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Phylogenetic studies of the Madagascan freshwater crabs (Potamoidea, Potamonautidae, Deckeniinae)

Rainee Stevens
Northern Michigan University, raineecristine@gmail.com

PHYLOGENETIC STUDIES OF THE MADAGASCAN FRESHWATER CRABS (POTAMOIDEA, POTAMONAUTIDAE, DECKENIINAE)

By

Rainee C. Stevens

THESIS

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Phylogenetic studies of the Madagascan freshwater crabs (Potamoidea, Potamonautidae, Deckeniinae)

This thesis by Rainee C. Stevens is recommended for approval by the student's Thesis Committee and Department Head in the Department of Biology and by the Interim Director of Graduate Education

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Committee Chair: Date

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First Reader: Date

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Second Reader (if required): Date

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Department Head: Date

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Dr. Lisa Eckert Date
Interim Director of Graduate Education

Abstract

Relationships within the Madagascan freshwater crab fauna were examined based on unstudied specimens from all parts of Madagascar. This study allowed the examination of the validity of existing genera and species, and identified potential new taxa. The new specimens provided phylogenetic and evolutionary data, as well as new insights into the distribution patterns of the Malagasy freshwater crab fauna. In addition, the large number of new localities from previously unsurveyed areas of Madagascar allowed the construction of updated distribution maps.

The present analysis included 62 unidentified specimens plus 13 identified species of Malagasy freshwater crab taxa that had already been sequenced. The gene targeted for constructing the phylogeny was a partial mitochondrial gene fragment (600 bp), cytochrome oxidase I (COI). Phylogenetic analyses, MP, ML, and BI, yielded 36 most parsimonious trees and recovered topologies with several highly-supported clades with similar topologies. The MP 50% majority-rule strict consensus tree was selected as the representative topology. Results indicate that the unidentified and identified Malagasy freshwater crab specimens included in this study show a great deal of diversification that falls into nine well-supported clades (Clades 1-9).

The distribution maps presented here based on comprehensive data from 500 identified individuals, 74 of which represent new localities, establish that freshwater crabs are found throughout all six provinces, and in all five ecoregions on the island. This includes new records from Mahajanga and Toliara Provinces. Improved taxonomic sampling for this study supports the validity of the current taxonomic assignment of seven of the eight currently recognized genera based on morphological and molecular studies.

Dedication

This thesis project is dedicated to the most influential women in my life. My grandmothers, Mary H. Hendricks, and Mary L. Sand. Daughters of immigrant families who fought and survived tribulations during pivotal times in America. Both were successful in their lives, despite their struggles, one held degrees in nursing and Russian and the other was one of the first women business owners of multiple saloons, cafes and diners in my hometown, Ogden City, Utah; and, to my mother, Deena M. Soter, a daughter of an immigrant coal miner, who despite her hardships raised me and my brother alone and was still a successful business owner. All three women were figures of strength, determination and of promise for me.

What I remember most from them are tic-tac size words of advice that shaped me into the person that I am today: “I’d rather be lucky than good any day”, “Please yourself, because it’s impossible to please everyone”, and mostly, “Life is not a dress rehearsal”. Had I not been raised by these women, with their level of wisdom, care, and love, nor heeded their advice, I would not be the confident woman nor the successful scientist that I am today. I am forever grateful, thank you.

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Antonya Begay (Weber State University, Ogden City, Utah), Laurel Hill (Minneapolis City, Minnesota) and Thomas Gable (Northern Michigan University, Marquette City, Michigan) for their cooperation and assistance through the mapping process using ArcGIS. Without their willingness or enthusiasm, a significant portion of my study would not have resulted in the data or the aesthetics that it did. And finally, most of all, I would like to thank my mother and brother (Deena and Zack) and friend (Rory Keefer) for the sacrifices they made to support me through my endeavors even though they were more than 2000 miles away. I could not have succeeded without their patience and considerate efforts emotionally, financially, or otherwise. Financial support for this project was funded by grants from NMU's Biology Department Developmental Fund, and the Dr. Louise M. Bourgault Scholarship.

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1. Introduction

Although it has been almost two centuries since the first descriptions of Madagascan freshwater crabs, until recently (Bott, 1965, Cumberlidge and Sternberg, 2002) the group had been generally overlooked by biologists. However, there is now increasing interest in the freshwater crabs of the Afrotropical region, particularly those found in the biodiversity-rich island of Madagascar. This renewed interest has been accompanied by a rise in the discovery of new species and two increasingly informative molecular phylogenetic studies, as well as studies on extinction threat levels and the need for conservation actions. As a result, the freshwater crabs of Madagascar have been recognized as a monophyletic group that is now included in a single family, the African Potamonautidae, as part of the wider Afrotropical fauna that is distributed throughout most of continental sub-Saharan Africa, Socotra, and the Seychelles Archipelago. The Madagascan freshwater crabs are 100% endemic at both the genus and species levels, and speciation on the island has produced 8 genera and 17 species (Table 1) with many more still awaiting discovery (Cumberlidge and Sternberg, 2002; Reed and Cumberlidge, 2006; Cumberlidge et al., 2007; Cumberlidge and Meyer, 2009; Meyer et al., 2014; Cumberlidge et al., 2015).

The origins of the Afrotropical freshwater crabs have long been uncertain, but recent molecular phylogenetic studies indicate that they may have evolved about 55 million years ago and since then have undergone a number of dispersal events that led to the present-day diversity and distribution patterns in Africa and Madagascar (Daniels et al. 2006; 2015). However, because freshwater crabs are never found in salt water the

question of how freshwater crabs which originated in Africa came to be found today on the island of Madagascar has only recently been resolved in favor of transoceanic dispersal on the island (rather than vicariance) (Daniels et al. 2006; 2015; Cumberlidge and Ng, 2009). The present study expands the taxonomic sampling of Madagascan freshwater crabs used by previous molecular phylogenetic studies (Daniels et al., 2006; 2015). The relationships within the island freshwater crab fauna will be examined here based on multiple new specimens from all parts of Madagascar that have been collected recently. The broader taxon sampling allows the examination of the validity of existing genera and species, and will flag any potential new taxa that may be included in the new material analyzed here. The new material will also provide a large number of new localities for freshwater crabs that will form the basis of updated distribution maps.

This study used a combination of molecular methods to reconstruct relationships in conjunction with morphological studies to understand evolutionary changes and to assist with identification, and classification, because this approach has become increasingly in systematic studies of freshwater crab faunas.

Table 1: Summary of the 17 species and 8 genera of Malagasy freshwater crabs with their taxonomic authorities prior to this study

Boreathelphusa uglowi (Cumberlidge and Sternberg, 2002)

Hydrothelphusa agilis A. Milne-Edwards, 1872

Hydrothelphusa bombetokensis (Rathbun, 1904)

Hydrothelphusa madagascariensis A. Milne-Edwards, 1872

Hydrothelphusa vencesi Cumberlidge, Marijnissen, and Thompson, 2007

Marojejy longimerus Cumberlidge, Boyko, and Harvey, 2002

Skelosophusa eumeces Ng and Takeda, 1994

Skelosophusa gollardi (Bott, 1965)

Skelosophusa prolixa Ng and Takeda, 1994

Madagapotamon humberti Bott, 1965

Malagasya antongilensis (Rathbun, 1905)

Malagasya goodmani (Cumberlidge, Boyko, and Harvey, 2002)

Foza ambohitra Cumberlidge and Meyer, 2009

Foza goudoti (H. Milne Edwards, 1853)

Foza manoni Cumberlidge, Klaus, Meyer, and Koppin, 2014

Foza raimundi Reed and Cumberlidge, 2006

Glabrithelphusa angene Meyer, Cumberlidge, and Koppin, 2015

1.1 Freshwater crab biology

True freshwater crabs (Potamoidea) belong to the infraorder Brachyura that are decapod crustaceans characterized by a reduced abdomen that is folded tightly under the thoracic sternum. There are more than 6,700 species of brachyuran crabs, and more than 1,350 of these species are primary (true) freshwater crabs that spend their entire life cycle in fresh water (Yeo et al., 2008; Daniels et al., 2015). There are more than 500 species of brachyuran crabs that spend a large proportion of their lives in freshwater habitats (e.g., Sesarmidae, Varunidae, and Hymenosomatidae) but many of these still require seawater to complete their life cycle: these are referred to as secondary freshwater crabs (Yeo et al., 2008; Schubart and Koller, 2005). There are five primary freshwater families: the Trichodactylidae Latrielle, 1828, and Pseudothelphusidae Ortmann, 1893 (both from Central and South America), the Potamonautidae Bott, 1970 (from the Afrotropical region), the Potamidae Ortmann, 1896 (from North Africa, southern Europe, and tropical Asia), and the Gecarcinucidae Rathbun, 1904 (from India, to Southeast Asia, as far south as Australasia) (Yeo et al., 2008; Cumberlidge and Ng, 2009). All primary freshwater crabs undergo direct development whereby their large yolky eggs (lecithotrophic) hatch directly into juvenile crabs and there are no free-living larval stages (Sternberg et al., 1999).

The primary freshwater crabs include species that are aquatic, semi-terrestrial, and terrestrial and are found in streams, lowland rivers, mountain streams, caves, karst, and water-filled leaf axils of the *Pandanus* palms in the rainforest canopy (Cumberlidge et al., 2002, 2005; Cumberlidge et al., 2003; Schubart et al., 2003). Aside from the ability to exploit diverse habitats, freshwater crabs also have physical and physiological character traits that set them apart from their marine relatives. The hemolymph of all freshwater

decapods has an ion concentration that is higher than their surrounding freshwater environment. This means that these animals must constantly osmoregulate by pumping ions into their bodies and pumping water out via a pair of antennal (green) glands (Cumberlidge et al., 2015).

Some species of Madagascan freshwater crabs are semi-terrestrial and have developed a highly vascularized branchiostegal membrane (a pseudolung) that helps them to breathe air as well as water (Cumberlidge, 1999). For example, *Madagapotamon humberti* is a primarily terrestrial species that lives in crevices and caves in limestone karst formations (tsingy) in northwest Madagascar. The semi-terrestrial species *Malagasya antongilensis* lives in phytotelmic freshwater pools up to 4 meters off the forest floor in water filled *Ravenala* leaf axils (Cumberlidge et al., 2005).

Structures of the male reproductive system (gonopods 1 (G1) and 2 (G2)) are important characters for freshwater crab taxonomy (Cumberlidge, 1999). Males release spermatophores containing spermatozoa into a space at the base of the copulatory organs (G1 and G2), and the spermatophores are introduced into the vulvae of the female via the gonopods during mating, assisted by the pumping action of G2. After mating, the male spermatophores are stored in female's spermathecae until favorable conditions for laying the eggs occur, when the eggs are fertilized as they pass through the spermathecae.

1.2 Madagascar

Madagascar is part of the Afrotropical region that comprises Sub-Saharan Africa, the Seychelles, Madagascar, and the island of Socotra (Cumberlidge et al., 2003). What was once part of Gondwana, Madagascar experienced multiple separation events as continental landmasses fragmented and migrated to their current geographic locations.

The last fragmentation occurred between India and Madagascar (84-96 Mya) (Daniels et al., 2006). Madagascar is the fourth largest island on Earth (Glaw and Vences, 2007) with a highly endemic flora and fauna that has evolved in complete isolation for as long as 80 my (Daniels et al., 2006; Samonds et al., 2011; Daniels et al., 2015; Cumberlidge et al., 2012). Madagascar's topography includes a forested mountain range running north-south in the eastern part of the island, savanna in the west and north, and desert in the extreme south (Cumberlidge et al., 2005; Rakotorison et al., 2012).

Freshwater crabs are found in most parts of the island and are especially diverse in the Antsiranana Province in the north of the island. For example, the Marojejy massif, home of the Marojejy National Park has four species of freshwater crabs belonging to three genera: *Marojejy longimerus*, *Skelosophusa eumeces*, *Hydrothelphusa madagascariensis*, and *H. vencesi*. Madagascar's ecosystems include rainforests (in the central and northern highlands that comprise mid-altitude humid evergreen forest above 800 m, and low altitude humid broadleaf forest below 800 m), seasonally dry deciduous forests, savanna in lowland river basins, and areas that are extremely arid (Cumberlidge et al., 2003).

1.3 Malagasy freshwater crabs

Bott (1960, 1965) wrote the first comprehensive taxonomic treatment of the freshwater crabs of Madagascar and recognized four species in three genera (*Madagapotamon*, *Hydrothelphusa,* and *Gecarcinautes*). Cumberlidge and Sternberg (2002) revised the classification of the freshwater crabs of Madagascar and recognized 12 species in six genera (*Hydrothelphusa, Malagasya, Madagapotamon, Skelosophusa, Boreathelphusa,* and *Marojejy*). Since then, two more genera (*Foza* and *Glabrithelphusa*)

and five more species have been described, bringing the number of species to date to 17 species in 8 genera (Reed and Cumberlidge, 2006; Cumberlidge and Meyer, 2009; Meyer et al., 2014; Cumberlidge et al., 2015). The most recent phylogenies of the Afrotropical freshwater crabs based on DNA sequences included a total sample size of ten and thirteen respectively (Daniels et al., 2006; 2015), and served to establish the monophyly of the Malagasy freshwater crabs. These studies also supported all the freshwater crab genera from Madagascar that have been recognized by recent authors (Cumberlidge and Sternberg, 2002; Reed and Cumberlidge, 2006; Meyer et al., 2014; Cumberlidge et al., 2015). The molecular trees also established that the Malagasy freshwater crabs are phylogenetically separate from other genera in the Deckeniinae that are found in the Seychelles, East Africa, and West Africa, and that this clade is a separate lineage from the many genera in the Potamonautinae on the mainland of Africa.

