciation and thus creating several endemic species. In contrast, Por (1989) suggests that no hydrographic connections between Africa and the Jordan Rift Valley existed and therefore all aquatic Ethiopian species, including the ancestors of *A. flavii josephi*, *T. simonis* and *T. sacra*, migrated from Africa through the Nile into the Israeli coastal rivers system, and from there entered the Jordan River system through a previously existing link. The Nile in its current form probably did not cut through Egypt to get to the Mediterranean until the middle Pliocene (Rzóska, 1976; Said, 1981) and therefore, according to Por (1989), migration probably occurred only during the late Pliocene or Pleistocene.

As vertebrate fossil sites from the middle Miocene to the late Pliocene in the southern Levant are rare (see Tchernov, 1992), the addition of genetic data to existing geological and palaeontological records may shed more light on dispersion events that occurred during that period. The genetic data may provide a time limit for dispersion events and thus may help match them more precisely with known environmental and geological events, as well as with the palaeontological record.

Astatotilapia flavii josephi is unique as it is the only non-African haplochromine cichlid species. Furthermore, *A. flavii josephi* is currently disconnected hydrographically from all other haplochromine cichlids, as the Jordan Rift Valley, *A. flavii josephi*'s distribution

area, is not connected with any African watershed. Whilst some cichlid species may have migrated from the Nile, along the Mediterranean coast and into the coastal streams of Israel (Por, 1989), these streams are at present disconnected from the Jordan Rift Valley. There is also no southern connection from the Jordan River that could enable aquatic species to migrate from Africa and into the Jordan Rift Valley. Genetic comparison of *A. flavii josephi* and other African haplochromines may associate the former with certain haplochromine groups and hence to a more specific area of origin, and may also help to date the separation between *A. flavii josephi* and the African haplochromines. The objective of this study was to better understand the dispersal of *A. flavii josephi* from Africa to the Levant, by adding genetic data to the existing geological and palaeontological records.

METHODS

THE STUDY SPECIES AND SPECIMEN COLLECTION

Astatotilapia flavii josephi is an endemic species to Israel, inhabiting Lake Kinneret (the Sea of Galilee), the central part of the River Jordan and numerous pools, springs, streams and canals that are connected to the River Jordan along its central part (Goren & Ortal, 1999). It represents a widely distributed, species-rich African group. The individuals for this study were sampled from two different locations within the above range (Fig. 1). The fish were collected using hand nets, small seines and electroshocking. *Astatotilapia flavii josephi* (both males and females), like all other haplochromine cichlids, bear colourful egg-spots on their anal fin that easily distinguish them from the rest of the Levantine cichlid species, which are all tilapiines. Therefore, each cichlid that was collected and showed egg-spots on its anal fin was identified as *A. flavii josephi*. No individual was harmed during collection. Tissue for DNA extraction was obtained by clipping the posterior tip of the dorsal fin, and fish were subsequently released. The tissue was stored in 95% alcohol solution until DNA extraction was performed.

DNA PREPARATION, AMPLIFICATION AND SEQUENCING

The fin tissue was digested in 0.5 mL buffer B, supplemented with 0.5 mg proteinase K and 50 µL of 10% (w v⁻¹) sodium dodecyl sulphate (Dierkes, Taborsky & Kohler, 1999) for 90 min at 56°C. DNA was then purified using three extractions: first with an equal volume of phenol, then with phenol:chloroform:isoamyl alcohol (25:24:1), and finally with chloroform (Dierkes *et al.*, 1999). Following ethanol precipitation DNA was redissolved in 35 µL of double-distilled water.

