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Phylogeographic relationships of Freshwater Crabs, *Potamonautes* Macleay, 1838, in Central Kenya in relation to similar species in Southern Africa highlands (Decapoda Potamonautidae)

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ABSTRACT Fresh water crabs, *Potamonautes* Macleay, 1838 (Decapoda Potamonautidae) occurring in highland drainages in Africa are endemic to the specific region due to their geographically restricted habitats. Phylogenetic studies indicate that *Potamonautes* species in East, Central and Southern Africa regions have close genetic affinities and may be represented by the same genetic stock. In this study, fresh water crabs were sampled from the Aberdare ranges rivers in the Central highlands of Kenya to further characterize their phylogeny. Ribosomal DNA sequences derived from the samples and similar dataset of Eastern and Southern Africa regions were employed in phylogenetic analysis to determine populations' affinities. The constructed phylogenetic trees show that the molecular affinities are geographically structured where populations in Eastern and Western Rift Valley have closer genetic relationships, while Southern Africa populations are more distantly related. Further, time tree phylogenetics indicated that Eastern Africa *Potamonautes* are evolutionary older stocks relative to populations in Southern Africa. Tajima-D population drift neutrality test was negative, suggesting that the geographically isolated *Potamonautes* crabs populations are experiencing purifying selection.

KEY WORDS *Potamonautes*; Phylogenetics; Kenya; southern Africa.

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INTRODUCTION

Africa's freshwater crab, Decapoda Potamonautidae, is highly endemic at family, genus, and species levels and has restricted geographical distributions (Avise et al., 1987; Cumberlidge et al., 2009; Cumberlidge & Meyer, 2010). It is observed in East Africa, where each highland area supports endemic or restricted species (Dobson, 2004; Darwall et al., 2005). Three species belonging to the genus *Potamonautes* Macleay, 1838 are endemic to the Central Kenya highlands and are regionally separated between Mount Kenya and Aberdares ranges.

Information to clearly determine the conservation status of East Africa freshwater crabs is inadequate due to lack of data on clearly defined species, habitat locality and population densities. In the International Union for Conservation of Nature

(IUCN) red list categories and Criteria (IUCN, 2001; Cumberlidge et al., 2009), Kenyan highlands freshwater crabs have been highlighted as a population whose greater majority of species may be endangered or vulnerable, but with little information available to make a realistic assessment of their conservation status (Darwall et al., 2005). Since *Potamonautes* crabs are endemic with restricted range of distribution, they are potentially vulnerable to fragmentation of their habitats and agricultural land encroachment to the forests and this could result in their rapid decline in numbers.

Most species of *Potamonautes* are variously detritivorous or omnivourous, with feeding choices based on individual species size and locally available food. They feed on the fresh water aquatic vegetation and prey on small invertebrates and mollusks (Okafor, 1988; West et al., 1991; Dobson, 2004). The crabs occur in flowing rivers and are adapted to a wide range of habitats, though most species are restricted to high gradient streams or sluggish flowing regions of the rivers (Dobson et al., 2007a). Two or more species may co-exist in the same river, normally with one species occupying the river itself among the rocks while others occur in riparian marginal habitats in the trickles, the stream bank or even humid forests where some species are also semi-terrestrial (Cumberlidge, 1999; Darwall et al., 2005).

The current classification of the Central Kenya highlands freshwater crab is based on a recent revision of taxonomic keys (Cumberlidge et al., 2009) from a previous study by Bott (1955). This classification identified three species: *P. jeanneli* (Bouvier, 1921) and *P. odhneri* (Colosi, 1924) that originally were synonymous, while *P. alluaudi* (Bouvier, 1921) is recognized as a valid species rather than as a subspecies of *P. suprasulcatus* (Hilgendorf, 1898). Related classification studies (Peer et al., 2017) on freshwater crabs in mountain streams in Kwa-Zulu highlands (South Africa) using cytochrome oxidase I gene and 16S ribosomal genes re-defined *P. danielsi* Peer, Gouws, Lazo-Wasem, Perissinotto et Miranda, 2017 as a separate species from the clade of closely related *P. sidneyi* (Rathbun, 1904) (Gouws et al., 2015). These studies highlight difficulties in taxonomic separation of closely related individuals based on morphological characters and the relevance of augmenting classification using molecular analysis. In this study, we applied ribosomal DNA sequences to analyze phylogenetics of freshwater crabs from rivers draining the Aberdare ranges in Central Kenya and related the regional crabs to similar species in Eastern and Southern Africa geographical drainages (Daniels et al., 2015).