Multiple authors (Bott, 1965; Ng and Takeda, 1994; Ng et al., 1995) based their higher classifications exclusively on characters of the mandibular palp and recognized at least two different families among the Madagascan freshwater crabs. Cumberlidge and Sternberg (2002) questioned the validity of the widespread use of mandibular palp characters for higher taxonomy in the Afrotropical freshwater crabs and were the first to recognize the monophyly of Madagascar's freshwater crab fauna. These studies were subsequently supported by comprehensive molecular studies that strongly supported the monophyly of the island's freshwater crab fauna (Daniels et al., 2006; 2015). Cumberlidge (1999) and Cumberlidge and Sternberg (2002) assigned the Malagasy freshwater crabs to the family Potamonautidae Bott, 1970, based on the common possession of multiple characters, including a two-segmented mandibular palp, a triangular abdomen, and a first gonopod with either a short or medium length terminal

article. The terminal segment of the mandibular palp shows an unusually large amount of variation among the genera of freshwater crabs found in Madagascar – with four states of this character recognized: (1) a simple segment with no anterior flap (*Madagapotamon*), (2) a segment with a small anterior ledge at the segment junction (*Skelosophusa, Boreathelphusa*), (3) a segment with a medium sized anterior flap (*Glabrithelphusa*), and (4) a segment with a large anterior flap forming a bilobed palp (*Hydrothelphusa, Malagasya, Marojejy, Foza*) (Cumberlidge, 1999; Cumberlidge and Sternberg, 2002; Cumberlidge and Reed, 2006; Cumberlidge and Meyer, 2009; Meyer et al., 2014) (Fig. 1). Over 25 morphological characters are used to distinguish between species and genera of Malagasy freshwater crabs including the epibranchial teeth, the postfrontal crest, the frontal margin, the thoracic sternum, the third maxilliped, gonopods 1 and 2, and the dactylus, propodus, carpus, and merus of the cheliped (Cumberlidge and Sternberg, 2002).

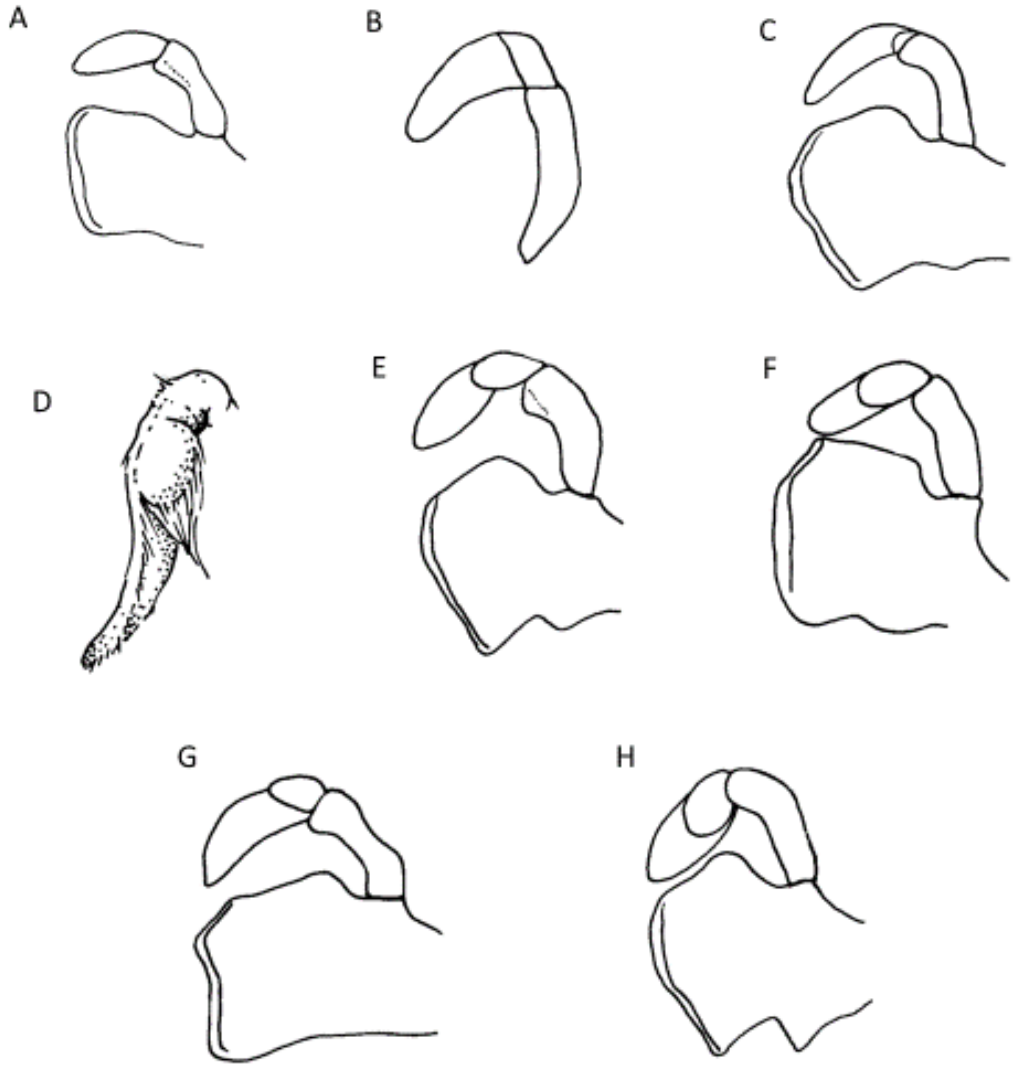

Fig. 1. Left mandibular palp of Madagascan freshwater crabs. Regarded as the simple state of character **A.** *M. humberti*. Regarded as the small ledge character state. **B.** *Skelasophusa*. **C.** *B. uglowi*. Character regarded as medium ledge or flap. **D.** *G. angene*. Character with large anterior flap. **E**. *Hydrothelphusa,* **F.** *Malagasya,* **G.** *Marojejy,* **H.** *Foza*. All sketches except letter D were reported by Cumberlidge and Sternberg, 2002; and, letter D was taken from Meyer et al., 2014.

1.3.1 Hydrothelphusa A. Milne-Edwards, 1872

Hydrothelphusa has been recognized as a distinct genus since its description in 1872, and currently includes four species: *H. agilis, H. madagascariensis, H. bombetokensis,* and *H. vencesi* (Cumberlidge and Sternberg, 2002; Cumberlidge et al., 2007). *Hydrothelphusa* is the most common genus of freshwater crabs on the island with a wide distribution ranging from the highlands of the north to the southern lowlands, and from the western to the eastern coasts. Species of this genus are sympatric with other species and genera of freshwater crabs. For example, *H. agilis* and *H. madagascariensis* have been collected together in the Marojejy National Park with species of *Marojejy, Foza,* and *Skelosophusa*. *Hydrothelphusa* is characterized by a large body size (adult size range CW 45-80 mm), a bilobed terminal segment of the mandibular palp, a discontinuous incomplete postfrontal crest that does not meet the anterolateral margins, a deep vertical sulcus on the ischium of the third maxilliped, a long flagellum on the exopod of the third maxilliped, and a Y-shaped sternal sulcus s3/s4 that almost reaches the anterior margin of the sternoabdominal cavity (Cumberlidge and Sternberg, 2002).

1.3.2 Boreathelphusa (Cumberlidge and Sternberg, 2002)

This is a monotypic genus with a restricted distribution in northwestern Antsiranana Province. *Boreathelphusa uglowi* is known from only six specimens collected from two localities – one on the mainland and one on the island of Nosy Bé about 8 km off the northwest coast. This species is characterized by small and blunt exorbital and epibranchial teeth, a smooth carapace surface, an ischium of the third

maxilliped that is smooth and lacking a vertical sulcus, a mandibular palp whose terminal segment has a small flat ledge at the junction between the segments, and a cheliped carpus with a large spine followed by a smaller spine, and a G1 with a slim, straight terminal article (Cumberlidge and Sternberg, 2002).

1.3.3 Madagapotamon Bott, 1965

Madagapotamon humberti is a distinctive species with a heart-shaped carapace that is wide anteriorly tapering sharply to a narrow posterior margin, with elongated thin legs, a mandibular palp with a simple terminal segment, and a third maxilliped that lacks a flagellum on the exopod. The species is found in Antsiranana Province in northwestern Madagascar in the Analamera and Ankarana National Parks and other localities on the north coast (Cumberlidge et al., 2015). The preferred habitat of *M. humberti* is tsingy (karst limestone formations) (Cumberlidge et al., 2003).

1.3.4 Malagasya Cumberlidge and Sternberg, 2002

Malagasya includes two species, *M. antongilensis* (Rathbun, 1905) and *M. goodmani* (Cumberlidge, Boyko, and Harvey, 2002). Members of this long-legged genus are distinguished by an elongated heart-shaped carapace, a distinct gap between the epigastric and postorbital crests, small but pointed exorbital and epibranchial teeth, and an anterolateral margin behind the epibranchial tooth that has five or more distinct pointed teeth, a bilobed mandibular palp, a third maxilliped ischium with a deep vertical sulcus, and a third maxilliped exopod with a long flagellum. The genus is distributed from the northern province of Antsiranana to the

southern Province of Fianarantsoa along the eastern mountain chain. This genus is also found along the eastern coastline of Toamasina Province north to Antongil Bay. *Malagasya goodmani* can be distinguished from *M. antongilensis* by the velvet-like covering of setae on the propodus of p2 through p5, and a v-shaped s3/s4 groove in the former species (vs. a propodus of p2-p5 is lacking velvety setae and a horizontal s3/s4 sternal groove in *M. antongilensis*).

1.3.5 Skelosophusa Ng and Takeda, 1994

This genus includes three species: *S. eumeces*, *S. gollhardi*, and *S. prolixa.* The genus is recognized by its medium size (its adult size range is between CW 24-35 mm), a transversely oval-shaped carapace, a small epibranchial tooth, a third maxilliped with a deep vertical sulcus, a mandibular palp with a small ledge at the junction between the segments, a carpus of p1 with a large sharp spine followed by a small sharp tooth, and a thoracic sternal groove 3/s4 that is reduced to two short notches at the lateral edges and faint in the middle. This genus is distributed in northwestern Madagascar in Antsiranana Province including the Marojejy National Park (Ng and Takeda, 1994; Cumberlidge and Sternberg, 2002).

1.3.6 Marojejy Cumberlidge, Boyko and Harvey, 2002

This is a monotypic genus characterized by a small body size (adult size range beginning at CW 24 mm), a bilobed mandibular palp, a conspicuously elongated cheliped merus, a transversely oval carapace, reduced exorbital and epibranchial teeth, an incomplete postfrontal crest, a third maxilliped exopod a short flagellum, and a third maxilliped ischium with a faint vertical groove, and shortened

eyestalks. (Cumberlidge and Sternberg, 2002). This species has a narrow distribution limited to the Marojejy National Park in Antsiranana Province (Cumberlidge et al., 2002). The Marojejy Mountains range to over 2,100m above sea level and comprise closed canopy forest, mountain woodland, ericoid bush, palms, ferns, orchids, and balsams (Cumberlidge et al., 2002).

1.3.7 Foza Reed and Cumberlidge, 2006

Foza includes four species: *F. raimundi*, *F. ambohitra*, *F. goudoti* and *F. manonae*. The genus is distinguished by epigastric crests that almost touch the frontal margin, a small, pointed epibranchial tooth, a faint incomplete post frontal crest that does not meet the anterolateral margins, a third maxilliped exopod with a shortened flagellum, a bilobed mandibular palp, and a carpus of the cheliped that has two sharp pointed teeth , and extremely long G2s each with a curving terminal article that forms a question mark and a mirror image of a question mark , and that together form a heart-shape when both are side by side.

1.3.8 Glabrithelphusa Meyer, Cumberlidge, and Koppin, 2014

Glabrithelphusa is a monotypic genus characterized by a smooth carapace, an incomplete postfrontal crest, small, weak epibranchial and exorbital teeth, a third maxilliped ischium with a deep vertical groove, a third maxilliped exopod with a long flagellum, and a mandibular palp with a reduced lobe at the junction between the segments (Meyer et al., 2014). There is no information available for the distribution or habitat preferences of this species, and all known specimens are

formalin-fixed and so this is the only genus that has not been included in the molecular data analysis.

1.4 Phylogenetic Relationships of the Malagasy Freshwater Crabs

The freshwater crabs of the world today are assigned to five families the Potamonautidae (Afrotropics), the Potamidae (Eurasia), the Gecarcinucidae (Asia and Australasia), and the Pseudothelphusidae and Trichodactylidae (Neotropics) (Cumberlidge and Ng, 2009). Molecular phylogenetic studies have revealed a great deal of new information about the relationships of the Afrotropical freshwater crab fauna, including those from Madagascar. Studies further support the more comprehensive molecular treatment of the Afrotropical freshwater crab fauna by Daniels et al. (2015). The two studies by Daniels et al. (2006, 2015) included Madagascan freshwater crabs, and provided the best hypotheses for the relationships between the genera and species on the island. However, the taxon sampling for both studies was far from complete and is in need of follow-up studies, such as the one presented here.

1.5 Biogeography of the Malagasy freshwater crabs

The ancient southern continent of Gondwana that included Madagascar began to split up during the mid-Mesozoic 158-165 million years ago (Mya) separating Madagascar, India, and Australasia from the African/South American plate (Daniels et al., 2006). Subsequent rifting resulted in the further separation of these landmasses until Madagascar was completely isolated from all other land masses about 68-65

Mya (Daniels et al., 2006). Politically, Madagascar is divided into six provinces (Antsiranana, Antananarivo, Mahajanga, Toamasina, Toilara, and Fianarantsoa) (Fig. 2), and it is divided ecologically into four ecoregions (Fig. 3) that share combinations of vegetation, climate, geology, altitude, and biological communities (Cumberlidge et al., 2003; Thieme et al., 2005). The four Madagascan ecoregions are: (1) the subhumid northwestern river basins that include the Bemanavy river basin, the eastern central and northern highlands above 800 m in Antsiranana Province that includes the Alaotra, Mangoro, and the eastern most region of the Betsiboka territories; and (2) the humid forests, where Marojejy National Park is located, where several specimens in this study were collected; and, the eastern lowlands below 800 m that includes the Namaza, Ihosy and Efaho River basins in the Toilara and Fianarantsoa Provinces; (3) the dry deciduous western river basins that includes the Mahajamba and Betsiboka Rivers where many specimens in this study were collected River; (4) southern river basins, where the spiny forests dominate, including the western coastal range that is host to three taxa in the present study.