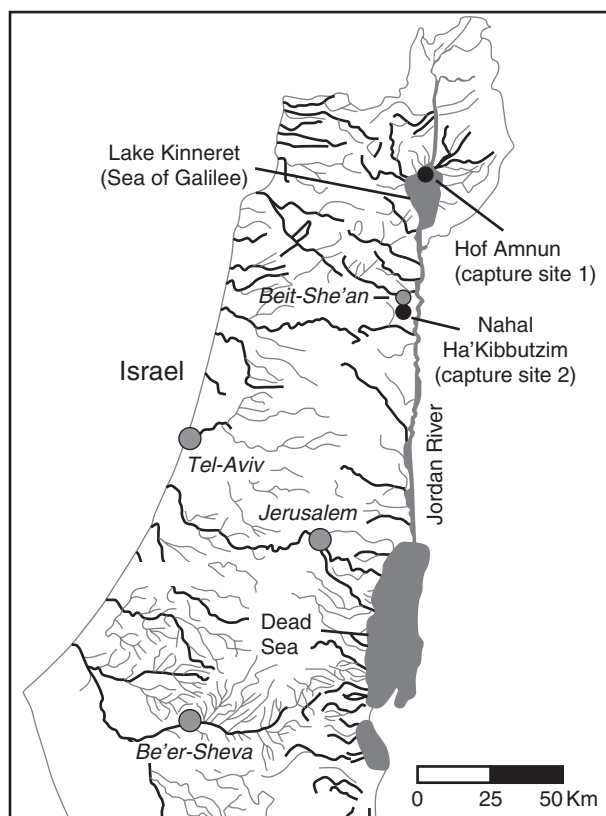


Figure 1. A schematic map of central and northern Israel including all historic and current watersheds. Major streams (permanent and seasonal) are marked by a darker colour and minor tributaries are marked by a lighter colour. *Astatotilapia flavijosephi* is endemic to Lake Kinneret, the central part of the Jordan River, and various streams and pools that drain to the Jordan River. The two sites where fish were collected for this study are marked.

Primers L15926 and H16498 (Meyer *et al.*, 1990) were used to amplify a portion of the mtDNA control region comparable to sequences obtained in previous studies (Sturmbauer & Meyer, 1992; Sturmbauer & Meyer, 1993; Sturmbauer, Verheyen & Meyer, 1994; Mayer, Tichy & Klein, 1998; Nagl *et al.*, 2001). L and H refer to light and heavy strands, respectively, and the numbers refer to the position of the 3' nucleotide of each primer in the human mtDNA sequence. Amplifications were performed in a total volume of 25 μ L containing 2 mM $MgCl_2$, 0.1 mM of each dNTP, 0.4 μ M of each primer, 2 μ L of template DNA solution and 1 unit of *Taq* polymerase. Amplification started with 2 min denaturation at 94°C, followed by 30 cycles of 45 s at 94°C (denaturation), 1 min at 55°C (annealing of primers) and 2 min at 72°C (polymerization), and ended with 5 min polymerization at 72°C. Sequencing of the amplified segments was performed using an ABI-377 automated sequencer.

PHYLOGENETIC ANALYSIS

In order to use the sequences obtained in this study for a phylogenetic reconstruction, we retrieved homologous sequences of additional African cichlids from the EBI database (European Bioinformatics Institute) (accession numbers and references are given in Fig. 2). These included several other *Astatotilapia* species from different regions of Africa as well as other haplochromines, and *Tropheus* sp. from Lake Tanganyika as an out-group to all haplochromines. Sequences were aligned using ClustalX (1.81) software (Thompson *et al.*, 1997). Phylogenetic reconstruction was carried out using maximum likelihood (ML) analysis, maximum parsimony and neighbour-joining through PAUP 4.0 software (Swofford, 1999). Additionally, a ML analysis was also executed through fastDNaml (1.0.6) to test for the significance of branch length (null hypothesis of zero length; 'global rearrangement' option invoked). For both types of ML, a transition/transversion ratio of 2 was calculated from the actual data. Five different methods were used to calculate coalescence time of sister groups, based on assumptions and coefficients suggested by previous studies (Parker & Kornfield, 1997; Nagl *et al.*, 2000; Sturmbauer *et al.*, 2001).

RESULTS

All sequences obtained in this study – three from the Sea of Galilee and one from Nahal Ha'Kibbutzim (Central Jordan River Rift Valley, Fig. 1) – proved identical. We therefore used a single representative for the phylogenetic analysis (EBI accession number AJ506160). Most other sequences that were retrieved from EBI for our phylogenetic analysis originated in the work of Nagl *et al.* (2000). Therefore, as would be expected, in our ML tree the haplochromine sequences (excluding Lake Malawi haplochromines) clustered into the seven haplogroups described by Nagl *et al.* (2000), numbering I–VII (Fig. 2). Other haplochromine sequences that originated in different studies also clustered into these haplogroups.

