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Savel R. Daniels

Department of Botany and Zoology, University of Stellenbosch, South Africa

Curtiss McLeod

Golder Associates Ltd., Edmonton, Canada

Corissa Carveth

Beacon Environmental, Peterborough, ON, Canada

Konan K. Mexim

Laboratory of Environment and Aquatic Biology, Nangui Abrogoua University, Côte d'Ivoire

Neil Cumberlidge

Northern Michigan University, ncumberl@nmu.edu

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EXAMINING THE EVOLUTIONARY RELATIONSHIPS AMONGST THREE SPECIES OF WEST AFRICAN FRESHWATER CRABS *LIBERONAUTES* BOTT, 1955 (BRACHYURA: POTAMONAUTIDAE) USING MTDNA SEQUENCE DATA

Savel R. Daniels^{1,*}, Curtiss McLeod^{2, 3}, Corissa Carveth³,
Konan K. Mexim⁴, and Neil Cumberlidge⁵

¹Department of Botany and Zoology, Private Bag X1, University of Stellenbosch, Matieland, 7602, South Africa

²Golder Associates Ltd., 107 Avenue North West, Edmonton, AB, Canada

³Beacon Environmental, 469 Water Street, Peterborough, ON, Canada K9H 3M2

⁴Laboratory of Environment and Aquatic Biology, Nangui Abrogoua University, Abidjan, Côte d'Ivoire

⁵Department of Biology, Northern Michigan University, Marquette, MI 49855-5301, USA

ABSTRACT

Evolutionary relationships amongst three species of freshwater crabs, *Liberonautes latidactylus* (De Man, 1903), *L. nimba* Cumberlidge, 1999, and *L. rubigimanus* Cumberlidge and Sachs, 1989, were examined using DNA sequence data of the mitochondrial cytochrome oxidase subunit I (COI) locus. The latter three species are listed in the IUCN Red List for 2008 as least concerned, vulnerable, and endangered, respectively. Specimens of the three species were collected in Liberia and neighbouring Guinea, West Africa. Phylogenetic results derived from maximum parsimony, maximum likelihood, and Bayesian inference revealed the monophyly of all three species. Moderate genetic variation was observed within *L. latidactylus*. Within the narrow endemic, montane species, *L. nimba* shallow divergence was observed, whereas marked genetic divergence was observed within *L. rubigimanus*. Three statistically well-supported clades were observed within the latter taxon, two of these clades were sympatric at Simandou, Liberia (clades A and B), and characterised by a 10% uncorrected sequence divergence for the COI locus, whereas the divergence between the latter two clades and the larger *L. rubigimanus* clade was 8%. Haplotype networks corroborate the absence of maternal gene flow amongst the three *L. rubigimanus* clades suggesting possible reproductive isolation and the presence of two novel species. Our results suggest that *Liberonautes* harbours undescribed, narrowly distributed species. The results are discussed in a conservation framework for the three *Liberonautes* species.

KEY WORDS: conservation, evolution, inland freshwater bodies

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INTRODUCTION

The Guinean Forest Belt region of West Africa is regarded as a global biodiversity hotspot (Myers et al., 2000). The region is geographically broad and heterogeneous and is comprised of two areas referred to as the upper and lower Guinean forest blocks (Myers et al., 2000). The upper block extends from southern Guinea into eastern Sierra Leone, Liberia, Côte d'Ivoire, Ghana, and western Togo, whereas the lower block extends from Nigeria into southwestern Cameroon. The region includes several mountainous areas including the Cameroonian Highlands and the Nimba Mountains. The biota of the region is diverse, with a high level of endemism including more than 9000 vascular plant species (approximately 20% endemic), 785 bird species (approximately 10% endemic), and 320 mammal species (approximately 20% endemic) (Myers et al., 2000). Similarly, the herpetofauna and the freshwater ichthyofauna exhibit high levels of endemism, although they are taxonomically understudied (Myers et al., 2000). Invertebrate biodiversity in the region is poorly studied hence estimates of alpha taxonomic diversity and endemism are largely unknown, although it

would be reasonable to assume that it is high. The notable exception is the freshwater crab fauna of the region (Decapoda: Potamonautidae) for which the alpha taxonomic diversity and endemism are relatively well documented (Cumberlidge, 1999).

Seven genera of freshwater crabs are present within the Guinean Forest Belt region (*Afrithelphusa* (Bott, 1959), *Globonautes* Bott, 1959, *Liberonautes* Bott, 1955, *Louisea* Cumberlidge, 1994, *Potamonemus* Cumberlidge and Clark, 1992, *Potamonautes* MacLeay, 1838, and *Sudanonautes* Bott, 1955), with the first five aforementioned genera being endemic (Cumberlidge, 1999). The epicentre of *Liberonautes* species diversity is Liberia and Guinea, and the genus consists of eight described species, all of which are on the Red List of the International Union for Conservation of Nature (IUCN) (Cumberlidge, 2008a-h). *Liberonautes nimba* Cumberlidge, 1999, is listed as vulnerable (VU), both *L. nanoides* Cumberlidge and Sachs, 1989, and *L. rubigimanus* Cumberlidge and Sachs, 1989, are listed as endangered (EN), whereas both *L. lugbe* Cumberlidge, 1999 and *L. grandbosa* Cumberlidge, 1999, are listed as critically endangered (CR) (Cumberlidge, 1999). The three remaining

* Corresponding author; e-mail: srd@sun.ac.za

species *L. chaperi* (A. Milne-Edwards, 1887), *L. latidactylus* (De Man, 1903), and *L. paludicolis* Cumberlidge and Sachs, 1989 are all in the IUCN Red List as least concern (LC) (Cumberlidge, 1999, 2008a-h).