Specimens of Malagasy freshwater crabs included in the present study were collected by Dr. S. Goodman between 2001 and 2015 and provide phylogenetic and evolutionary data, as well as new insights into the distribution patterns of the Malagasy freshwater crab fauna. These newly-collected specimens originate from the western river basins, and the southwestern and southern ecoregions that are previously unsurveyed areas of the island. The most recent comprehensive survey of the freshwater habitats throughout the island was conducted by Jean-Marc Elouard and colleagues from the Institute de Recherche pour le Development (IRD, ex-ORSTOM)

and the Centre national de Recherche sur l'Environment Malgaches. This biotic survey sampled over 100 different localities and collected freshwater crabs wherever they were encountered. Also included in the analysis of these data by Cumberlidge et al. (2003) were freshwater crabs collected by S. M. Goodman (FMNH) which included specimens of *H. agilis*, *H. madagascariensis*, H. *goudoti*, *Madagapotamon humberti*, *Marojejy longimerus*, and *B. uglowi* (Cumberlidge et al., 2003). These combined data allowed for the most comprehensive analysis of the Malagasy freshwater crabs that had ever been done (Cumberlidge et al., 2003). Those authors provided maps of every known species and genus and noted the high species richness in the northern province of Antsiranana. In addition, the freshwater crabs of Madagascar are found to be 100% endemic at the species and genus levels, and some species such as *B. uglowi* and *M. longimerus* were established as being extremely rare (Cumberlidge and Sternberg, 2002; Cumberlidge et al., 2003; Cumberlidge et al., 2005).

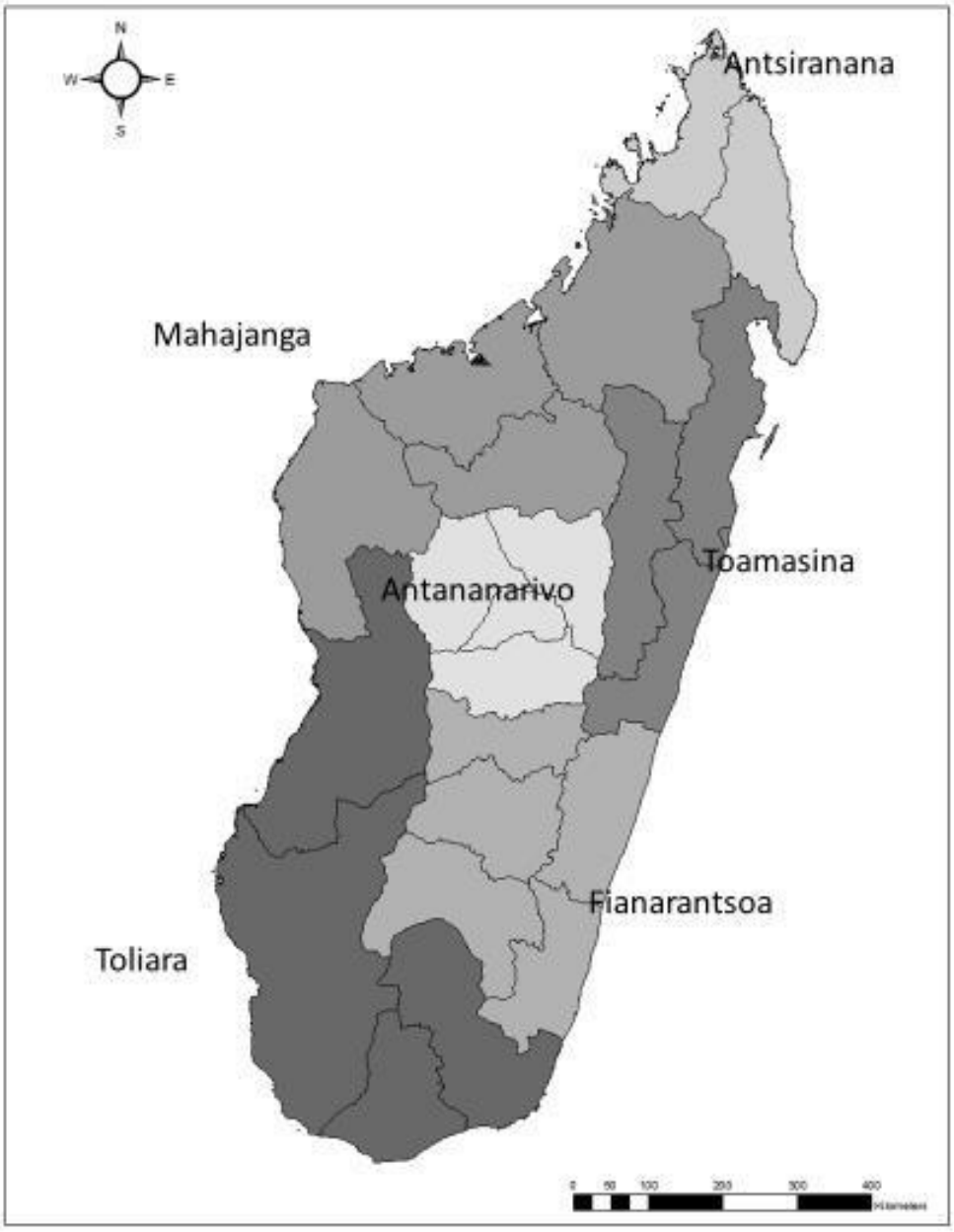

Fig. 2 . Political map of Madagascar, includes six provinces.

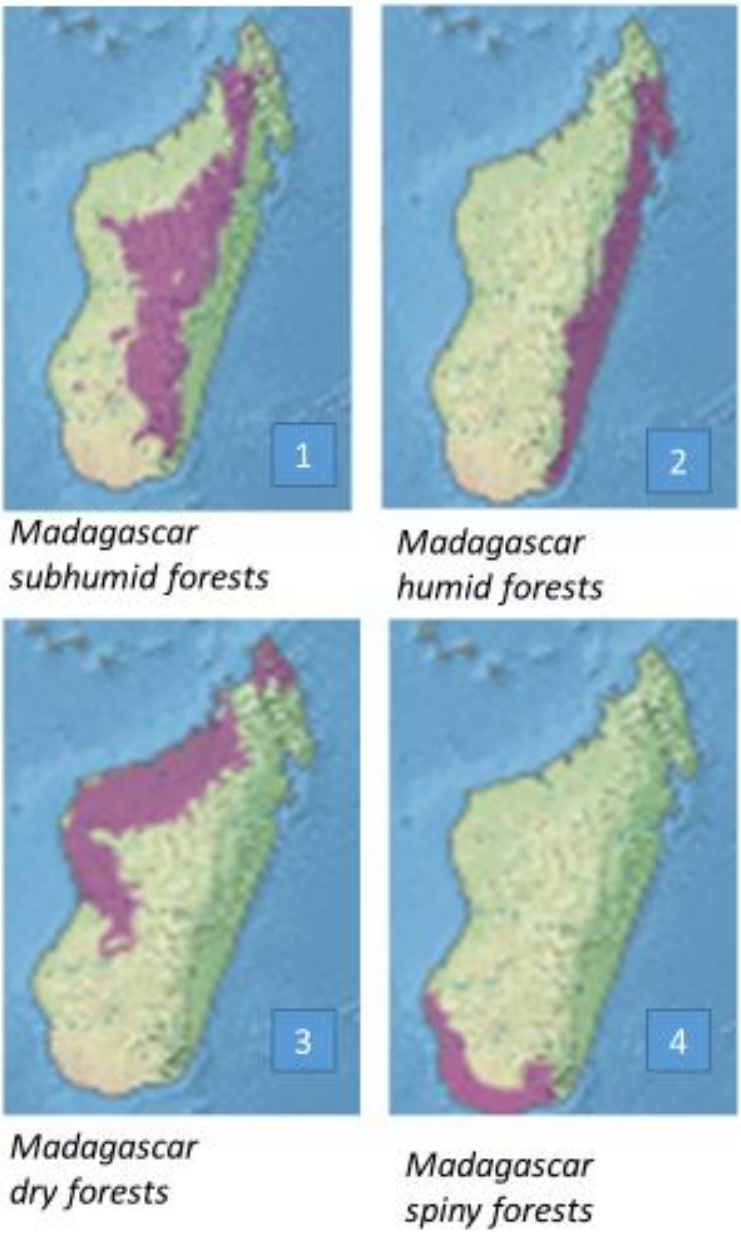

Fig. 3 . Political map of Madagascar with labeled ecoregions. 1.Subhumid forests. 2. Humid forests. 3. Dry forests. 4. Spiny forests.

2. Materials and Methods

This study used a combination of molecular methods to reconstruct relationships in conjunction with morphological studies to understand evolutionary changes and to assist with identification, and classification, because this approach has become increasingly in systematic studies of freshwater crab faunas. A list of specimens of the different taxa sequenced in this study together with their museum catalog numbers and localities is provided in Table 2, along with the 13 Malagasy freshwater crab taxa that have already been sequenced by Daniels et al. (2006; 2015). The majority of these samples are part of the collection of the Field Museum of Natural History (FMNH) in Chicago, Illinois. Other specimens are held in the crustacean collection at Northern Michigan University (NMU). All measurements of specimens were made with digital calipers and are given in mm. The terminology is adapted from Cumberlidge (1999) and Cumberlidge and Sternberg (2002). Morphological analyses consisted of a detailed examination of important taxonomic characters of the carapace, mouthparts, carpus, merus, chelipeds, sternum and gonopods. Maps were constructed using ArcGIS/ArcMap 10.3.1 and HamsterMap.com.

Table 2. List of sequences used in 2016 study with museum catalogue number; including 13 samples from Daniels et al., (2006; 2015). Clade and taxon designation correlates to placement on phylogeny and current taxonomic placement. OG represents the outgroup taxa. Samples with new data referred to with N/A await for proper GenBank assignment.

No.	Museum No.	Clade	Taxon	Reference	GenBank Accession
1	FMNH 7579	1	*1.1*	This study	N/A
2	FMNH 7592	1	*1.1*	This study	N/A
3	FMNH 7589	1	*1.1*	This study	N/A
4	FMNH 12645	2	*Ml. sp1*	This study	N/A
5	FMNH 12648	2	*Ml. sp1*	This study	N/A
6	FMNH 12646	2	*Ml. sp1*	This study	N/A
7	FMNH 12668	2	*M. goodmani*	This study	N/A
8	M. goodmani (unaccessioned)	2	*M. goodmani*	2015	KP 640493
9	NMU PN12.3.2003	2	*M. antongilensis*	This study	N/A
10	FMNH 13934	2	*M. antongilensis*	This study	N/A
11	NMU 29_M. antongilensis	2	*M. antongilensis*	This study	N/A
12	FMNH 5769	3	*3.1*	This study	N/A
13	FMNH 5731.2	3	*3.2*	This study	N/A
14	FMNH 5727.2	3	*3.2*	This study	N/A
15	FMNH 5727.1	3	*3.2*	This study	N/A
16	FMNH 5464	3	*3.2*	This study	N/A
17	FMNH 6643	4	*4.1*	This study	N/A
18	FMNH 5745	4	*4.2*	This study	N/A
19	FMNH 5759.1	4	*4.3*	This study	N/A
20	FMNH 5759.2	4	*4.3*	This study	N/A
21	FMNH 5758	4	*4.3*	This study	N/A
22	FMNH 5712	4	*4.3*	This study	N/A
23	FMNH 12526	4	*4.4*	This study	N/A
24	FMNH 5732	4	*4.4*	This study	N/A
25	FMNH 13939	4	*4.4*	This study	N/A
26	FMNH 5748	4	*4.4*	This study	N/A
27	MNHN PB27556	4	*4.4*	This study	N/A
28	AMNH 17531	5	*5.1*	This study	N/A
29	FMNH 4652	5	*6.1*	2006/2015	AY803579
30	NMU 1987	5	*7.1*	This study	N/A

Table 2. List of sequences used in present study with museum catalogue number; including 13 samples from Daniels et al., (2006; 2015). Clade and taxon designation correlates to placement on phylogeny and current taxonomic placement. OG represents the outgroup taxa. Samples with new data referred to with N/A await for proper GenBank assignment.

No.	Museum No.	Clade	Taxon	Reference	GenBank Accession
31	FMNH 7598	6	*M. longimerus*	2006/2015	AY803582
32	FMNH 7599	6	*M. longimerus*	This study	N/A
33	FMNH 7581	6	*M. longimerus*	This study	N/A
34	FMNH 4656	6	*M. longimerus*	2006	AY 803579
35	NMU 17	7	*F. ambohitra*	This study	N/A
36	FMNH 13931	7	*F. ambohitra*	This study	N/A
37	FMNH 11046	7	*F. ambohitra*	This study	N/A
38	MNHN 30154	7	*F. ambohitra*	This study	N/A
40	FMNH 5732	8	*B. uglowi*	2015	KP 640470
41	FMNH 7597	8	*S. eumeces*	This study	N/A
42	FMNH 7596	8	*S. prolixa*	2015	KP 640495
43	FMNH 5729	9	*H. agilis*	2015	AY803578
44	FMNH 5804	9	*H. agilis*	This study	N/A
45	FMNH 7573	9	*H. agilis*	This study	N/A
46	FMNH 7585	9	*H. bambetokensis*	This study	N/A
47	FMNH 5764	9	*H. bambetokensis*	This study	N/A
48	FMNH 5771	9	*H. bambetokensis*	This study	N/A
49	FMNH 5772	9	*H. bambetokensis*	This study	N/A
50	FMNH 5723	9	*H. bambetokensis*	This study	N/A
51	FMNH 5471	9	*H. bambetokensis*	This study	N/A
52	FMNH 7438	9	*H. madagascariensis*	This study	N/A
53	FMNH 7584	9	*H. vencesi*	This study	N/A
54	FMNH 7589	9	*H. vencesi*	This study	N/A
55	FMNH 7569	9	*H. vencesi*	This study	N/A
56	FMNH 13940	9	*H. vencesi*	2015	KP 640473
57	FMNH 7567	9	*H. vencesi*	This study	N/A
58	FMNH 6637	9	*Hsp1*	This study	N/A
59	Unaccessioned	OG	*D. mitis*	2015	KP 640471
60	SAMA48220	OG	*S. alluaudi*	2015	JF 799368
61	SAMA48235	OG	*S. mahafregate*	2015	JF 799319
61	SAMA48226	OG	*S. silhouette*	2015	JF 799351
62	NMU 25.IV.2005.C	OG	*A. monodosa*	2015	KP 640469

The following abbreviations are used:

A = abdominal somite
a7/a6 = sutures between abdominal somites
CH = carapace height measured at maximum height of cephalothorax (mm)
CL = carapace length along median line from anterior to posterior margin (mm)
CW = carapace width measured at widest point (mm)
e = thoracic episternite
FMNH = Field Museum of Natural History, Chicago, USA
FW = front width measured along anterior frontal margin between orbits (mm)
G1 = first gonopod
G2 = second gonopod
p1-p5 = pereiopods 1–5
s = thoracic sternite
s4/s5 = sternal sulci between adjacent thoracic sternites
s4/e4 = episternal sulci between adjacent thoracic sternites and episternites

2.1 Specimens

The present analysis includes 66 individual specimens representing identified and unidentified taxa. There were 13 sequences used from (Daniels et al., 2006; 2015), five of those are outgroup taxa; giving a total of 53 newly sequenced individuals. The two genera missing from the molecular part of this study are *Glabrithelphusa* and *Madagapotamon*, but both of these genera were included in the morphological assessment. The outgroup taxa consisted of genera that were previously identified as sister taxa to the Malagasy freshwater crab lineage in the subfamily Deckeniinae (Daniels et al., 2015). Locality data provided for this study includes over 100 localities in previously unexplored parts of Madagascar; 64 of the localities are from new specimens. All specimens used in this study have been identified based on morphological characters by Dr. Cumberlidge who wrote the latest identification keys for the freshwater crabs of this island (Cumberlidge and

Sternberg, 2002; Reed and Cumberlidge, 2006; Cumberlidge and Meyer, 2009; Meyer et al., 2014; Cumberlidge et al., 2015).