Astatotilapia flavijosephi, however, did not fit into any of the haplogroups mentioned above and instead was positioned in an independent group. *Astatotilapia flavijosephi* differs from all other haplochromines by an insertion of the nucleotide A at position 264 (numbering follows Nagl *et al.*, 2000; Fig. 2) and a substitution at position 15 (T instead of the common C). Our ML tree suggests that *A. flavijosephi* is a sister group to a monophyletic group that includes haplogroups I, III, VI and VII. These haplogroups share the nucleotide G in position 16, a substitution of the C that is found in the two flanking clades (i.e. *A. flavijosephi* and the haplogroups II and IV clade).

The five different methods that were used to calculate coalescence times for *A. flavijosephi* and its sister

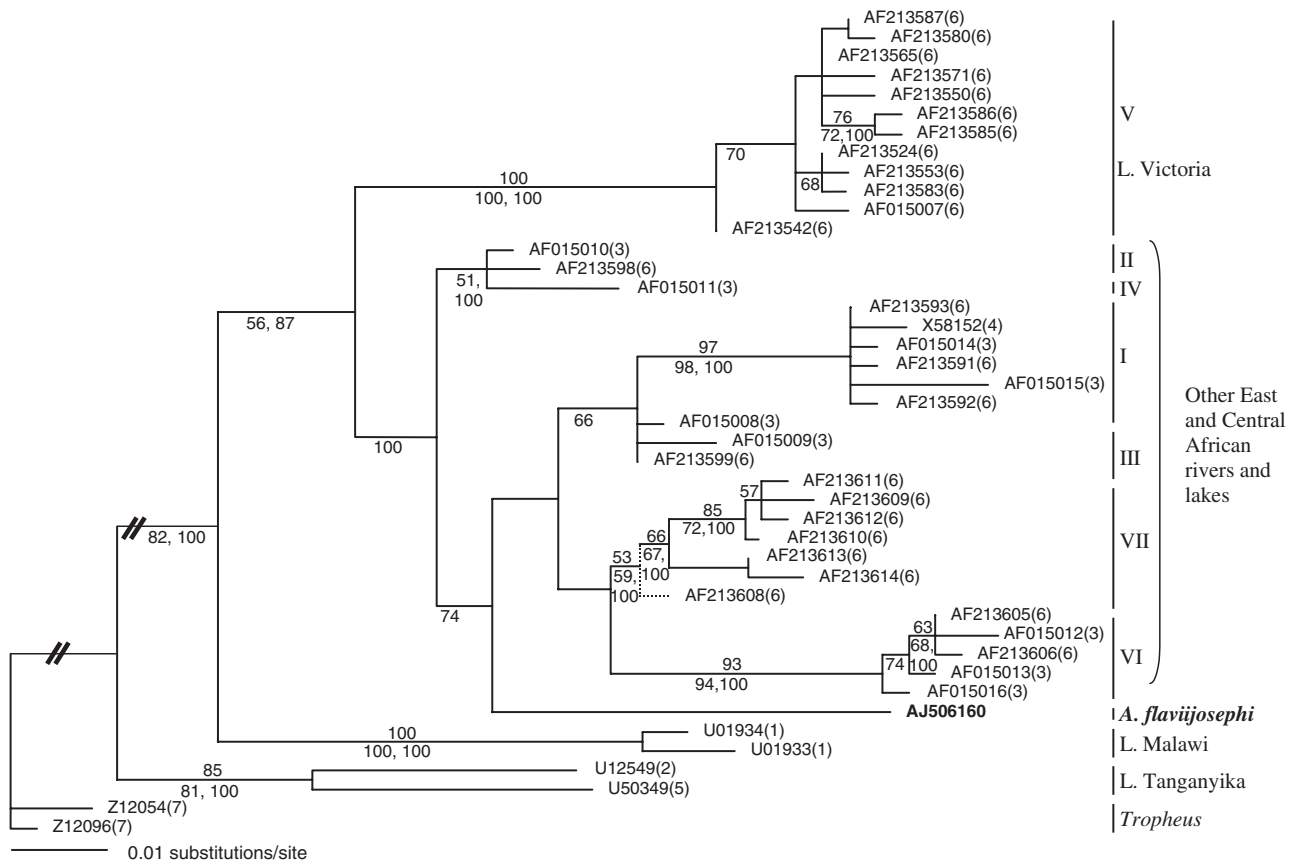


Figure 2. Phylogenetic reconstruction: the presented tree (topology and branch length, note scale bar) was obtained by a maximum likelihood (ML) analysis using PAUP 4.0 (Swofford, 1999), except for the dotted branch. This precise topology was supported by a neighbour joining analysis. All branches shown correspond to branches found significantly longer than zero by analysis with fastDNaml ($P < 0.01$; see Material and Methods). The two branches crossed by double lines were shortened by 0.01 substitutions site⁻¹ for graphic presentation purposes. The numbers above the branches indicate the percentage of 1000 bootstrap replications using ML analysis in which the group to the right of the value occurred. Numbers below the branches refer to a maximum parsimony analysis: the first and second number indicate the percentage of 1000 bootstrap replications and the percentage of all 3024 equally parsimonious trees in which the group to the right of the value occurred, respectively; only the latter is indicated where only one number appears; only values >50% are shown. Roman numerals and names on the right identify haplotype groupings, and indicate the location of *Astatotilapia flavijosephi*, cichlids from other lakes and the outgroup (*Tropheus* sp.). References are given by numbers in parentheses: (1) Bowers, Kocher & Stauffer (EBI entries only); (2) Lee *et al.* (1995); (3) Mayer *et al.* (1998); (4) Meyer, Kocher & Allen (1991); (5) Meyer, Knowles & Verheyen (1996); (6) Nagl *et al.* (2000); (7) Sturmbauer & Meyer (1992).

group yielded somewhat variable results. Using Parker and Kornfield's estimation of 1×10^{-8} substitutions base pair⁻¹ year⁻¹ (Parker & Kornfield, 1997) we obtained a coalescence time of 3.13 Mya. Nagl *et al.*'s (2000) calibration of Kimura's two-parameter model (Kimura, 1980) for the haplochromine control region gave us a coalescence time of 1.63–3.26 Mya. When using the same method with transversions only (Nagl *et al.*, 2000), we obtained a coalescence time of 1.33–2.66 Mya. The estimation offered by Nagl *et al.* (2000) for sequence divergence in the control region ($5.6\% \times 10^{-6}$ substitutions site⁻¹ year⁻¹) indicates a coalescence time of ~1.12 Mya. Finally, from the estima-