MATERIAL AND METHODS

Crabs sample collection

The crabs were sampled from various streams and tributaries draining the main central Kenyan highlands restricted to Muranga and Kiambu administrative boundaries. These streams border the highland farming lands and forested river line areas of the Aberdare ranges.

Sixty crab samples representative of each of the streams draining into the main rivers were collected from the streams and tributaries. The samples were stored alive and transported in containers with river water and specimens preserved at -20 °C until use.

DNA extraction

DNA was extracted from leg muscle tissue (Sambrook, 1987). The muscle tissue was incubated in 50 μg/ml proteinase K, 1 percent SDS in STE buffer (150 mM NaCl, 100 mM EDTA, 10 mM Tris–HCl, pH 7.4) at 55 °C for 3 h. The DNA was extracted from the lysate by the phenol:chloroform method and precipitated from the aqueous phase by adding 2–3 volumes of absolute ethanol. The pellet was suspended in 50–100 μl TE (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). DNA concentration was measured by absorbance at 260/280 nm and the quality analyzed by electrophoresis in 1 percent agarose gel in 1× TAE buffer (40 mM Tris acetate, 1 mM EDTA, pH 8.0). Respective tubes with DNA were appropriately labelled and stored at -20 °C.

Polymerase chain reaction (PCR) and geneclean procedures

In this study, 36 DNA samples representing fresh water crabs from Kenyan Central highlands were analyzed.

PCR was carried out using the following parameters: denaturation at 94 °C, 1 min annealing at 56 °C, 1 min and extension at 72 °C for 1 min 30 sec. The primers flanking region of 16S ribosomal RNA gene of *Potamonautes* species were applied. The amplification products were verified on 1% agarose gel and the fragment excised from the gel, solubilized in sodium iodide solution then bound to (silica) column in the gene clean procedure. Bound DNA was eluted in 30 µl nuclease-free ddH_2O.

Sequencing

Gene cleaned DNA of the amplified fragments was sequenced at Macrogen Inc., Netherlands, using the Applied Biosystems Sanger's dye terminator method. Each of the analyzed samples was independently sequenced three times and the raw sequences with non ambiguous consensus selected. Representative consensus sequences were deposited in NCBI nucleotide database (GenBank Accession ID: KU847922–KU847957).

Phylogenetic and diversity analysis

Ribosomal DNA sequences derived from the present study were combined with related sequences obtained from NCBI's nucleotide database. Sequences were aligned using the Clustal-W program in BioEdit (Version 7.05) and the phylogenetic relationships inferred from the aligned nucleotide sequences by the Neighbour-Joining method at Boostrap 1000 replicates using Phylip program (Felsenstein, 1985) as implemented in the MEGA6 version suite (Tamura et al., 2013). In the analysis, multiple identical lineages were deleted to simplify data presentation. Analysis of number of haplotypes, allele diversity and population divergence was done in DNA sequence polymorphism statistics packages implemented in dnaSP V5 software (Librado & Rozas, 2009). Divergence time in the evolutionary history was inferred using Neighbour-Joining method (Saitou & Nei, 1987). The branching points in the tree topology were calculated with the RelTime method implemented in MEGA6 software. Haplotype network analysis was constructed using the Median-joining method in PopART program (Leigh & Bryant, 2015).