2.2 DNA extraction

Original sequence data for a standard COI DNA marker were collected from each of the 53 newly collected samples. Gill tissue or pereiopod muscle tissue was excised from each freshwater crab specimen that had been preserved in 70% ethanol and that had been collected recently (from one year to 15 years ago). A tissue sample from each specimen was placed into a 1.5mL tube with 200 proof ethanol and sent for DNA sequencing to the University Stellenbosch, South Africa (Daniels et al., 2015). Purification of tissue samples included washing multiple times with sterilized water followed by a standard DNA extraction using a Machery and Nagel DNA extraction kit (Daniels et al., 2006; 2015). DNA extraction of the specimens was conducted with a blank sample (containing reagents) as a control. To ensure maximization of elution, DNA from the specimens was treated with elution buffer. DNA samples were stored at -20° C until needed to carry out the polymerase chain reaction (PCR) (Daniels et al., 2015).

DNA was isolated from each freshwater crab specimen used in the molecular analysis. The gene targeted for constructing the phylogeny was a partial gene fragment, cytochrome oxidase I (COI) with a pair of primers, amplified using the polymerase chain reaction (PCR), and sequenced at Microgen (Microgen Inc., Amsterdam, The Netherlands). Original DNA sequences (COI marker) of each the specimens included in the study were combined with COI sequences from 7 genera

and 12 species of Malagasy freshwater crabs downloaded from GenBank from the study of Daniels et al. (2015).

2.3 Polymerase chain reaction, sequencing, and alignment

Amplification with PCR followed protocols in Daniels et al. (2015). Prior to PCR a 1µ of 1:20 dilution was made (DNA; millipore water). Amplification of the partial gene used mitochondrial DNA (mtDNA) COI primer pairs 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). To compare existing data, this DNA marker was chosen because previous analyses gave strong support for species relatedness, genetic structure, and subgeneric divisions (Raupach et al., 2015; Carvallo-Batista et al., 2014; Daniels et al., 2002) including studies of Afrotropical freshwater crab relationships by Daniels et al. (2015). The present study included COI sequences from GenBank from the studies by Daniels et al. (2006; 2015) for 13 species. This study also provided original COI sequence data that were previously unavailable for *Madagapotamon humberti*, *Foza ambohitra*, and *Hydrothelphusa bombetokensis*. Three described species, *Foza manonae, Glabrithelphusa angenei,* and *Skelosophusa gollhardi* have no DNA sequence data available and were not included in the molecular study, but were included in the morphological part of this study.

The following PCR protocol was followed based on techniques from Daniels et al. (2015). A 25 µl reaction was used that contained Millipore water, 2.5 µl $MgCl_2$, 10 x Mg^{2+} free buffer, 10mM dNTP solution, 10mM forward and reverse primers, 0.1 µl of

Taq polymerase and 2.5 µl of the 1:19 template DNA dilution. PCR conditions COI: 94°C (4 min), (94°C [30 s], 42°C [40 s], 72°C [45 s]) for 36 cycles, with final extension for all PCR conditions was 72°C (10 min). PCR products were electrophoresed (3h in a 1.5% agarose gel containing ethidium bromide) and the PCR products were then gel purified with the BioFlux purification kit (Bioer Technology Co., Ltd), after which they were sent to Macrogen for sequencing (Macrogen Inc., Amsterdam, The Netherlands). Sequences were checked for ambiguities and initially aligned with MUSCLE (Edgar 2004). Due to a program update, multiple alignment was executed in both MEGA6 and MEGA7 (Tamura et al., 2013; Kumar et al., 2016). The COI sequence is a protein-coding genetic locus, and was translated to amino acids and checked for stop codons using EMBOSS-Transeq (http://www.ebi.ac.uk/emboss/transeq/). All new sequences arising from the present study will eventually be deposited in GenBank when the results of this thesis are published. A COI DNA sequence alignment for freshwater crab fauna was constructed by combining the new COI DNA sequences with those of previously sequenced taxa taken from GenBank (Daniels et al., 2015).

2.4 Phylogenetic analysis

The DNA sequencing and initial tree building for this MS thesis project were completed in the molecular biology lab headed by Dr. Savel Daniels at Stellenbosch University in South Africa. Initial reconstructive phylogenetic analysis was performed using a 600bp COI a protein-coding locus. The Madagascan freshwater crab phylogeny was reconstructed using maximum parsimony (MP), maximum likelihood (ML), and Bayesian inference (BI) methods. MP analyses were executed in PAUP*4.0 version beta 10 (Swofford, 2002). For MP analyses, trees were

generated using the heuristic search option with TBR branch swapping using 100 random taxon additions, with gaps treated as a fifth character or missing data.

Phylogenetic confidence in the nodes recovered from MP was estimated with bootstrapping 1000 replicates (Felsenstein, 1985). Analyzing the data set was accomplished with MrBayes v. 3.2.3 (Ronquist et al., 2012) using Bayesian inferences coupled with Markov chain Monte Carlo (MCMC) with the General Time Reversible (GTR) substitution model, invariable proportion (I), and gamma distribution (Γ). For the ML and BI analyses an appropriate nucleotide substitution model was recovered using MODELTEST version 2.1.6 (Darriba and Posada, 2014; Guindon and Olivier, 2003). The GTR+I+G model was chosen using the Akaike Information Criterion (AIC). To generate a representative history of the Malagasy freshwater crabs, the ML analysis was run using GARLI (Zwickl, 2006). The robustness of nodal support was assessed with 1000 bootstrap replicates, the final tree search was conducted under the GTR+I+G model. The Bayesian phylogenetic reconstruction was executed in MrBayes v3.2.2 (Ronquist et al., 2012), analysis was also conducted under the Markov Chain Monte Carlo (MCMC) with chains 10×10^7 generations with a sampling frequency of 1000 generations and a burn-in of 25%. To illustrate the bootstrap values, a consensus tree restricted to bootstrap values ≥50% was generated using PAUP* 4.0, and FigTree v1.4.0 (Rambaut & Drummond, 2009) was used to visualize the consensus tree. Of the sampled parameters, convergence, and the potential autocorrelation (effective sampling size/ESS for all parameters > 200) was examined in Tracer v1.6 (Rambaut, 2009).

3. Results

3.1 Molecular Tree: COI Phylogeny

Phylogenetic analysis performed on the partial mitochondrial gene Cytochrome Oxidase I (COI) fragment (600 bp) recovered topologies with several highly supported clades. The MP, ML, and BI analyses all recovered similar topologies and grouped taxa into the same major clades with only minor differences. The MP analysis recovered supported relationships where a MP value of ≥60% was considered to be strong nodal support (Daniels et al., 2006). The MP 50% majority-rule strict consensus tree summarizing the results of the three independent analyses (MP, ML, and BI) is shown in Fig. 4 and was selected as the representative topology with strong nodal support for the principle lineages recovered.

The outgroup taxa *Deckenia mitis* from Tanzania, East Africa, *Seychellum alluaudi, S. mahafregate, S. silhouette* (all from the Seychelles), and *Afrithelphusa monodosa* from Guinea, West Africa formed a clade that was sister to the Malagasy clade which is consistent with previous Afrotropical freshwater crab molecular analyses (Daniels et al., 2015). Deep nodal support for these relationships is strong (100%). The consensus tree of the COI phylogeny recovered a topology that indicates that the Malagasy freshwater crabs form a monophyletic group, and that there are nine well-supported clades or lineages within the island's fauna.

3.1.1 Maximum Parsimony

Phylogenetic analysis yielded 36 most parsimonious trees with 1,000 bootstrap replicates. The shortest tree had 1,232 steps (with gaps treated as

missing), 236 informative parsimonious characters, 335 constant characters, and 29 variable parsimonious uninformative characters. The maximum parsimony tree indicates that the unidentified and identified Malagasy freshwater crab specimens included in this study shows a great deal of diversification within the Malagasy freshwater crabs that falls into four major groups (numbered here Groups I-IV) and nine well-supported clades (numbered here Clades 19). The groups and clades were clustered on the tree as follows: Group I (Clade 1), Group II (Clades 2-5), Group III (Clades 6-8), and Group IV (Clade 9).

Significantly, the six genera and ten species that were already identified from previous studies were each recovered as well-supported separate lineages on a particular clade. At the genus level, the following lineages were supported: *Malagasya* (Clade 2), *Marojejy* (Clade 6), *Foza* (Clade 7), *Skelosophusa* and *Boreathelphusa* (Clade 8), and *Hydrothelphusa* (Clade 9). At the species level, ten-existing species were recovered as well-supported lineages within one of the nine clades: *M. antongilensis* and *M. goodmani (Clade 2), M. longimerus (Clade 6), Foza ambohitra (Clade 7), S. eumeces* and *B. uglowi* (Clade 8), and *H. madagascariensis, H. agilis, H. bombetokensis,* and *H. vencesi* (Clade 9).

Group I includes the most basal lineage on the tree (Clade 1) which had strong statistical support (100% BPP) and comprises three unidentified individuals collected from the Marojejy National Park (F7579, F7592, and F7589). None of the identified genera or species from anywhere in Madagascar were included in this isolated clade, and these unknown specimens remain unidentified, and are referred to here as Taxon 1.1.

The topology of the rest of the tree is dominated by three major groups (lineages): Group II (Clades 2-5), Group III (Clades 6-8), and Group IV (Clade 9) each of which show a great deal of internal diversification that is discussed below. Group II (Clades 2-5) includes a basal clade (Clade 2) that was retrieved as monophyletic with strong statistical support (96% BPP) that comprised three well-supported lineages. Two of these are described species of *Malagasya*, while the third lineage is unknown. *Malagasya antongilensis* is represented on Clade 2 by one previously identified and sequenced specimen (F13934_M_anton.) and by two other previously unidentified specimens from this study (N29_M_anton. and N12_M_anton.). *Malagasya goodmani* is represented in Clade 2 by one previously sequenced specimen (F_M_goodmani) and one other from this study (F12668 Ml. taxa1). Interestingly, three of the unidentified specimens in the present study formed a third well-supported lineage within Clade 2 with 100% BPP support (*Ml. taxa 1*, F12645, F12648, and F12646). Given their position on the tree it is likely that these specimens belong to *Malagasya*, and because of their separation from the two-known species on this part of the tree it is likely that these may prove to be new to science upon further investigation. Clades 3, 4, and 5 of Group II form a sister group to Clade 2 (*Malagasya*) and each show further internal diversification. For example, Clade 3 was retrieved as a monophyletic group with strong statistical support (100% BPP) and comprised three well supported lineages. No previously named specimens are included in Clade 3, making it of great taxonomic interest. The most basal lineage within this clade is represented by a large-bodied specimen (F5464, referred to here as Taxon 3.1). The other lineage within Clade 3 is formed by four specimens Taxon

3.2 (F5727_1, F5727_2, F5731_2, and F5769) with strong nodal support (95% BPP). The group formed by Clades 4 and 5 has 71% support. Clade 4 itself has strong nodal support (98% BPP) and includes several unidentified specimens (referred to here as Taxa 4.1, 4.2, 4.3, and 4.4), that are each found on separate well-supported branches within the clade (Fig. 4). The most basal is Taxon 4.1 (F6643) (98% BPP), the next is Taxon 4.2 (F5745), followed by Taxon 4.3 (F5759_1, F5759_2, F5758, and F5712) (80% BPP), and Taxon 4.4 (F12526, F5732, F13939, F5748, and PB27556) (92% support). Clade 5 (100% BPP) is sister to Clade 4, and includes three individuals (A17531, F4652, and N1987) that are referred to here as Taxon 5.1. Interestingly, one of these three specimens that are included in Taxon 5.1 (FMNH 4652) was previously identified as '*Foza goudoti'* and this finding throws this genus assignment into doubt.

Group III (100% BPP) includes Clades 6, 7, and 8, each of which has an identified genus within the clade. The most basal is Clade 6 (99% BPP) that includes *Marojejy longimerus* (F4656) and this is sister to a group comprising three unidentified specimens (F7581, F7589, and F7598) (91% BPP) that are referred to here as Taxon 6.1 (Fig. 4), which given its position on the tree is most likely an unidentified species of *Marojejy*). Clade 7 (100% BPP) is formed by four specimens (N_17_F_amb, F13931_F_amb, F11046, and P_B_1054) that are all part of the unidentified new material, and are all tentatively identified here as *Foza ambohitra*. Clade 8 (79% BPP) comprises three specimens that belong to three identified taxa in two genera, *Boreathelphusa uglowi* (F5732_1), *Skelosophusa prolixa* (F7596), and *S. eumeces* (F7597).