tion suggested by Sturmbauer *et al.* (2001) we obtained a coalescence time of 0.71–0.96 Mya.

DISCUSSION

AREA OF ORIGIN

Astatotilapia flavijosephi is positioned inside the haplochromine phylogenetic tree. By the time it diverged from the rest of the haplochromines, the separation between Lake Malawi and Lake Victoria haplochromines had occurred and several East African haplochromine haplogroups had also already diverged. Within the tree, *A. flavijosephi* forms a sister-group to

a monophyletic group that includes haplogroups I, III, VI and VII. These haplogroups include specimens that were captured in rivers and small lakes in the area between Lake Tanganyika and the East African coast, currently corresponding to northern Tanzania and the south-eastern corner of Kenya (Fig. 3). The sister group relationships between these haplogroups and *A. flavijosephi*, suggested by our ML tree (Fig. 2), may therefore indicate that this area is the area of origin of *A. flavijosephi*. Northern Tanzania is positioned almost at the bottom of both divisions of the African Rift Valley. The eastern and western divisions of the valley circle the Lake Victoria basin. At present the western division incorporates, from south to north, Lakes Tanganyika, Kivu, Edward, George and Albert. The eastern division incorporates Lake Malawi, a

series of small salt lakes and Lake Turkana and then cuts eastward through the Ethiopian plateau towards the Bab-el-Mandab straits (Fig. 3).

ROUTE AND TIME OF DISPERSAL

Coalescence time for *A. flavijosephi* and its sister group was estimated by several methods to range between 1.12 and 3.26 Mya or 0.71–0.96 Mya. This time span covers the middle Pliocene to middle Pleistocene. By the middle Pliocene the Saharo-Arabian desert belt was already developed and thus formed a major barrier for the dispersal of organisms from Central Africa northwards. Nevertheless, two possible routes were suggested for crossing the desert belt (Tchernov, 1992; Wood & Turner, 1995; Bar-Yosef & Belfer-Cohen, 2001). One route was a northward progression along the Nile valley, which at various times was connected to both divisions of the lower African Rift Valley (Fig. 3). The alternative route was by crossing the occasionally dry Bab-el-Mandab straits, from East Africa to Arabia, and progressing northward along Arabia's western shore. As the suggested area of origin of *A. flavijosephi* may have at one time been connected to both divisions of the Rift Valley, either division could have served as a migration route northwards.