RESULTS

Potamonautes *phylogenetic clusters*

Ribosomal DNA gene sequence from samples across Eastern and Southern Africa showed few monophyletic lineages (Fig. 1: bold lines) that give rise to distinctive clusters of *Potamonautes* crabs by geographic regions. Sequences are derived from Kenyan highlands crabs representative clustered by watershed that drain to the Indian ocean and also to the closely related clusters representing crab populations in highland watershed that drain Western Lift Valley (Fig. 1: clusters in italic). The population of crabs in highlands watershed that drain to Lake Victoria and those in Lake Tanganyika showed different clusters (Fig. 1, bracket I). The crabs in the southern part of Lift Valley in Malawi are more closely related to species found in South Africa Drakensburg and Kwa-Zulu highlands (Fig. 1: bracket II, III).

Departure from Neutrality

Tajima-D test of population drift departure from neutrality showed that the Kenyan highland derived crabs had Tajima-D values comparable to those obtained by analyzing samples representing the larger Africa region south of equator (Table 1). When a population is at equilibrium neutrality, the nucleotide diversity (π) and the number of nucleotide segregating sites (Θ) are indistinguishable and the Tajima D value is near zero. The analysis of the two populations of *Potamonautes* crabs showed a weak negative Tajima's D test (-0.402999 and -0.415967) for Kenyan and Southern Africa populations, respectively.

Population divergence analysis of Kenyan derived crabs in relation to the rest of Southern Africa group showed a comparable number of haplotypes, but Kenyan population had relatively higher nucleotide diversity (Table 2). There were 65 shared mutations but no fixed difference in the samples analyzed.

Haplotype network

Haplotype network of evolutionary relationships among populations of *Potamonautes* from Eastern and Southern Africa was based on rDNA sequence applied in determining haplogroups. Fourty-seven haplotypes of Eastern and Southen Africa *Potamonautes* were analyzed using median-joining haplotype network (Fig. 2) implemented in popPART. The visualized genealogical relationships showed similar clusters separation observed in phylogenetic tree where regional populations are separated though some haplotypes from Lake Malawi region appear to share genealogy with Kenya highlands derived samples.

Evolutionary time distance in Eastern and Southern Africa Potamonautes

Molecular time divergence analysis of the *Potamonautes* show that the Eastern African population represents and older genetic stock with early branching lines approximately 40 to 55 million years ago that further diverged 10 to 30 million years ago while the southern African stock is 1 to 5 million years ago, a comparatively recent time (Fig. 3). The Western Rift Valley and Lake Malawi stocks are phylogenetically closer to Southern Africa *Potamonautes* population (Fig. 3: in italic, brackets II and II).