There has been no molecular systematics studies of *Liberonautes*, and no recent geographic surveys have been undertaken on its species, rendering it likely that novel species exist in hitherto unsampled regions. The three *Liberonautes* species under study are morphologically and ecologically distinct. *Liberonautes nimba* is a small-size montane species with an enlarged right dactylus, *L. rubigimanus* has flattened chelipeds adapted for burrowing, whereas *L. latidactylus* is a large-size, robust, river-dwelling species. Recent work on the systematics of potamonautid crabs in a supposedly well-studied region such as southern Africa has revealed the presence of several novel species (e.g., Daniels and Bayliss, 2012, 2014; Phiri and Daniels, 2013, 2014; Peer et al., 2015; Wood and Daniels, 2016). A similar pattern of increased alpha taxonomic diversity is likely for *Liberonautes*. For example, the widely distributed riverine species, *L. latidactylus*, occurs from Senegal along the West African coast and adjacent interior to as far south as Ghana, covering an area of approximately 2000 km² (Cumberlidge, 1999). This species is the ideal candidate taxon with which to explore the possible presence of cryptic lineages. Research has revealed remarkable drainage phylogeny and cryptic speciation amongst Afrotropical freshwater crabs (Daniels et al., 2006; Phiri and Daniels, 2014). A phylogeographic study of the common Cape River crab *Potamonautes perlatus* (H. Milne Edwards, 1837) *sensu lato*, in the Western Cape province of South Africa, demonstrated the presence of three lineages within the species, two of which were considered novel (Daniels et al., 2006; Phiri and Daniels, 2014). In addition to defining species boundaries, molecular genetic studies are useful for determining the best management practise for taxa that require conservation. The endangered *L. rubigimanus* is known from only a few localities, including Mount Gibi, Margibi County, and Balloon creek Grand Gedah county of Liberia, and the Nimba Mountains in both Guinea and Liberia (Cumberlidge, 1999). Similarly, the vulnerable *L. nimba* is presumably endemic to the Nimba Mountains that traverses the border between Guinea and Liberia, although the species was historically only known from a couple of sites on the slopes of Mount Nimba in Guinea (Cumberlidge, 1999).

The continued survival of freshwater crabs in the Guinean Forest belt hotspot is threatened mostly by habitat loss and degradation. Significant increases in human populations accompanied by intensive slash and burn agricultural practices, have negatively affected water quality and habitat availability for freshwater species, including crabs. The Mount Nimba region is similarly under considerable economic developmental pressure due to its high-quality iron ore deposits. Open cast iron ore mining has occurred since the mid-1970s, and has negatively impacted both terrestrial and freshwater habitats and their associated biodiversity. Freshwater crabs (mainly the large-body species such as *L. latidactylus*) are also frequently consumed as a source of inexpensive protein by rural communities although the impact on the species biomass remains unquantified (SRD, pers. obs.).

We investigated the genetic differences amongst the three aforementioned *Liberonautes* species. We hypothesised that *L. nimba* should exhibit limited genetic differentiation given its narrow mountain distribution range in Mount Nimba. In contrast, *L. rubigimanus*, should display marked genetic differentiation similar to what has been observed in other burrowing freshwater crabs (Daniels et al., 2002; Daniels, 2011), whereas the widespread *L. latidactylus* is likely comprised of several evolutionary lineages (Daniels et al., 2006; Phiri and Daniels, 2014). Results from the present study also aim to help make informed conservation management recommendations for the two species (i.e., *L. nimba* and *L. rubigimanus* listed as vulnerable and endangered, respectively) in the ICUN Red List, and provide insight into evolutionary relationships of the widespread taxon (i.e., *L. latidactylus*). Mitochondrial DNA sequence markers are frequently used to define conservation management units within species, while nuclear DNA markers when congruent with mtDNA is used to define evolutionary significant units (Crandall et al., 2000). This investigation represents the first genetic study of *Liberonautes*.

MATERIALS AND METHODS

Sample Collection

Liberonautes latidactylus, *L. nimba*, and *L. rubigimanus* were collected primarily from rivers and streams around the Nimba Mountains in Liberia and Guinea, focusing on the area that will be severely impacted by proposed open iron-ore mining (Fig. 1; Table 1). Additional specimens of *L. latidactylus* and *L. rubigimanus* were collected from the Simandou region, Liberia, a 100 km range of mountains in southeastern Guinea. Specimens were identified using the dichotomous key in Cumberlidge (1999). Crabs were killed by freezing and a pereopod was broken off, and preserved in absolute ethanol, and stored at 4°C in a refrigerator. Five *L. latidactylus* specimens from the collection of NC at Northern Michigan University (NMU) were also included. For the latter specimens, gill tissue was removed from the branchial chambers to minimize the destruction of specimens. Gill tissue from museum specimens is preferred over pereopod muscle tissue as it appears to preserves better and yielded higher-quality DNA and result in limited the damage to specimens.

DNA Sequencing

DNA was extracted from pereopod muscle or gill tissue using a Machery Nagel tissue DNA extraction kit, following the manufacturer's protocol. Extracted DNA was stored in a refrigerator until required for the polymerase chain reaction (PCR). The protein-coding mitochondrial gene, cytochrome oxidase subunit one (COI) was amplified and sequenced. The primer pair LCOI-1490 (5'-GGT CAA CAAA TCA TAAA GAT ATTG-3') and HCOI-2198 (5'-TAAA CTT CAG GGT GAC CAAA AAA TCA-3') (Folmer et al., 1994) was used to amplify a partial fragment of the COI locus. This locus has been used extensively in phylogeographic studies of freshwater crabs (Daniels et al., 2006; Gouws et al., 2016; Phiri and Daniels, 2016; Wood and Daniels, 2016). Nuclear DNA sequence markers in freshwater crabs evolve slow and is of limited utility in freshwater crab phylogeography (Phiri and Daniels, 2016).