Group IV comprises Clade 9 (97% BPP) with 15 specimens among which are four identified species of *Hydrothelphusa.* This clade clearly represents diversification within the genus *Hydrothelphusa*. Six specimens in Clade 9 (F7585, F5764, F5771, F5723, F5772_1, and F5471) form a lineage with 96% support that includes specimens identified as *H. bombetokensis* that cluster together with several of the previously unidentified specimens that are here all referred to this same species. This lineage is sister to a clade that includes identified specimens of *H. agilis* (F5729, F5804, and F7573) together with unidentified specimens here referred to this same species. Clade 9 also includes a well-supported lineage (89% BPP) that has identified specimens of *H. vencesi* that group closely with previously unidentified specimens that are here referred to this species (F7584, F7589_2, F7569, F13940, and F7567). The most basal lineage within Clade 9 comprises a single unidentified specimen (F6637_H.sp.1) that is referred to here as Taxon 9.1, and this may prove to be a new species of *Hydrothelphusa* upon further investigation.

3.1.2 Maximum Likelihood

The best tree recovered by the maximum likelihood analysis in GARLI 2.0 had a log-likelihood score of -8452 resulting phylogram was visualized using FigTree v4.1.2 (Rambaut, 2009) with branch lengths in units of substitutions per site (Fig. 5). Bootstrap values were obtained in GARLI 2.0 and are illustrated. The relationships presented here were recovered in over half of the bootstrap replicates. The ML analysis recovered a tree with a different overall topology from the MP tree, but it clearly includes the nine distinct clades found in the MP analysis. In addition, each of these nine clades included the same taxa as the MP analysis (Fig. 4). Notable

differences between the MP and ML trees (Figs. 4, 5) was that the ML analysis did not include a polytomy for Clades 2-9, and that the position of the nine lineages on the tree was not in the same. For example, although the most basal clade in both trees was Clade 1, in the ML tree Clade 1 (100% ML) was followed by a group that included Clades 6, 7, and 8 (representing *Marojejy, Foza, Skelosophusa,* and *Boreathelphusa).* Next was Clade 9 (representing *Hydrothelphusa*) (100% support) that included poor support (≤60%) for the sister group relationship between *H. agilis* and *H. vencesi* (designated by an asterisk (*). Clade 9 was sister to a group comprising Clades 2-5, with Clade 2 (representing *Malagasya*) separate from Clades 3-5, and Clades 3, 4 and 5 (representing several unidentified specimens).

3.1.3 Bayesian Inference

The .p files from the BI analysis were combined in Tracer 1.6 (Rambaut et al., 2014) and the ESS values were all >250 which indicate an effective independent search of the sample size during the MCMC analysis. The posterior probabilities (PP) generated in MrBayes 3.2.3 from the consensus tree are mapped out in Fig. 4. One consensus tree was retained and the analysis recovered the same 9 clades as the ML and MP trees. Clade 1 was most basal and separate to the other clades (2-9). Clades 2-4 (*Malagasya* and unidentified specimens) grouped together, Clades 6-8 (*Marojejy, Foza, Boreathelphusa,* and *Skelosophusa*) grouped together and Clade 9 (*Hydrothelphusa*) formed a single lineage.

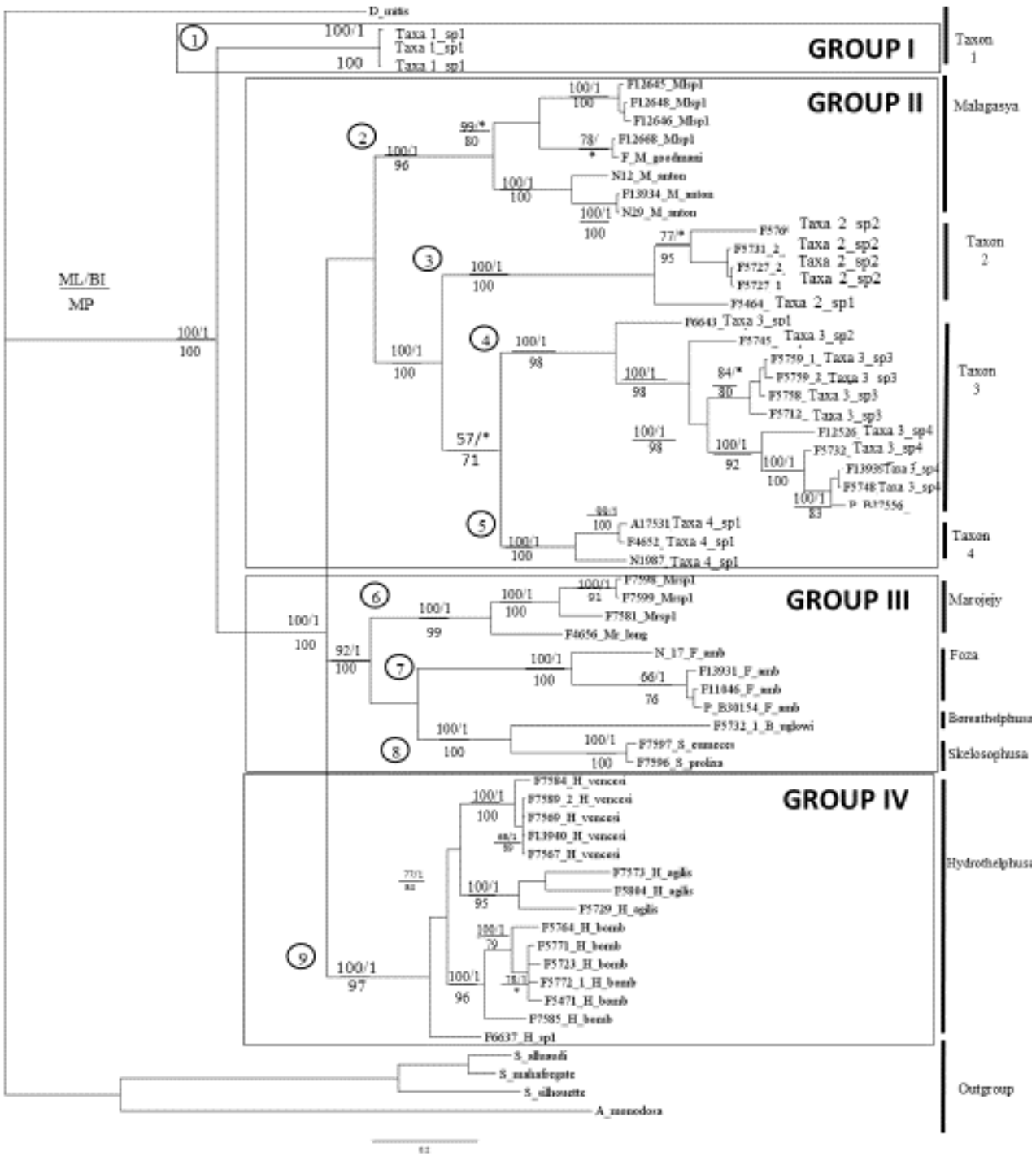

Fig. 4. A ML topology derived from the model GTR + I + Γ. The tree was rooted using five outgroups (Deckenia mitis, Afrithelphusa monodosa, Seychellum alluaudi, Seychellum mahafregate, and Seychellum silhouette). The values listed above the nodes are values for ML and posterior probability for Bayesian inferences, while the values below each node represent the MP bootstrap values. Each clade is denoted with a corresponding number surrounded by a circle at the base of the clade's branch. An (*) represents a value <60%.

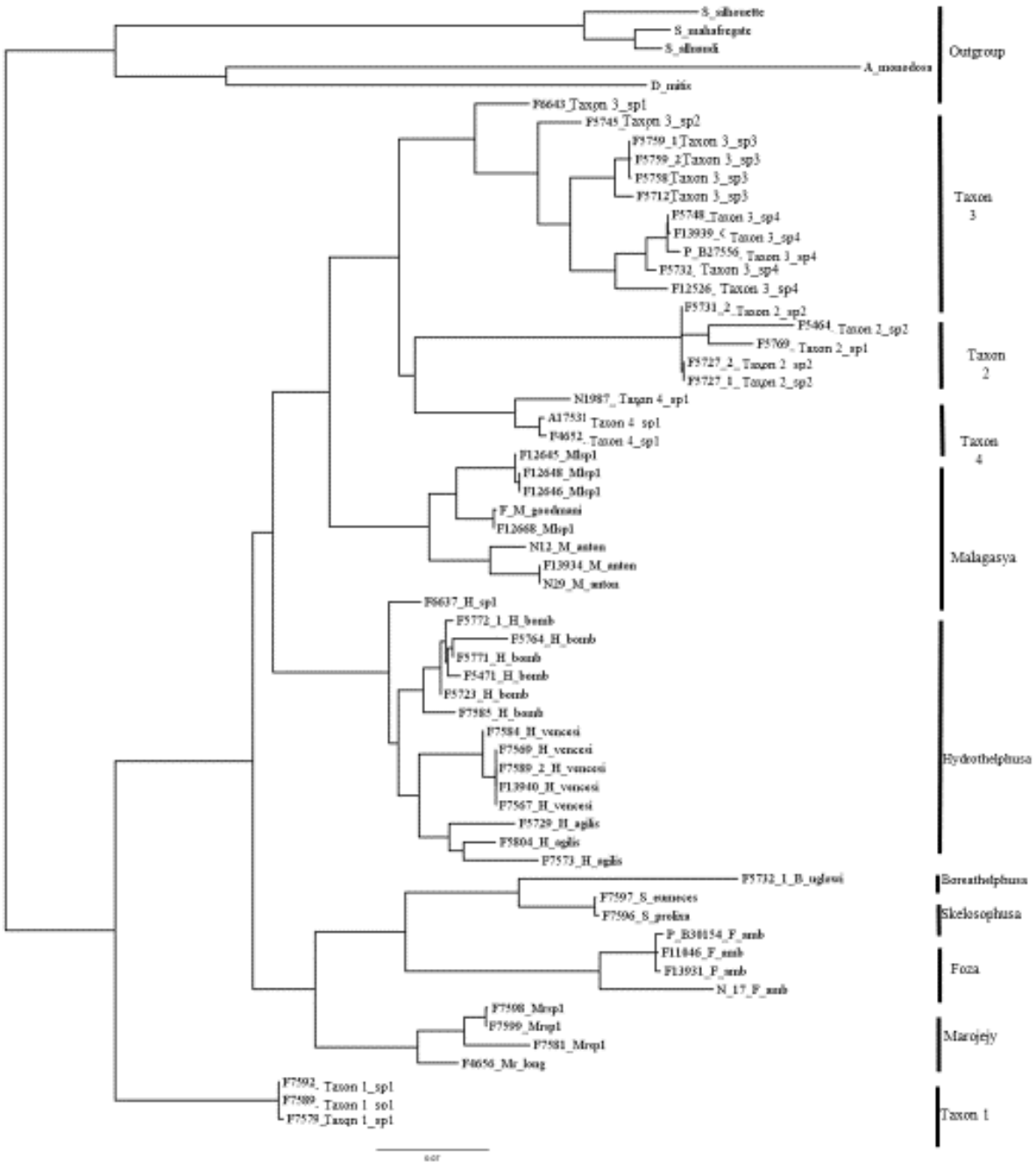

Fig. 5. A ML topology derived from the model GTR + I + Γ, using GARLI for the analysis and FigTree to visualize the results. The tree was rooted using five outgroups (Deckenia mitis, Afrithelphusa monodosa, Seychellum alluaudi, Seychellum mahafregate, and Seychellum silhouette).

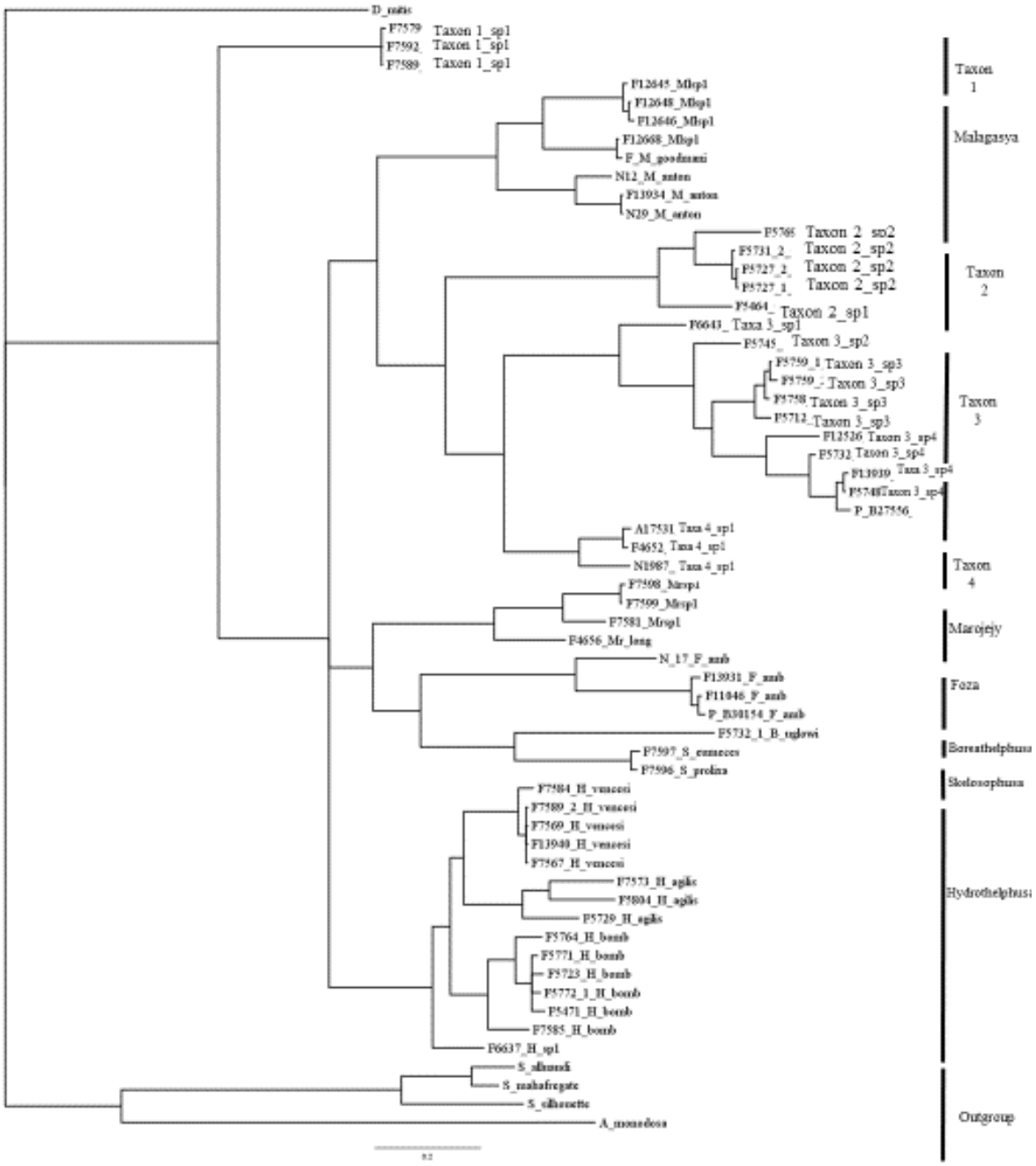

Fig. 6. A topology derived from the model GTR + I + Γ, using MrBayes for the analysis and FigTree to visualize the results. The tree was rooted using five outgroups (Deckenia mitis, Afrithelphusa monodosa, Seychellum alluaudi, Seychellum mahafregate, and Seychellum silhouette).