The Bab-el-Mandab straits, together with the rest of the Red Sea, were flooded during the early Pliocene and remained so for the rest of the period (Tchernov, 1992). Following their flooding, however, sea level changes that occurred during the Pliocene and more frequently so during the Pleistocene (Haq, Hardenbol & Vail, 1987) sometimes exposed the relatively shallow straits, hence making them a possible dispersal route. Nonetheless, in order for aquatic organisms to reach the Levant via the latter route a freshwater link between Africa and Arabia would have had to exist when the straits were exposed and, furthermore, a continuous, or at least semicontinuous, freshwater link between the south-western tip of Arabia and the Levant was needed to enable their northward migration. As the Saharo-Arabian desert belt was well developed by the late Pliocene (Rea, 1994) we suggest that such a link was unlikely, making the Bab-el-Mandab straits a less probable migration route for *A. flavijosephi*. Support for our suggestion comes from the fact that since the Oligocene there is no evidence for the presence of cichlids in the Arabian Peninsula (Roberts, 1975; Murray, 2001). In contrast to the straits, the Nile, when flowing, formed a continuous freshwater link that crossed the Sahara and reached the Levant (see Introduction) and therefore enabled Central African aquatic elements to disperse northwards. In a recent study Seehausen *et al.* (2003) used nuclear markers, rather than mtDNA, to reveal

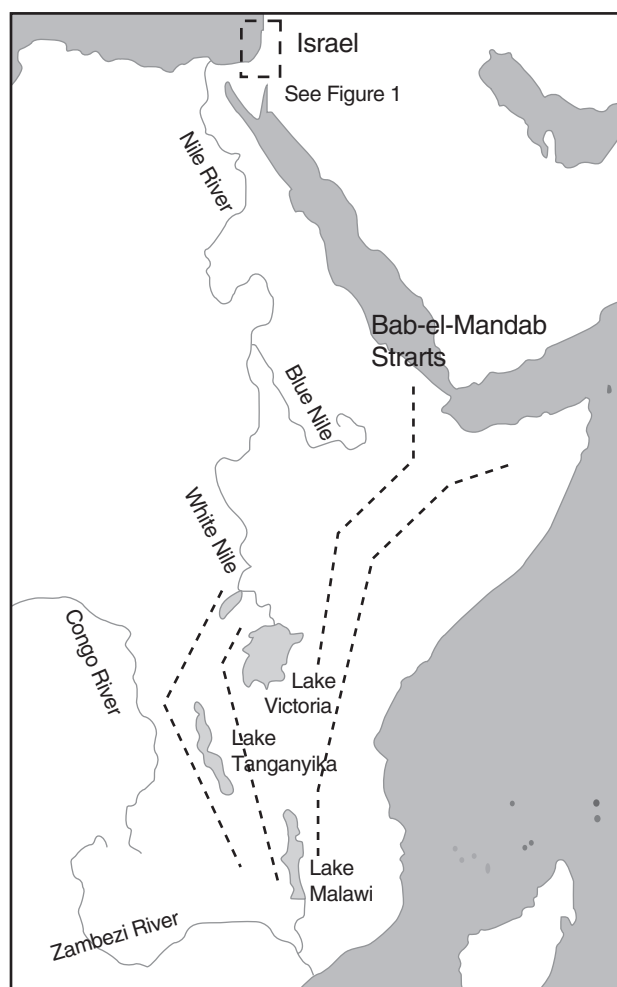


Figure 3. A schematic map of the African Rift Valley. The two divisions of the rift are marked by dashed lines. The rift continues northwards, via the Red Sea, the Arava valley, the Dead Sea, the Jordan River valley, Lake Kinneret, and ends in Syria (see Fig. 1).

some surprising changes in the phylogeny of African cichlids. Despite certain discrepancies between the findings of the study by Seehausen *et al.* (2003) and those of our own study on phylogenetic reconstruction, both studies found *A. flavijosephi* to be more closely related to the Lake Victoria haplochromines than to the haplochromines of Lake Malawi. Furthermore, Seehausen *et al.* (2003) found *A. flavijosephi* to be most related to haplochromines from the upper Nile (Lake Edward, Lake George), thus supporting our suggestion for a Nilotic dispersal of *A. flavijosephi* from Africa to the Levant.