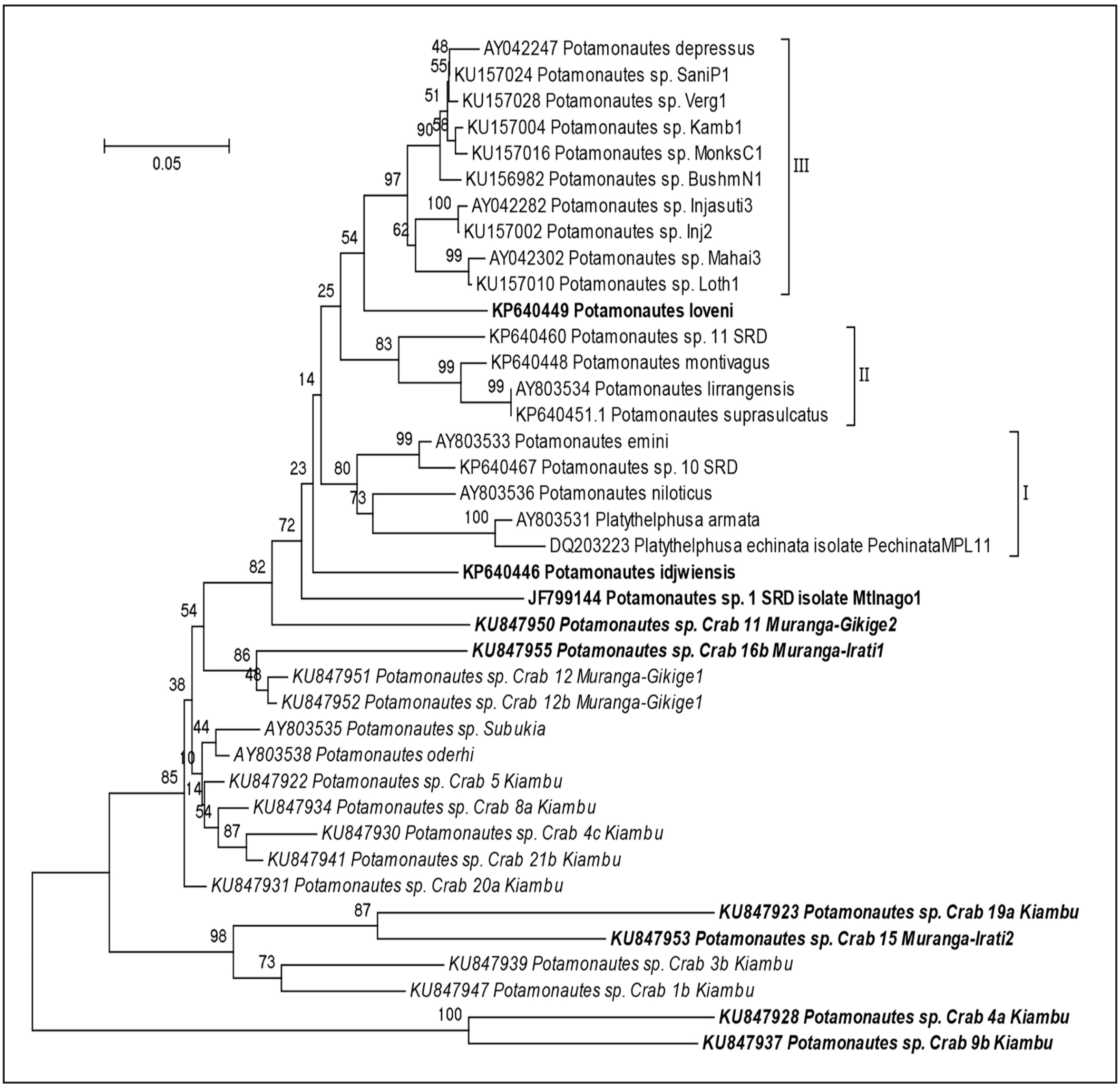

Figure 1. Evolutionary relationships of *Potamonautes* crabs from Eastern and Southern Africa. The evolutionary history was inferred using the Neighbor-Joining method. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances and the percentage boostrap (1000 replicates) are shown at the branches. Kenya region derived crabs are in italic. Evolutionary analyses were conducted in MEGA6.

DISCUSSION

Africa's *Potamonautes* fresh water crabs are found throughout the non-arid areas of the continent with high diversity of the species occurring in forested areas of equatorial Africa and in lower diversity in savanna regions. The crabs are found in slow flowing rivers in over a wide range of habitats. Two or more species may co-exist in the same river, with one species occupying the river and others occurring in adjacent riparian environment, so the humid semi-terrestrial environments, can be as important to them as the aquatic environment (Bott, 1955; Cumberlidge, 1999; Cumberlidge et al., 2009).