Polymerase chain reactions (PCRs) were performed on a GeneAmp PCR System Thermocycler (Applied Biosystems, Foster City, CA, USA). For each PCR, a 25 µl reaction was performed under standard conditions. This reaction contained 14.9 µl of Millipore water, 3.5 µl of 25 mM MgCl₂, 2.5 µl of 10 × Mg²⁺-free buffer, 0.5 µl of a 10 mM dNTP solution and 0.5 µl of the primer sets at 10 mM, 0.1 unit of *Taq* polymerase and 1 to 3 µl of template DNA. Standard protocols were used to cycle sequence-purified PCR products, and the sequencing was performed on an ABI 3730 XL automated machine at Stellenbosch University Central Analytical Facility.

Phylogenetic Analysis

SEQUENCE NAVIGATOR (Applied Biosystems, Waltham, MA, USA) was used to check for ambiguities and compute a consensus sequence from

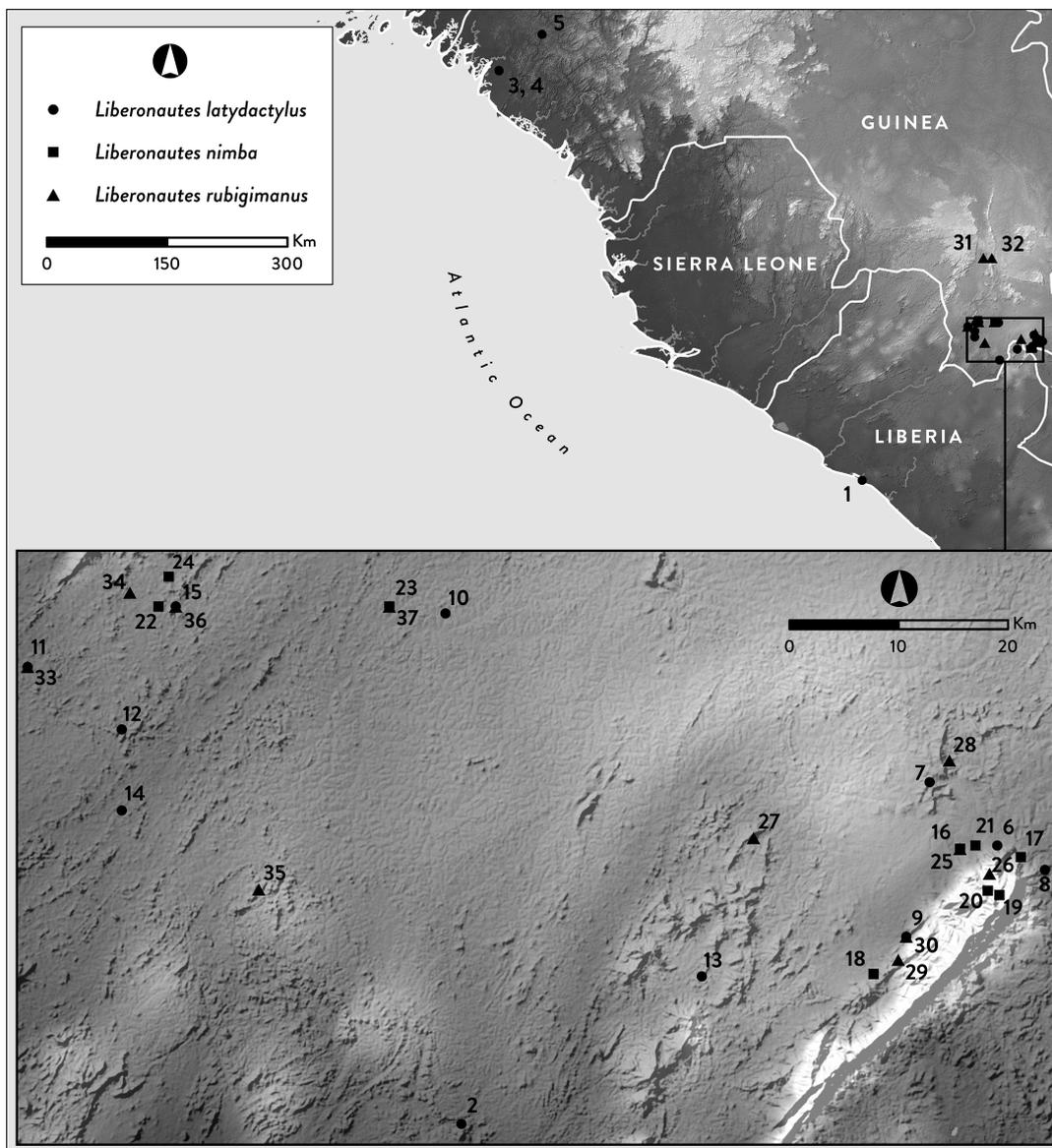


Fig. 1. Sample localities in Liberia and Guinea, West Africa for the three freshwater-crab species of *Liberonautes*. The numbers on the map correspond to those in Table 1.

forward and reverse strands, while CLUSTAL X (Thompson et al., 1997) was used to determine the sequence alignment. The COI sequences were converted to amino acids to test for the presence of stop codons. No stop codons were observed hence the sequences were regarded as functional and not as pseudogenes. Phylogeny analyses using Bayesian inference (BI), maximum likelihood (ML) and maximum parsimony (MP) was completed for the COI data. The optimal tree space for the COI data set was investigated through Bayesian inferences using MRBAYES 3.2 (Ronquist et al., 2012). The best-fit substitution model was determined using the Akaike information criteria (AIC) (Akaike, 1973) in MODELTEST (Posada and Crandall, 1998), which was used to resolve the tree topology for BI and ML, as this reduces the number of parameters that contribute little to describing the data by penalizing more complex models. For each Bayesian analysis, ten Monte Carlo Markov chains were run, with each chain started from a random tree and 5 million generations generated, sampling from the chain every 1000th tree. A 50% majority rule consensus tree was generated from the trees retained after the burn-in trees were discarded, with posterior probabilities (pP) for each node estimated by the percentage of time the node was recovered. Posterior probabilities (pP) < 0.95 were regarded as poorly supported. For the ML analyses in PAUP* version 4.010b (Swofford,

2002), heuristic searches using tree-bisection-reconnection (TBR) branch swapping with 100 random taxa additions were performed. For the MP analysis in PAUP* version 4.010b (Swofford, 2002), a heuristic search option was used to generate trees with TBR branch swapping, utilizing 100 random taxon stepwise additions. MP analyses were obtained from a bootstrap analysis of 1000 pseudo-replicates (Felsenstein, 1985) and 100 pseudoreplicates for ML due to computational constraints. In the present study, bootstrap values >75% were regarded as strong statistical support for a clade for both ML and MP analyses.