The Nile, termed Palaeo-Nile in that period, cut through Egypt during the earlier part of the time frame set by our study (corresponding to the middle to late Pliocene). At the beginning of the Pleistocene, around 1.85 Mya, this traversal ceased, and did not start again until ~0.7 Mya (Rzóska, 1976; Said, 1981). The rejuvenated Nile, or Proto-Nile, started flowing again at ~0.7 Mya, but was never connected to sub-Saharan Africa (Rzóska, 1976; Said, 1981). The Proto-Nile was followed by the Pre-Nile that did connect to sub-Saharan watersheds, but, based on archaeological findings, the onset of its flowing was suggested to be ~0.5 Mya (Butzer, 1980), which is a younger date than our youngest coalescence time. We therefore suggest that the younger coalescence times that were obtained from our results are less likely, which leaves the possible coalescence time ranging between 1.8 and 3.26 Mya.

During the late Pliocene, when the Nile cut through Egypt to reach the Levant, its sources were probably within Egypt itself (Said, 1981). This obviously would not have allowed the migration of Ethiopian elements to the Levant through the Nile. However, during the early to middle Pliocene the Nile was probably connected to Central African and Ethiopian waterways as evident from early Pliocene Egyptian malacofauna (Said, 1981) and the mineralogy of early Pliocene Nile valley sediments (Tateo, Sabbadini & Morandi, 2000). This dispersal route is also supported by the presence of cichlid fossils, and other primarily sub-Saharan freshwater fishes, in the late Pliocene palaeontological site of Wadi Natrun at the Nile delta (Greenwood, 1972). The Nile delta, as well as the rest of the Nile Valley all the way south to Aswan, was flooded by the sea from the early Pliocene to the middle of the period (Rzóska, 1976; Said, 1981). Therefore, the presence of late Pliocene cichlid fossils in the delta most probably reflects a northward dispersal of Central African cichlids during that period rather than a relict of a Miocene Nilotic population. After the entry of *A. flavijosephi* into the Nile system, during the mid to late Pliocene, which corresponds with the earlier dates obtained in our study, a disjunction between the Nile and Central

African and Ethiopian watersheds occurred, causing the separation of *A. flavijosephi* from its congeners. At the same time, the marine transgression of the Nile canyon during the early Pliocene retreated, giving way to the freshwater Nile (Rzóska, 1976; Said, 1981). This could have enabled aquatic elements to disperse northwards towards the Levant. Thus, based on geological evidence, we suggest that the coalescence time should be 'pushed back' to the earlier part of the suggested range (2.5–3.3 Mya). This suggestion is supported by recent genetic work (Durand *et al.*, 2002) that implies a similar date for the separation of sub-Saharan and Mesopotamian/North-African clades of cyprinids.

Our findings suggest that *A. flavijosephi* is most closely related to the haplochromines that currently occupy the area between Lake Tanganyika and the Indian Ocean. This area constitutes the lower part of both the eastern and the western branches of the African Rift Valley. The extremely varied haplochromine clade that occupies Lake Victoria basin, situated between the two rift divisions (it is not a part of the Rift Valley), diverged prior to the divergence of *A. flavijosephi* from the haplochromine phylogenetic tree. It is also suggested that *A. flavijosephi* diverged from the other haplochromines during the middle or late Pliocene and that *A. flavijosephi* dispersed northwards towards the Levant via the Nile.

The late Pliocene has been previously described as a time of major dispersal events between Eurasia, the Levant and Africa, including that of *Homo* sp. However, the scarcity of palaeontological sites from that period found along the suggested dispersal routes (the Nile and the Arabian coast) hinders the possibility of clearly separating between the alternative, possible dispersal routes. Our study suggests that during the middle to late Pliocene the Nile River may have been an important dispersal route for aquatic Ethiopian elements from Africa to the Levant. If indeed this freshwater link existed, then both semiaquatic and terrestrial animals may have also followed this same route out of Africa during that period.

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