The crabs are highly endemic at the family, genus, and species levels (Cumberlidge, 1999). In East Africa, each highland area supports endemic or restricted species within linked rivers and streams (Dobson, 2004), but there occurs admix-

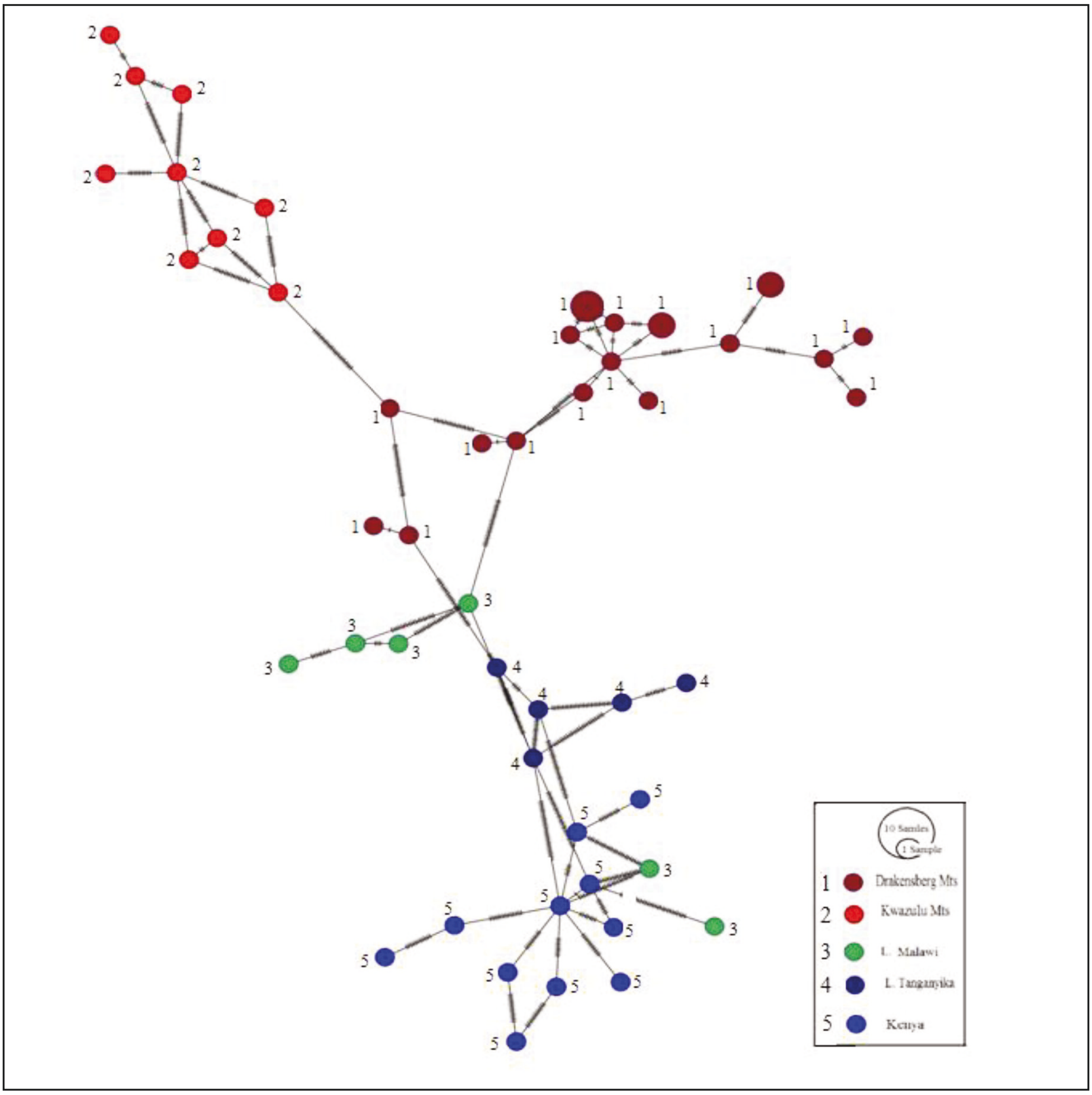

Figure 2. Network tree for Eastern and Southern Africa *Potamonautes* populations. The size of the circles correspond to numbers of individuals in different haplotypes indicated by colors. Divergence between haplotypes is illustrated by the number of hatch marks between haplotypes.