Uncorrected sequence divergence “p” distance values for the COI locus were calculated in PAUP* version 4.010b (Swofford, 2002). The Potamonautinae is monophyletic (Daniels et al., 2015). Hence two representative species, one *Potamonautes* species from the island of São Tomé in West Africa (*Potamonautes* sp.; GenBank accession number KP640499) and one species of *Potamonemus* (*P. asylos* Cumberlidge, 1993, GenBank accession number KP640489) were selected as outgroups. Uncorrected sequences have been widely used in studies of freshwater crabs, thus allowing for easy comparisons of our results with the published literature (Daniels, 2011; Peer et al., 2015). A haplotype network was constructed for each of the three *Liberonautes* species using TCS 1.21 set at 95% confidence

Table 1. Samples of the species of *Liberonautes* that were sequenced. *N* represents the number of samples per site.

Sample locality	Species	N	Country	Drainage/Locality	GPS coordinates	
					Latitude	Longitude
1	<i>Liberonautes latidactylus</i>	1	Liberia	North Creek (NMMU 12.8.1998)	6.129187	-10.359586
2	<i>Liberonautes latidactylus</i>	5	Liberia	Lugbe	7.683333	-8.651667
3	<i>L. latidactylus</i> and <i>L. rubigimanus</i>	1, 1	Guinea	Mount Tokadeh B/A	7.879167	-9.2975
4	<i>L. latidactylus</i> and <i>L. rubigimanus</i>	2, 1	Guinea	Gbapa B/A	7.950833	-9.296389
5	<i>Liberonautes latidactylus</i>	5	Guinea	Blue Lake	7.980556	-9.035278
6	<i>Liberonautes latidactylus</i>	4	Guinea	Bento A	8.026944	-9.282778
7	<i>Liberonautes latidactylus</i>	1	Guinea	Yepole Creek	8.076389	-9.0775
8	<i>Liberonautes latidactylus</i>	1	Guinea	Guinea Forestiere (NMMU-PN 26-29.6.2003)	7.483333	-8.816667
9	<i>Liberonautes latidactylus</i>	1	Guinea	Bangue River A	7.666667	-8.483333
10	<i>Liberonautes latidactylus</i>	2	Guinea	Yeblo River	7.70791	-8.336154
11	<i>Liberonautes latidactylus</i>	1	Guinea	Gouan River A	7.713705	-8.394677
12	<i>Liberonautes latidactylus</i>	1	Guinea	Ye River	7.683333	-8.433333
13	<i>Liberonautes latidactylus</i>	1	Guinea	Sonogolon (NMMU 28.4.2005)	10.739691	-14.432883
14	<i>Liberonautes latidactylus</i>	1	Guinea	Sarabaya-Kaveto (NMMU 27.4.2005)	10.745088	-14.434084
15	<i>Liberonautes latidactylus</i>	1	Guinea	Djiljedidi Creek (NMMU 9.5.2005)	11.15	-13.95
16	<i>Liberonautes nimba</i>	3	Guinea	Zougue River B	7.710781	-8.406397
17	<i>Liberonautes nimba</i>	2	Guinea	Siha River	9.012222	-6.441944
18	<i>Liberonautes nimba</i>	1	Guinea	Gbin River	7.234722	-9.105278
19	<i>Liberonautes nimba</i>	3	Guinea	Zie River	7.733333	-8.366667
20	<i>Liberonautes nimba</i>	2	Guinea	Zougue River A	7.710781	-8.406397
21	<i>Liberonautes nimba</i>	2	Guinea	Gouan River C	7.713705	-8.394677
22	<i>Liberonautes nimba</i>	5	Liberia	Mount Gangara A	8.075833	-9.076667
23	<i>Liberonautes nimba</i>	5	Liberia	Mount Nimba A	8.075833	-9.020833
24	<i>Liberonautes nimba</i>	5	Liberia	Mount Yuelliton	7.965	-9.173611
25	<i>Liberonautes rubigimanus</i>	1	Guinea	Zougue River B	7.710781	-8.406397
26	<i>Liberonautes rubigimanus</i>	5	Guinea	Gouan River A	7.713705	-8.394677
27	<i>Liberonautes rubigimanus</i>	1	Guinea	Bangue River B	7.666667	-8.483333
28	<i>Liberonautes rubigimanus</i>	4	Guinea	Bento A	8.026944	-9.282778
29	<i>Liberonautes rubigimanus</i>	5	Guinea	Mount Buton	8.006389	-9.185
30	<i>L. rubigimanus</i> and <i>L. latidactylus</i>	4, 1	Guinea	Cool Water B/A	7.837222	-8.985278
31	<i>Liberonautes rubigimanus</i>	4	Guinea	Mount Gangara B	8.075833	-9.076667
32	<i>Liberonautes rubigimanus</i>	4	Guinea	Mount Nimba B	8.075833	-9.020833
33	<i>Liberonautes rubigimanus</i>	2	Liberia	Monmih River	8.9675	-5.972222
34	<i>Liberonautes rubigimanus</i>	2	Liberia	Yoho River	9.109167	-6.208889
35	<i>Liberonautes rubigimanus</i>	1	Liberia	Izelo River	8.754167	-6.228889
36	<i>Liberonautes rubigimanus</i>	4	Liberia	Simandou A	9.907778	-5.029722
37	<i>Liberonautes rubigimanus</i>	5	Liberia	Simandou B	9.907778	-5.339722

(Clement et al., 2000). We opted not to perform an analysis of molecular variance (AMOVA) due to the small sample sizes collected for each *Liberonautes* species, since no biologically meaningful results would be obtained from such an AMOVA.