3.2 Morphological analysis

Clade 1 (F_7579, F_7592, F_7589) was phylogenetically distinct from all other lineages recovered in this study. Members of this clade (here referred to as Taxon 1.1) are small-bodied crabs that have a textured carapace surface (that is granulated anterolaterally and with carinae posterolaterally), a bilobed mandibular palp that has a small rounded lobe ¼ the length of the terminal segment at the junction between segments, a third maxilliped with an ischium that has a deep vertical sulcus and with a short flagellum on the exopod, a thoracic sternal sulcus s3/s4 that is deep in the center and faint at the edges, and slender ambulatory legs. This combination of morphological characters (Table 3) together with the phylogenetic isolation of these specimens on the COI tree may support the recognition of these specimens as new to science in future studies.

Clade 2 includes several specimens that had been identified in previous studies as belonging to two described species (*M. antongilensis* NMU 29.4.11.2001B, *M. goodmani* FMNH unaccessioned). Clade 2 therefore represents the genus *Malagasya* and the three different lineages correspond to three different species: *M. antongilensis* (N_29, F_13934, and N_12), *M. goodmani* (F_M_goodmani and F_12668), and the group of unidentified specimens (F_12645, F_12646, and F_12648) that may prove on further investigation to be a new species of *Malagasya. Malagasya* has a heart-shaped carapace and a distinctly bilobed mandibular palp with a large anterior lobe at the junction between the segments. The anterolateral margin of the carapace is lined with 5-6 small teeth, and the carapace has a highly deflexed frontal margin.

Table 3. List of morphological characters of *Taxon 1.1*.

Character	Taxon 1
Holotype Meaasurements	c.w. 38.8. c.l. 28.6. c.h. 12.3. f.w. 12.5.
Carapace outline	Transversly oval
Carapace texture	Punctate
PFC	Incomplete and Faint
Frontal Margin	Deflexed, lined with granules
Exorbital tooth	Small, low, broad
Epibranchial tooth	Reduced to a small granule
Cervical grooves	Faint U-shaped
AL surface of carapace	Granulated
PL surface of carapace	Carinae
AL margin	Granulated
Suborbital margin	Granulated
Suborbital region	Smooth with few small granules
Subhepatic region	Granules present and carinae
Pterygostomial region	Close to 3rd maxilliped smooth, granules distally
3rd MxP	Deep vertical groove
3rd MxP flagellum	Short
MP junction between segments	Small flap, ¾ length of terminal article
P1 Carpal teeth	1st is large, 2nd small/ low
P1 Merus inferior margins	Smooth, lined with faint granules
Major Cheliped (fixed finger of propodus)	2 small teeth followed by large fused molar
s3/s4	Deep at edges, faint in the center
s6/s7 meets a5	Meets a5/ a6
G1 terminal article	Slim straight flagellum, terminal article slightly curved, new.
G2 terminal segment	straight
P4 walking legs	Longest and slender, longer than P3
Sternoabdominal cavity	No setae present
Notes	Merus margin and inferior margin smooth with a low distal meral tooth.

Malagasya antongilensis (from Masoala and Mangoro in Toamasina Province) is distinguished from *M. goodmani* (from Tampolo in Toamasina Province) by the margins of the propodus of pereiopods p2 to p5 (toothed in *M. antongilensis* and with dense setae in *M. goodmani*) but the unidentified branch of this lineage (from the Bobankora Forest in Antsiranana Province) has smooth margins of the propodus of pereiopods p2 to p4, but p5 has patches of setae on the margins.

The two well-supported lineages in Clade 3 include no previously identified specimens, and these large bodied crabs are referred to here as Taxon 3.1 (F5464) and Taxon 3.2 (F5727_1, F5727_2, F5731_2, and F5769) and are morphologically different from the other recognized freshwater crab taxa on the island (Table 4 and Table 5). All individuals have an evenly curved anterolateral margin lined with small granules, an incomplete postfrontal crest that is faint and does not reach the anterolateral margins, and an incomplete sternal sulcus s3/s4 that does not reach the anterior margin of the sternoabdominal cavity, and a mandibular palp that has a small ledge at the junction between the segments.

The second largest clade in the phylogeny (Clade 4) is represented by 11 specimens that form four distinct branches within this clade. These are referred to here as Taxon 4.1 (F_6643), Taxon 4.2 (F_5745), Taxon 4.3 (F_5712, F_5758, F_5759.1, F_5759.2) and Taxon 4.4 (F_12526, F_5732, F_13939, F_5748, P_B27556) and are morphologically different from other Malagasy freshwater crabs (Table 6).

Table 4. List of morphological characters of Taxon 3.1

Character	Taxon 3
Holotype Meaasurements	c.w. 44.3. c.l. 35.3. c.h. 17.6. f.w. 15.0.
Carapace outline	Transversly oval
Carapace texture	Smooth with faint carinae
PFC	Incomplete and advanced
Frontal Margin	Moderately delfexed
Exorbital tooth	Small
Epibranchial tooth	Small
Cervical grooves	Deep not reaching epibranchial tooth
AL surface of carapace	Granulated
PL surface of carapace	Smooth
AL margin	Smooth with faint carinae
Suborbital margin	Small granules
Suborbital region	Smooth with few granules
Subhepatic region	Faint carinae
Pterygostomial region	Granulated except for inferior medial region which is smooth
3rd MxP	Deep vertical groove, slight 45° angle
3rd MxP flagellum	Long
MP junction between segments	Small with ¼ length flap
P1 Carpal teeth	Large pointed tooth followed by few small granules
P1 Merus inferior margins	Smooth with faint granules
Major Cheliped (fixed finger of propodus)	Distinct tooth pattern; dactylus matching pattern
s3/s4	Deep edges and does not meet sac
s6/s7 meets a5	Meets a5/ a6
G1 terminal article	Cone shape with lump
G2 terminal segment	Terminal article slender with slight curve
P4 walking legs	Average
Sternoabdominal cavity	No setae present
Notes	

Table 5. List of morphological characters of *Taxon 3.2*.

Character	Taxon 3
Holotype Meaasurements	CW 24.2, CL 18.2, CH 9.34 FW 7.1
Carapace outline	Transversly oval
Carapace texture	Smooth
PFC	Faint, incomplete and granulated
Frontal Margin	Moderately deflexed
Exorbital tooth	Low and broad, exorbital angle no tooth
Epibranchial tooth	Small
Cervical grooves	Deep and does not meet epibranchial tooth
AL surface of carapace	Faint carinae
PL surface of carapace	Light carinae
AL margin	Granulated
Suborbital margin	Smooth with light granules
Suborbital region	Smooth with light granules
Subhepatic region	Smooth with faint carinae
Pterygostomial region	Smooth with distince granules in pattern
3rd MxP	Deep with vertical groove
3rd MxP flagellum	Long
MP junction between segments	Ledge
P1 Carpal teeth	1st carpal tooth large and pointed, followed by 3 sharp teeth
P1 Merus inferior margins	Smooth with small granules laterally
Major Cheliped (fixed finger of propodus)	3 teeth increasing in size, followed by small sharp teeth
s3/s4	Faint edges, V-shape, does not meet sac
s6/s7 meets a5	Meets a5/ a6
G1 terminal article	Broad, cone shape with lump
G2 terminal segment	Straight and flagellum-like
P4 walking legs	Average
Sternoabdominal cavity	No setae present
Notes	

Table 6. List of morphological characters of *Taxon 4.1*.

Character	Taxon 4
Holotype Meaasurements	c.w. 45.0. c.l. 33.3. c.h. 18.5. f.w. 12.7.
Carapace outline	Transversly oval and high
Carapace texture	Smooth
PFC	Complete, meets epibranchial tooth and is distinct
Frontal Margin	Delfexed
Exorbital tooth	Small and low
Epibranchial tooth	Long ending just at epibranchial tooth
Cervical grooves	Deep, almost reach epibranchial tooth
AL surface of carapace	Smooth
PL surface of carapace	Carinae
AL margin	Smooth raised and granulated
Suborbital margin	Granules
Suborbital region	Smooth with few granules near longitudinal groove
Subhepatic region	Smooth
Pterygostomial region	Smooth with a ventrally granulated strip
3rd MxP	Deep vertical groove
3rd MxP flagellum	Long
MP junction between segments	Large flap ½ length with setae on terminal article
P1 Carpal teeth	Large pointed tooth followed by few small granules
P1 Merus inferior margins	Smooth lined with small granules
Major Cheliped (fixed finger of propodus)	3 teeth followed by one large tooth
s3/s4	Deep V-shape, meets sac
s6/s7 meets a5	Meets a5/ a6
G1 terminal article	Slender, cone shape with extended tip
G2 terminal segment	Flagellum-like
P4 walking legs	Average
Sternoabdominal cavity	No setae present
Notes	Purchased from a market in Antananarivo

It is possible that the lineage represented by Clade 4 will prove to include new taxa (possibly a genus with four species), but this will take a great deal of further investigation. All of the individuals that comprise Clade 4 have a transversely oval shape carapace, with faint carinae. The post frontal crest is incomplete, advanced, and slightly faint; the exorbital tooth is small, epibranchial tooth is small. The cervical grooves are deep and don't reach the post frontal crest. The anterolateral surface is of carapace has faint granules, and the anterolateral margin is smooth with faint carinae. The posterolateral surface is smooth, both the subhepatic and suborbital region and margin is smooth with a few distinct granules. The third maxilliped has a deep vertical groove in the ischium, and the flagellum of the exopod is long. These crabs also have a distinct tooth pattern of the cheliped, the propodus has three teeth proximally, followed by a large tooth, two small teeth, one large tooth, and followed by a series of small teeth. G1 and G2 are unique in that the terminal article along the medial side of G1 has a lump, and G2 is slender with a slight bend medially.

The three unidentified specimens in Clade 5 (NMU_1987, FMNH 4652, A17531) are phylogenetically different from other Malagasy freshwater crabs and are referred to here as Taxon 5.1. Morphological differences that set these taxa apart from the rest of the Malagasy freshwater crabs include a carapace that is smooth anterolaterally and with carinae posterolaterally, a smooth carapace sidewall suborbital region, a postfrontal crest that meets the anterolateral margins, deep and long cervical grooves, and a GO1 terminal article that is slender with an extended tip (Table 7).

Table 7. List of morphological characters of *Taxon 5.1*.

Character	Taxon 5
Holotype Meaasurements	c.w. 15.1. c.l. 10.8. c.h. 5.6. f.w. 5.0.
Carapace outline	Transversly oval
Carapace texture	Smooth
PFC	Faint and incomplete
Frontal Margin	Delfexed
Exorbital tooth	Small, round and low
Epibranchial tooth	Rounded to granule-like
Cervical grooves	Shallow and smooth
AL surface of carapace	Smooth
PL surface of carapace	Light carinae
AL margin	Small granules
Suborbital margin	Smooth
Suborbital region	Smooth
Subhepatic region	Smooth
Pterygostomial region	Smooth
3rd MxP	Faint groove
3rd MxP flagellum	Short
MP junction between segments	Large flap ½ length of terminal article, with slight setae
P1 Carpal teeth	1st carpal tooth pointed followed by distinct pattern of 2 granules and small tooth
P1 Merus inferior margins	Small granules
Major Cheliped (fixed finger of propodus)	Small distinct teeth pattern
s3/s4	Very faint, wide V-shape
s6/s7 meets a5	Meets a5/ a6
G1 terminal article	Slender with a lump present
G2 terminal segment	Slender , slightly curved
P4 walking legs	Average
Sternoabdominal cavity	Tuft of setae present
Notes	Specimen is damaged, extremely fragile

Clade 6 includes the genus *Marojejy* and is represented on the tree by the identified species *M. longimerus* (FMNH 4656). Interestingly there is another well-supported branch within this clade (99% BPP) that includes unidentified specimens (FMNH 7581, FMNH 7599, FMNH 7598) that may prove to be an undescribed species of *Marojejy*. The genus *Marojejy* is recognized by an oval- shaped smooth carapace, a faint postfrontal crest, a third maxilliped with a faint vertical sulcus and an exopod with a distinctly shortened flagellum, a shallow V-shaped s3/s4 sternal sulcus, and sternal sulcus s6/ s7 meets the abdomen at the junction between a5 and a6. Both GO1 and GO2 are slender with a lump present at the distal end of the terminal article. The specimens that form a sister group to *M. longimerus* are morphologically different in several ways. For example, the exorbital tooth of *M. longimerus* is small and rounded (vs. low but no tooth in the unidentified specimens) and the subhepatic region of the carapace sidewall of *M. longimerus* is granular (vs. smooth in the unidentified specimens).