tures via translocation of these crabs within narrow geographic regions. Morphological examination of the type of crab specimens from Mt Kenya and Aberdares (Cumberlidge et al., 2009; Dobson et al., 2007a; 2007b) identified three species: *P. odhneri*, *P. jeanneli*, and *P. alluaudi*, in which *P. odhneri* was the most abundant. However, as the taxonomy of this group is poorly understood, there has been a re-description of the three species on basis of sizes and other morphological parameters. *Potamonautes alluaudi* adult size ranges at cross-section width from 44 to 55 mm, while *P. jeanneli* or *P. odhneri* adults are at cross-section width 22 and 32 mm respectively, but distinguishing juvenile sizes versus adult sizes is problematic.

The species of *Potamonautes* from Central Kenya highlands have not been defined at molecular level. In this study, ribosomal DNA sequences were applied for phylogenetic analyses (Saitou & Nei, 1987) of crabs collected in randomly selected rivers and streams draining the Aberdare ranges. The results show that these Kenyan specimens relate with *P. odhneri* (Figs. 1, 3) and the closely related to *Potamonautes* Subukia isolate previously described by Daniels et al. (2015). This is conceivable since rivers in Subukia region drain to Rift Valley, westwards from the Aberdare ranges. There are possibly four major clusters defined from the sequence data from Aberdare ranges isolates

Tajima's Neutrality Test

	m	S	p_s	Θ	π	D
***Potamonautes* (Kenya)**	36	189	0.658537	0.158807	0.141806	-0.402999
***Potamonautes* (Southern Africa)**	81	194	0.769841	0.155039	0.136187	-0.415967

Table 1. *Potamomautes* from Kenya. The analysis involved 36 nucleotide sequences. There were a total of 287 positions in the final dataset *Potamonautes* from Southern Africa. The analysis involved 81 nucleotide sequences. There were a total of 252 positions in the final dataset. Abbreviations: m = number of sequences, n = total number of sites, S = number of segregating sites, Ps = S/n, Θ = Ps/a1, π = nucleotide diversity, and D is the Tajima test statistic (π and S/a1 both estimate Θ, where E (expected) E [π] = Θ, E [S] = a1Θ), software default significant at P < 0.10. Analyses were conducted in MEGA6.

Divergence between Populations	
Population 1: *Potamonautes* **(Kenya)** Number of sequences: 39	**Population 2:** *Potamonautes* **(Southern Africa)** Number of sequences: 42
Number of Haplotypes, h: 36	Number of Haplotypes, h: 37
Nucleotide diversity (per site), Pi: 0.14304	Nucleotide diversity (per site), Pi: 0.08732
Between populations: Mutations polymorphic in population 1, but monomorphic in population 2: 192 Mutations polymorphic in population 2, but monomorphic in population 1: 66 Shared Mutations: 65 Number of fixed differences: 0	

Table 2. Diversity differences and genetic divergence between *Potamonautes* in Kenya versus Southern Africa population conducted using DnaSP V5 software. (Significance: P < 0.10).

(Fig. 1: lineages in italic), each restricted to separate river drainages, suggesting that further revision of species in reference to *P. alluaudi*, commonly reported along Aberdare ranges, need to be done combining molecular data with morphological classification. Cross analysis with previously published sequences indicate that *P. suprasulcatus* belong to a distant clade found in southern Tanzania/Malawi highlands linked to the Western Rift Valley and geographically separated from Kenyan Central highlands. Phylogenetic analysis (Fig. 1) is indicative of monophyletic lineages that expanded to distinctive clusters in their respective highland drainages. Most freshwater organisms such as crabs and crayfish are isolated to specific aquatic inland systems with either restricted or no dispersal between drainages and can therefore be used to examine hydrological patterns over evolutionary time (Avise & Felley, 1979; Nicolas et al., 2011; Phiri & Daniels, 2013).