RESULTS

Phylogenetics

We sequenced a total of 106 *Liberonautes* specimens for a 637 base pair fragment of the COI locus. These sequences have been deposited in GenBank (accession numbers KX377204-KX377309). The substitution model for the locus was GTR + I + Γ ($-\ln L = 4125.06$; AIC = 8270.13). The base frequencies were A = 31.35%, C = 18.82%, G = 14.26%, and T = 35.58%; the proportion of invariable sites was 0.51 while the Γ rate was 1.29. The rate matrix was R(a)[A-C] = 2.92, R(b)[A-G] = 6.58, R(c)[A-T] = 1.72, R(d)[C-G] = 0.26, R(e)[C-T] = 12.10 and R(f)[G-T] = 1.00. The MP analyses recovered 1000 trees with a tree length of 663 steps, a consistency index (CI) of 0.53 and a retention index (RI) of 0.94 from 211 parsimony informative characters. The tree topology produced by MP, ML and Bayesian inference methods were highly congruent, hence only the Bayesian tree topology is shown and discussed (Fig. 2). All three species were retrieved as monophyletic. *Liberonautes nimba* formed a basal clade and specimens were characterised by short internal branches suggesting recent divergence between the samples. *Liberonautes rubigimanus* was retrieved as sister to *L. latidactylus*. Three divergent clades were observed within *L. rubigimanus*. We observed two clades (A and B) at Simandou, Liberia, which were characterised by a 10% uncorrected “p” distance for the COI locus. The latter result suggests the presence of two distinct maternal gene pools at Simandou. The two Simandou clades were retrieved as sister to the remainder of the *L. rubigimanus* clade and that was characterised by long internal branches. The uncorrected “p” sequence divergence between the Simandou clades and the remainder of the *L. rubigimanus* was 9.5%. Within *L. latidactylus* two clades were observed that were characterised by a 4% uncorrected “p” distance.

The TCS haplotype network was congruent with the tree topology. We obtained 15 COI haplotypes (Appendix A and B in the online version of this journal, which can be accessed via <http://booksandjournals.brillonline.com/content/journals/1937240x>) from the 28 *L. nimba* specimens sequenced. With the exception of *L. nimba* specimens from the Gouan and Shi rivers, Guinea that formed a haplocluster, all the remaining specimens could be connected into a single network demonstrating shallow genetic divergences. We obtained 32 COI haplotypes from the 46 *L. rubigimanus* specimens that were sequenced, characterised by six haplocluster and showed marked genetic differentiation (see Appendix A and B). Haplocluster one was exclusive to the Monmihi River, Liberia; the second haplocluster included samples from Mount Buton, Guinea; the third and fourth haploclusters were exclusive to samples from Simandou, Liberia, whereas the fifth contained samples from Bento, Guinea, and the sixth contained the remainder of the sampled sites (see Appendix A and B). We retrieved 20 haplotypes that comprised five distinct haploclusters from the

32 *L. latidactylus* samples that were sequenced (see Appendix A and B). The presence of several distinct haploclusters within both *L. rubigimanus* and *L. latidactylus* suggests the absence of widespread maternal gene flow.

DISCUSSION

The phylogenetic results obtained provide corroborative evidence for the initial hypothesis by observing limited genetic variation within *L. nimba*, moderate differentiation within *L. latidactylus*, and marked differentiation within *L. rubigimanus*. The degrees of genetic differentiation for the three *Liberonautes* species likely reflect differences in the dispersal capabilities and the ecological preferences, together with the geographic coverage sampled during the present study. Montane freshwater crabs generally tend to be small-bodied, narrowly distributed, and adapted to life in mountain streams, conditions that result in limited genetic differentiation (SRD, unpublished), hence the result observed for *L. nimba* is not surprising. Daniels et al. (1998) observed limited genetic variation, using allozyme electrophoresis, in the small-bodied freshwater crab, *Potamonautes parvispina* Stewart, 1997, endemic to first-order mountain streams along the Berg and Olifants rivers in South Africa. A similar pattern has also been documented for *P. parvicorpus* Daniels, Stewart and Burmeister, 2001 on the Cape Peninsula of South Africa (Wood and Daniels, 2016). *Liberonautes nimba* is frequently collected sympatrically with the larger burrowing species, *L. rubigimanus*, in the high-elevation streams around Nimba Mountains (SRD, pers. obs.), whereas the lowland streams and rivers are populated by the large-size *L. latidactylus*. It is likely that competitive exclusion from the main drainage by the latter two large-size species might potentially limit the dispersal capabilities of the smaller montane *L. nimba*. The latter hypothesis needs to be tested within an ecological framework.