Clade 7 represents *Foza* (97% BPP) and is shown on the tree by one identified species, *F. ambohitra* (for P_B30154, FMNH 11046_F_amb, FMNH 13931_F_amb, and NMU_17_F_amb). Although there are four species in this genus the specimens included in this study all appear to belong to *F. ambohitra* based on their affinities with this recognized species and did not include any representatives of *F. raimundi, F. goudoti,* and *F. manonae.* All species of *Foza* have a mandibular palp that is bilobed, and a G1 terminal segment that is tubular tapering to a broad tip. Members of the species *F. ambohitra* have a faint postfrontal crest, a narrow frontal carapace margin, an epibranchial tooth positioned forward on the margin that almost

touches the exorbital tooth, exceptionally long cervical grooves that resemble the outline steer horns, and carapace sidewalls with smooth suborbital and subhepatic regions.

Clade 8 consists of two recognized genera *Skelosophusa* and *Boreathelphusa*. Some of the specimens had been identified in previous studies, and here represent three described species (*B. uglowi* (FMNH 5732), *S. eumeces* (FMNH 11059), *S. prolixa* (FMNH 7596)). Three of the unidentified specimens included in the present study included within this clade are identified here based on their affinities with already recognized species. Interestingly, one of the unidentified specimens did not group with any of the four-recognized species and formed a separate lineage. For example, *S. eumeces* (F_7597) and *S. prolixa* (F_7596) represent two species of *Skelosophusa* (with 100% support for this genus), and one specimen (B. uglowi_F_5732_1) represents *B. uglowi* (with79 % support for this monotypic genus). Members of the genus *Skelosophusa* are recognized by a smooth carapace that is transversely oval, an incomplete postfrontal crest not reaching the anterolateral margin, an epibranchial tooth that is low and almost granular, smooth carapace sidewall suborbital and subhepatic regions, a sternal sulcus s3/ s4 that is faint, a 2-segmented mandibular palp is 2-segmented and that is not bilobed but has a small hard ledge at the junction between the segments, a third maxilliped ischium with a deep vertical sulcus and an exopod with a long flagellum, and a cone-shaped G1 terminal article. Both specimens used in this study, (F_M_7596, and F_M_7597) expressed all of these characters. Species-level differences within this genus are as follows. Characters unique to *S. eumeces* (and shown by F_7597) include two large molariform teeth on the fixed fingers (propodi) of both chelipeds.

Characters unique to *S. prolixa* (and shown by F_7596) include an epigastric region that has small cristae that are low almost smooth and a long and shallow cervical groove.

Members of the genus *Boreathelphusa* are recognized by the following characters that are also shown by the identified specimen included in this study of the species (*B. uglowi* F_5732.1). The carapace outline is transversely oval, the anterior carapace surface is smooth, the posterolateral regions of the carapace have short and light carinae, the postfrontal crest is faint and does not reach the anterolateral margins, the epibranchial tooth is blunt, low and continuous with the anterolateral margin, the pterygostomial region of the carapace sidewall is smooth except for a few sparse setae and a few small granules medially near the third maxillipeds, the mandibular palp is two segmented and not bilobed but there is a small hard ledge at the junction between her two segments, the third maxilliped ischium is smooth and the exopod has a long flagellum, medium sized exopod with a long flagellum. Major cheliped has series of small teeth, the dactylus is narrow with series of small teeth interspersed with larger teeth, a large molar tooth on the fixed fingers (propodi) of both chelipeds.

The largest of all the clades (Clade 9) with 15 individuals has strong support (97%) and represents the genus *Hydrothelphusa* that is one of the most widespread genera on Madagascar. Several of these 15 specimens had been identified to species in previous studies (Daniels et al., 2015), and here represent four described species: *H. agilis* (FMNH 5729, FMNH 5804, FMNH 7573), *H. bombetokensis* (FMNH 5764, FMNH 5771, FMNH 5772, FMNH 5723, FMNH 5471), *H. madagascariensis* (FMNH

7438), and *H. vencesi* (FMNH 7584, FMNH 7589_2, FMNH 7569, FMNH 13940, FMNH 7567).

Three of the unidentified specimens included in the present study that group within this clade are identified here based on their affinities with already recognized species. Interestingly, one of the unidentified specimens (F6637_Hsp1) did not group with any of the four-recognized species and formed a separate lineage. This finding warrants further study to test the hypothesis that this specimen may represent a taxon that is new to science. All species are large bodied crabs with an adult carapace width ranging from 40–44 mm to 61.9 mm, a distinctly indented frontal margin, an incomplete postfrontal crest that does not reach the anterolateral margins, a two segmented bilobed mandibular palp with a small flap roughly ⅓ the size of the terminal segment, a third maxilliped ischium that has a deep vertical sulcus and an exopod with a long flagellum, a thoracic sternal sulcus s3/s4 that is deep and y-shaped, and a GO2 terminal segment that is long, straight and flagellum-like. Species-level differences within this genus are as follows. Characters unique to *H. agilis* (and shown by F7573, F5804, and F5729) include a toothed and deeply indented frontal margin, a toothed suborbital margin, and a toothed margin of the p1 ischium. Characters unique to *H. madagascariensis* (that are shown by F6637_Hsp1) include a granulated (but untoothed) frontal margin, exorbital and epibranchial teeth that are both large and pointed; a heavily granulated carapace sidewall in the suborbital and subhepatic regions, and a granulated margin of the p1 ischium. Characters unique to *H. bombetokensis* (that are shown by F7585_H_bomb, F5471_H_bomb, F5772.1_H_bomb, F5723_H_bomb, F5771_H_bomb, and F5764_H_bomb) include

large, wide, triangular exorbital and epibranchial teeth, a large molariform cluster of three or four fused teeth on the fixed fingers (propodi) of both chelipeds, and a p1 carpus with a very short proximal tooth. Characters unique to *H. vencesi* include posterolateral regions of carapace that are marked by granules and short carinae, distinctly raised and separate epigastric crests, a distinct gap between the epigastric and postorbital crests, large, pointed exorbital and epibranchial teeth, and heavily granulated suborbital and pterygostomial regions of the carapace sidewall, and subhepatic regions with distinct short carinae.

3.3 Species distribution patterns

The new locality data included here support the findings of previous studies (Cumberlidge et al., 2003) that established that freshwater crabs are found throughout all six provinces in Madagascar, that they are particularly species rich in the northernmost Antsiranana Province, and although primarily in the east, that they are found in all five ecoregions (Figs. 7, 8). The new material included in this study has provided several new localities and has allowed updates to the species distribution maps for most species (Fig. 9), including new records from western Mahajanga Province and southwestern Toliara Province. Figure 10 illustrates the distribution of the unidentified taxa that are new to science.

The new locality data from 64 new specimens allow the updating of the distributions of many of the existing genera and species. Figure 9 include species data from over 100 localities in previously unexplored parts of Madagascar. Figures 11- 19 show specimens are grouped on the maps based on their membership within

one of the nine well-supported lineages recovered in the present study. In addition, the present work provides new locality data for existing species (for example, *H. bombetokensis* and *H. vencesi* are recorded here for the first time from the western part of Mahajanga Province). The present work also includes new locality data for *Malagasya antongilensis* that expand the range of this species westward to include the forested western region of Antananarivo Province (from a previous range that included the eastern forested highlands from the Masoala Peninsula in the north to Fianarantsoa Province in the south). The present study also includes two new records for *Foza ambohitra* from Marojejy National Park and one new record for *F. raimundi* Reed and Cumberlidge, 2006, from a locality 100 km inland in Toamasina Province. The specimens included in Clade 3 of the present study (referred to here as Taxa 3.1, 3.2, and 3.3) were collected from new localities Toliara Province and represent the first records of freshwater crabs from this part of the country. The only taxon not included in the distributional range maps here is *Glabrithelphusa angenei* due to a complete lack of label information beyond 'Madagascar' for the only known specimens (Meyer et al., 2014).

Fig. 7. Distribution map illustrating all localities of freshwater ecosystems with open circles where freshwater crabs were surveyed, reported for the Cumberlidge et al., 2003 study. Malagasy freshwater crabs are found in all five ecoregions of the island.

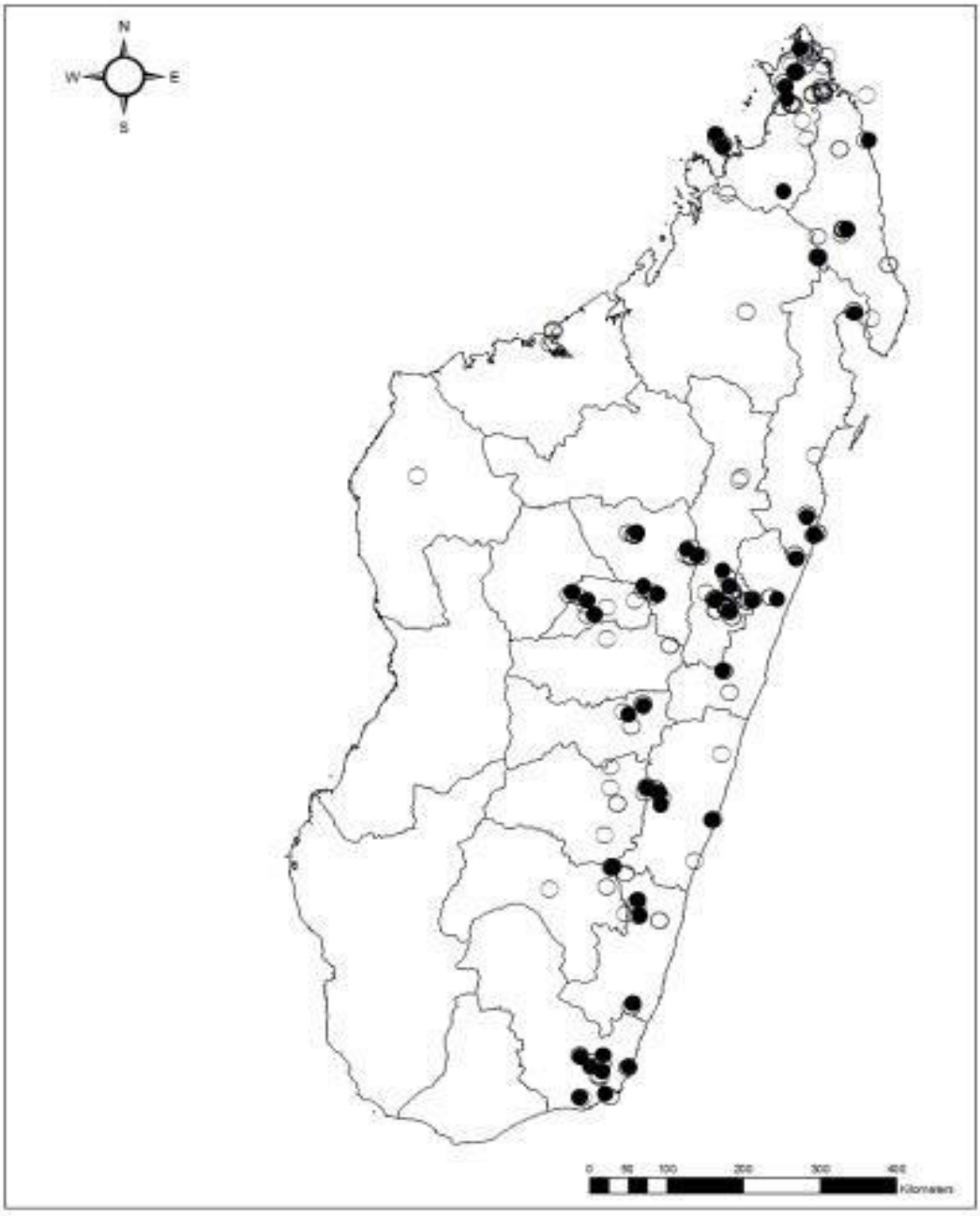

Fig. 8. Distribution map illustrating all localities of Malagasy freshwater crabs survey, dark circles represent localities where a freshwater crab was collected, for the Cumberlidge et al., 2003 study. Malagasy freshwater crabs are found in all five ecoregions of the island.