The highland fresh water crabs in Eastern and Southern Africa are phylogenetically distinct with endemic species in each of the separated highlands. Tajima-D test of genetic drift from population neutrality (Tajima, 1989; Nei & Kumar, 2000) show that these crabs inhabiting these highlands are at weak negative drift (-0.42 and -0.40) for Southern Africa region and Kenyan isolates, respectively (Table 1). This indicates few mutations that accumulate at silent sites but low average heterozygosity possibly as a result of being long time geographically isolated populations that are experiencing purifying selection over evolutionary time. Population divergence analysis of *Potamonautes* crabs sampled within Kenyan compared to Southern Africa population showed a comparable number of haplotypes, but Kenyan population had relatively higher nucleotide diversity (Table 2). The data showed 65 shared mutations, but no fixed difference in the samples analyzed. This may indicate a common ancestral stock that has been separated by geographic barriers over evolutionary time, but is experiencing comparable habitat conditions.

The high altitude *Potamonautes* in Africa are relatively small in size (Cumberlidge et al., 2009; Dobson et al., 2004; Daniels et al., 2006), that is sometimes interpreted as convergent adaptation to the habitat, but evidence of gene sequence phylogenetic relationship indicate a singular ancestral stock. For example, haplotype network analysis (Fig. 2) showed that *Potamonautes* stocks in Western Rift Valley and Southern Tanzania are presumably the geneological link between Kenya derived populations and those from Malawi and Southern Africa (Peer et al., 2017). Ribosomal DNA sequence data obtained in this study (Figs. 1, 3) for *Potamonautes* in central Kenya highlands, Tanzania highlands, Malawi and Southern Africa highlands show genetic distance separation coupled to geographic distance between them. While each regional highland clade is distinct, the genetic distance separation increases with geographic distance. The regional distinctive clusters are reflective of isolated aquatic populations with either restricted or no dispersal between drainages (Avise & Felley, 1979; Avise et al., 1998; Phiri & Daniels, 2013; Daniels & Klaus, 2018), providing evidence for phylogeographic drainage patterns across the continent of Africa. The estimated divergence times suggests that the Afrotropical Potamonautidae diverged during the Eocene to Oligocene period 25 to 55 million years ago (Daniels et al., 2015; Daniel & Klaus, 2018), that coincided with the geological formation of the Eastern Africa Rift Valley and re-direction of regional river drainages, while the Southern Africa highlands *Potamonautes* clades are relatively recent, arising in Miocene to Pleistocene period approximately 1 to 20 million years ago (Phiri & Daniels, 2013; 2014). The evolutionary time tree constructed using sequence data in this study (Fig. 3) demonstrates approximately a similar time divergence of *Potamonautes* clusters in Eastern Africa and Southern Africa highlands as observed in the previous studies.

The Kenyan samples in this study are from Aberdare ranges drainages to rivers leading to the Eastern coastline (Indian Ocean), while other rivers drain westwards to rift valley lakes. The previously described sample of the Subukia species (GenBank ID AY803535) (Daniels et al., 2015) is presumably from Central Kenya highlands draining westwards to the Lift Valley, hence the lineage affinities to crabs originating from these highlands (Fig. 1). The Lake Victoria catchment includes rivers from Western Kenya highlands and northern Tanzania have a distinctive *Potamonautes* species stock represented by *P. niloticus* and *P. emini*, while lake Tanganyika restricted drainage within the Western Rift Valley

Figure 4. Evolutionary time tree relationships of *Potamonautes* from Eastern and Southern Africa. The evolutionary history was inferred using the Neighbor-Joining method. Divergence times for all branching points in the topology were calculated with the RelTime method using the branch lengths contained in the inferred tree. Relative times were optimized and converted to absolute divergence times (shown next to branching points). The analysis involved 56 nucleotide sequences. Evolutionary analyses were conducted in MEGA6. Abbreviations: S.A. (Southern Africa), W.R.V. (Western Rift Valley).

has distinctive endemic stock of *P. platynotus* and species on the genus *Platythelphusa* A. Milne-Edwards, 1887 (Cumberlidge et al., 1999; Marijnissen et al., 2004).

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