The three clades observed for *L. rubigimanus* were characterized by strong topological statistical support, high-uncorrected distance values ($>9.5\%$), and the absence of shared haplotypes, suggesting the presence of three maternally discreet gene pools and the presence of three genetically and possibly reproductively-isolated taxa. The use of COI sequence divergence values is not optimal for the recognition of novel taxonomic operational units, but the latter approach has been used to describe several new potamonautid freshwater-crab species. Peer et al. (2015), for example, showed a sequence divergence that ranges 7.9% for the COI locus between *P. lividus* Gouws, Stewart and Reavell, 2001 and *P. isimangaliso* Peer, Perissinotto, Gouws, Miranda, 2015. Similarly, three genetically distinct clades were identified using mtDNA sequences in the Seychelles freshwater crab, *Seychellum alluaudi* (A. Milne-Edwards and Bouvier, 1893) (Daniels, 2011). The uncorrected COI sequence divergence values between clade one (from Silhouette Island, Seychelles) was 8.20% from clade two, whereas clade two (from Praslin Island sister to La Digue Island) was 4.01% divergent from clade three (Mahe Island sister to Fregate Islands). All three of the clades were allopatric, and two of these clades were subsequently described as new species of *Seychellum* Ng, Števcic and Pretzmann, 1995 (Cumberlidge and Daniels, 2014). Daniels and Bayliss (2012) reported an

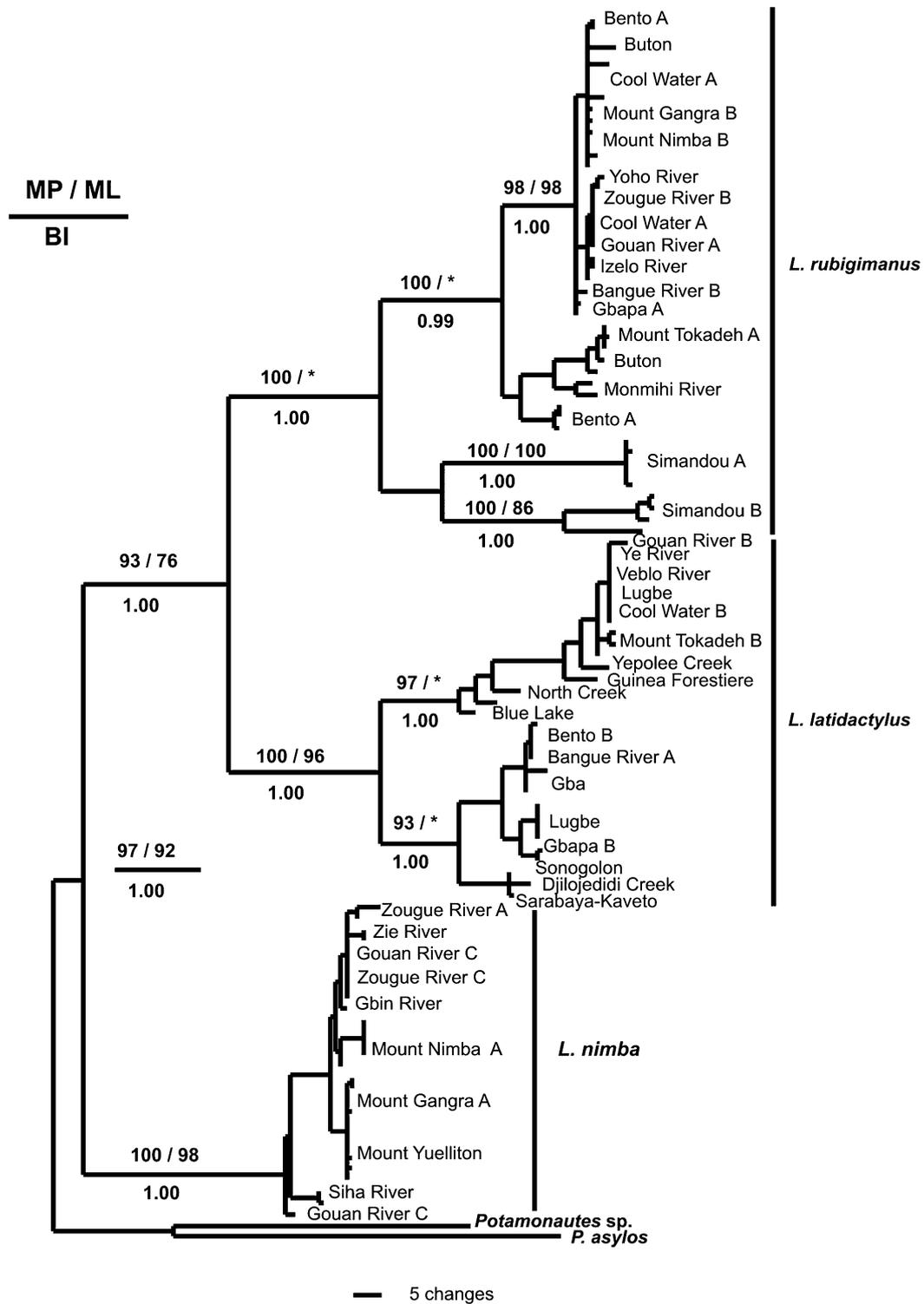


Fig. 2. A Bayesian phylogram for the COI mtDNA sequence data based on the GTR + I + Γ model for the three *Liberonautes* species. The nodal values represent the bootstrap values (>75%) for MP/ML and posterior probability (pP) values for BI (>0.95 pP). Values that are not statistically supported are marked with an asterisk *.

uncorrected sequence divergence value of 12.31% using the COI locus for the burrowing freshwater crab *Potamonautes obesus* (A. Milne-Edwards, 1868) from Zanzibar, Tanzania mainland, Kenya, and Malawi, typifying the marked genetic differentiation evident amongst burrowing freshwater crabs. The latter species has a broad distributional range along the East African coast (Reed and Cumberlidge, 2006) and likely represents a species complex. The sequence divergence values retrieved between the three *L. rubigimanus* clades fall within the range for the recognition of novel taxa and compares favourably to divergence values between sister species.

The question now arises as to whether the three *L. rubigimanus* clades are reproductively isolated. We would argue that collectively, there is sufficient evidence for the reproductive isolation at the Simandou, Guinea site, where two of the clades (A and B) appear sympatric. We observe evidence for genetic isolation between the two sympatric gene pools at Simandou. The two Simandou clades represent the novel taxa nested within *L. rubigimanus* since the type locality for *L. rubigimanus* is Sengbe Creek on the slopes of Mount Gibi, Liberia. A morphological examination of specimens from the three clades are currently being undertaken to provide corroborative evidence for the recognition of a new species. Examination of the taxonomically important first and second gonopods for the three *L. rubigimanus* clades will be conducted when male specimens becomes available.

Marked genetic differentiation was observed within *L. latidactylus* alluding to the possible presence of cryptic lineages. We observed a low frequency of shared haplotypes amongst sample sites suggesting the capability of the species to disperse across drainages (Appendix A and B in the online version of this journal, which can be accessed via <http://booksandjournals.brillonline.com/content/journals/1937240x>). *Liberonautes latidactylus* is a large-size species that is frequently observed in the terrestrial environment suggesting that it likely moves easily between drainages. Considering the evidence of the two clades and the wide geographic distribution of *L. latidactylus* we suggest that a comprehensive sampling endeavor be undertaken to explore species boundaries in the species. Species distributed across broad geographic ranges are likely to harbor distinct lineages that could often be cryptic. One example is the widely distributed Cape River crab, *Potamonautes perlatus*, Phiri and Daniels (2014) described two additional cryptic lineages. The three clades nested within *P. perlatus* showed very distinct drainage phylopatry, indicating that freshwater crabs exhibit limited dispersal capabilities, and are unlikely to disperse over high-lying mountains in the region. Furthermore, these clades could be separated by marked COI sequence divergence values. Clade 1A (Gamtoos and Gouritz rivers drainage systems) and 1B (Berg and Breede rivers drainage systems) showed a mean of 10.7% difference, whereas clade 1A and clade 2 (Berg, Eerste and Olifants Rivers and Cape Peninsula) showed a mean of 10.7%, while between clade 1B and clade 2 it was 4.8%. In the Natal River crab, *P. sidneyi* (Rathbun, 1904), phylogenetic analyses similarly revealed two distinct clades and seven independent networks suggesting substantial genetic structure (Gouws et al., 2016). These two *P. sidneyi* clades were 12.2%-14.3% divergent based on the COI lo-

cus. The latter author suggests the presence of two distinct lineages within *P. sidneyi*.

Our results suggest the presence of one and two genetically discreet lineages within *L. latidactylus* and *L. rubigimanus* respectively. This result has considerable implication for the conservation of *L. rubigimanus* as it clearly indicates that within the latter species there are two undescribed species. *Liberonautes nimba*, despite being listed as vulnerable in the IUCN Red List appears to be present in well conserved regions such as the Nimba Mountain Nature Reserve and exhibits limited genetic structure, suggesting that the possible loss of a couple of localities in the newly proposed mine area will not be detrimental to the prolonged survival of the species. Since the species is restricted to high-lying mountains that are currently under conservation they should be minimally impacted by highly localized iron mining. At Mount Nimba in the East Nimba Nature reserve, Liberia, this species was collected at sites previously mined by ArcelorMittal suggesting that the species can recover well after mining. Although the species was only known from Mount Nimba in Guinea, the current study has extended its distribution range to include the slopes of Mount Nimba in Liberia. We observed two additional clades at Simandou (A and B) within *L. rubigimanus*, suggesting the presence of two species that requires formal description and inclusion in the IUCN Red Listsince our results suggest they are narrow endemics. A revision of the IUCN Red List is required following more extensive sampling of *Liberonautes* species in the region. We recommend a widespread genetic study of the species to explore possible cryptic differentiation, as well as widespread sampling and quantification of population sizes to formulate a more accurate reflection of their distribution ranges. The two most recently described *Liberonautes* species (*L. lugbe* and *L. grandbosa*) were described by Cumberlidge (1999) based purely on morphological characters. Considering that species of freshwater crabs often exhibit limited and subtle morphological characters it is likely that a molecular systematic approach for delineating species boundaries would be ideal in providing diagnostic value for species recognition as has been demonstrated in the present study. Furthermore, our results imply that both of the two novel species nested within *L. rubigimanus* might have a narrow distribution range, implying a higher IUCN listing. A solid taxonomic study of *Liberonautes* species, however, is required for this. In addition, none of the five endemic West African freshwater crab genera (*Afrithelphusa*, *Globonautes*, *Louisea*, *Potamonemus* and *Sudanonautes*) have not been subjected to recent taxonomic study, and most are devoid of molecular systematic attention and should be targeted in future taxonomic studies since they potentially harbor several undescribed species.

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APPENDIX A. DISTRIBUTION OF HAPLOTYPES (H) IN THE THREE SPECIES OF THE FRESHWATER CRAB *LIBERONAUTES*Table A1. Distribution of haplotypes (H) in the freshwater crab *Liberonautes nimba*. H, number of haplotypes, corresponds to Appendix B.

Locality	H														
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Mount Gangra A	4			1											
Mount Yuelliton		2	1		1										
Mount Nimba A						4					1				
Zougue River A							1	1		3					
Zie River									2	1					
Gouan River C										1			1		
Gbin River												1			
Siha River														1	1

Table A2. Distribution of haplotypes (H) in the freshwater crab *Liberonautes rubigimanus*. H, number of haplotypes, corresponds to Appendix B.

Locality	H																				
	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	
Cool Water A	1													1	1						1
Buton		1																			
Bento A			1	2								1									
Simandou A					1	1	1														
Simandou B								1	3	1	1										
Mount Gangra B												1		1		1			1		
Mount Nimba B													1	2					1		
Bangue River B																	1				
Gbapa A																				1	
Gouan River B																					2
Zougue River A																					1

Table A3. Distribution of haplotypes (H) in the three species of the freshwater crab *Liberonautes rubigimanus*. H, number of haplotypes, corresponds to Appendix B.

Locality	H											
	36	37	38	39	40	41	42	43	44	45	46	47
Mount Gangra B												1
Gouan River A			1	1								
Izelo River	1											
Monmihi River				1	1							
Yoho River						1	1					
Buton								1	1		1	1
Mount Tokadeh A										1		

Table A4. Distribution of haplotypes (H) in the three species of the freshwater crab *Liberonautes latidactylus*. H, number of haplotypes, corresponds to Appendix B.

Locality	H																			
	48	49	50	51	52	53	54	55	56	57	58	59	60	61	62	63	64	65	66	67
Ye River	1																			
Veblo River	2																			
Blue Lake	1						1	1	1											
Lugbe	1															4				
Cool Water B		1																		
Mount Tokadeh B			1																	
Guinea Forestiere				1																
North Creek					1															
Yepole Creek						1														
Bangue River A										1										
Gba											1									
Bento B												1	3							
Gbapa														1	1					
Gouan River B																	1			
Djiljedidi Creek																		1		
Songolon																			1	
Sarabaya-Kaveto																				1

APPENDIX B

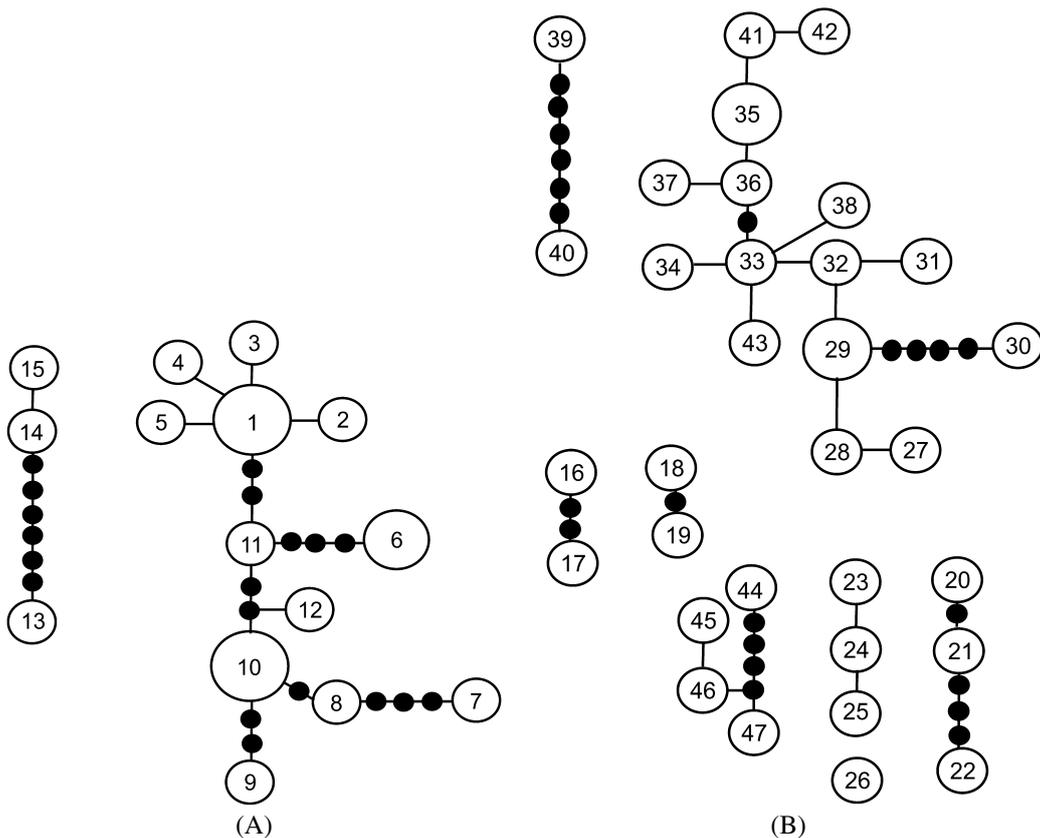


Fig. B1. Haplotype networks for each of the three *Liberonautes* species: A, haplotypes for *L. nimba*; B, haplotypes for *L. rubigimanus*; C, haplotype network *L. latidactylus*. The haplotype numbers correspond to those in Appendix A. Black closed circles represent missing or unsampled haplotypes.

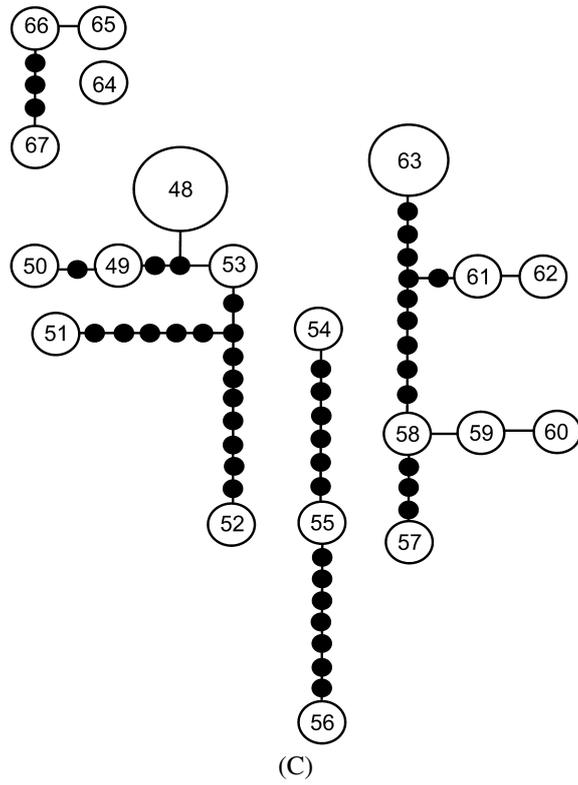


Fig. B1. (Continued.)