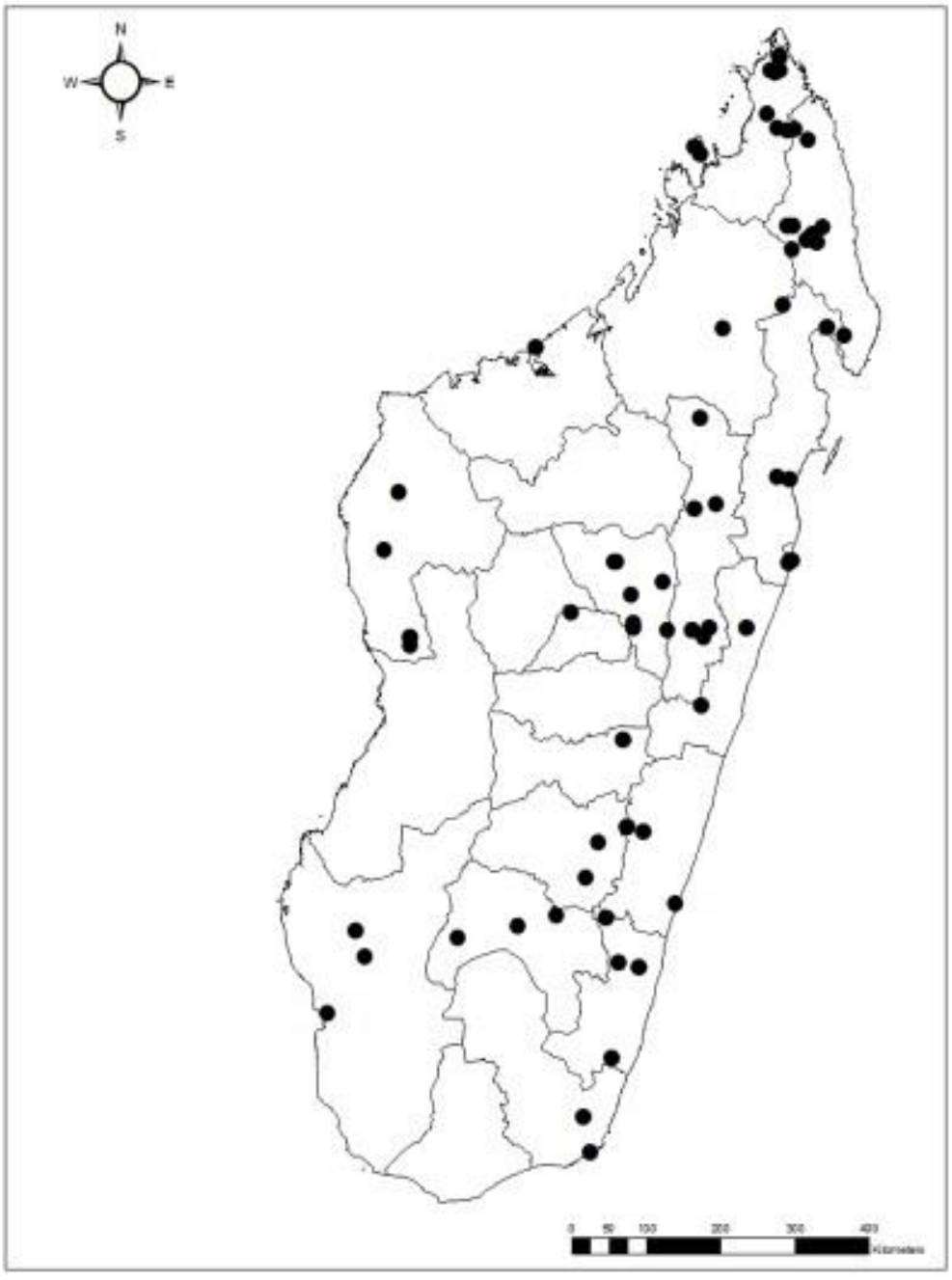

Fig. 9. Distribution map illustrating localities of newly collected Malagasy freshwater crab taxa. These points are all newly recorded for distributions and data collected from molecular assays.

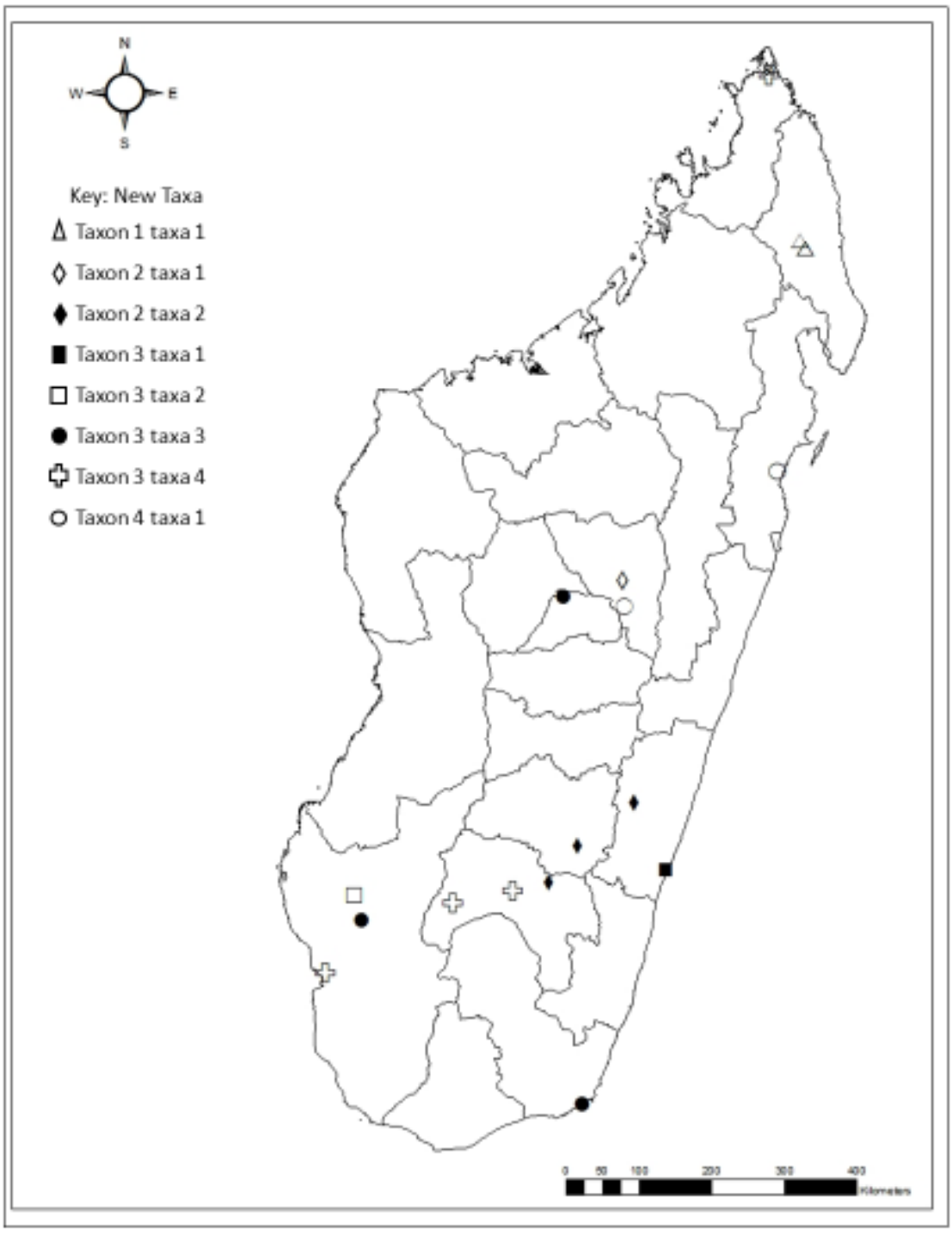

Fig. 10. Distribution map illustrating localities of new freshwater crabs that are potentially new to science.

Key:
● *Taxon 1.1*

Fig. 11. Distribution data of Clade 1 featuring *Taxon 1.1*.

Key:
● *Malagasya*

Fig. 12. Distribution data of Clade 2 featuring *Malagasya prior this study.*

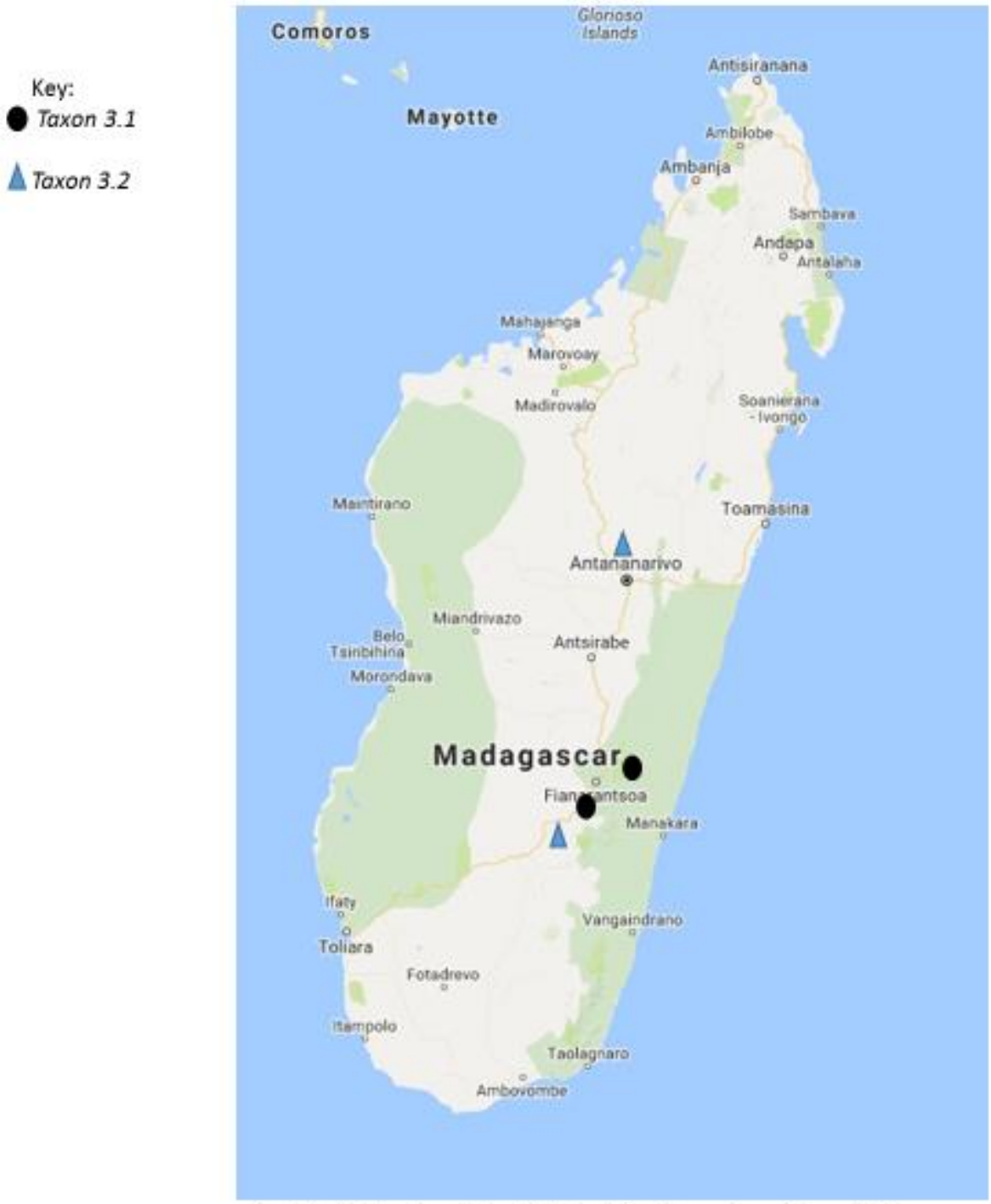

Fig. 13. Distribution data of Clade 3 featuring *Taxa 3.1 and 3.2*

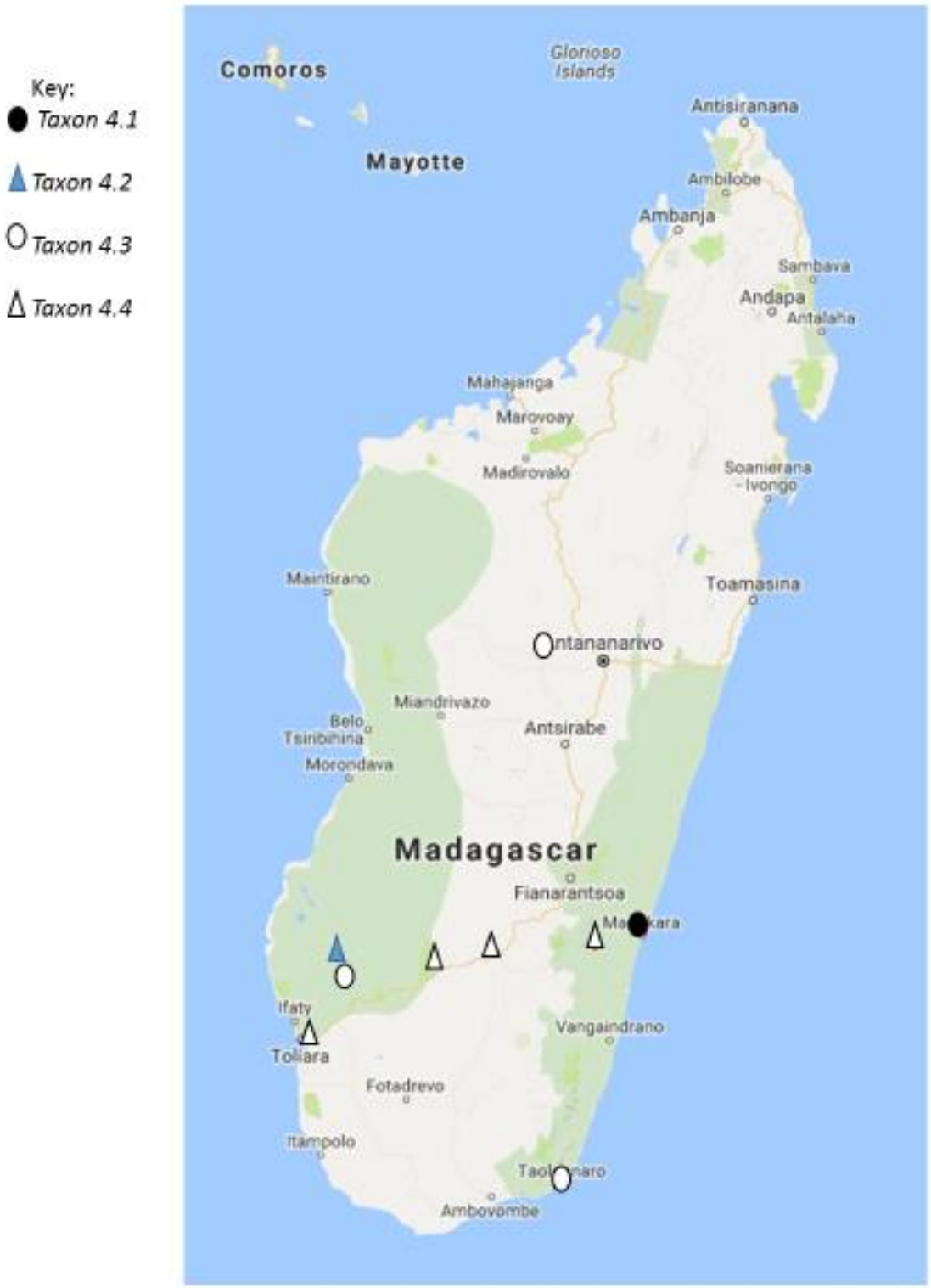

Fig. 14. Distribution data of Clade 4 featuring *Taxa 4.1, 4.2, 4.3 and 4.4.*

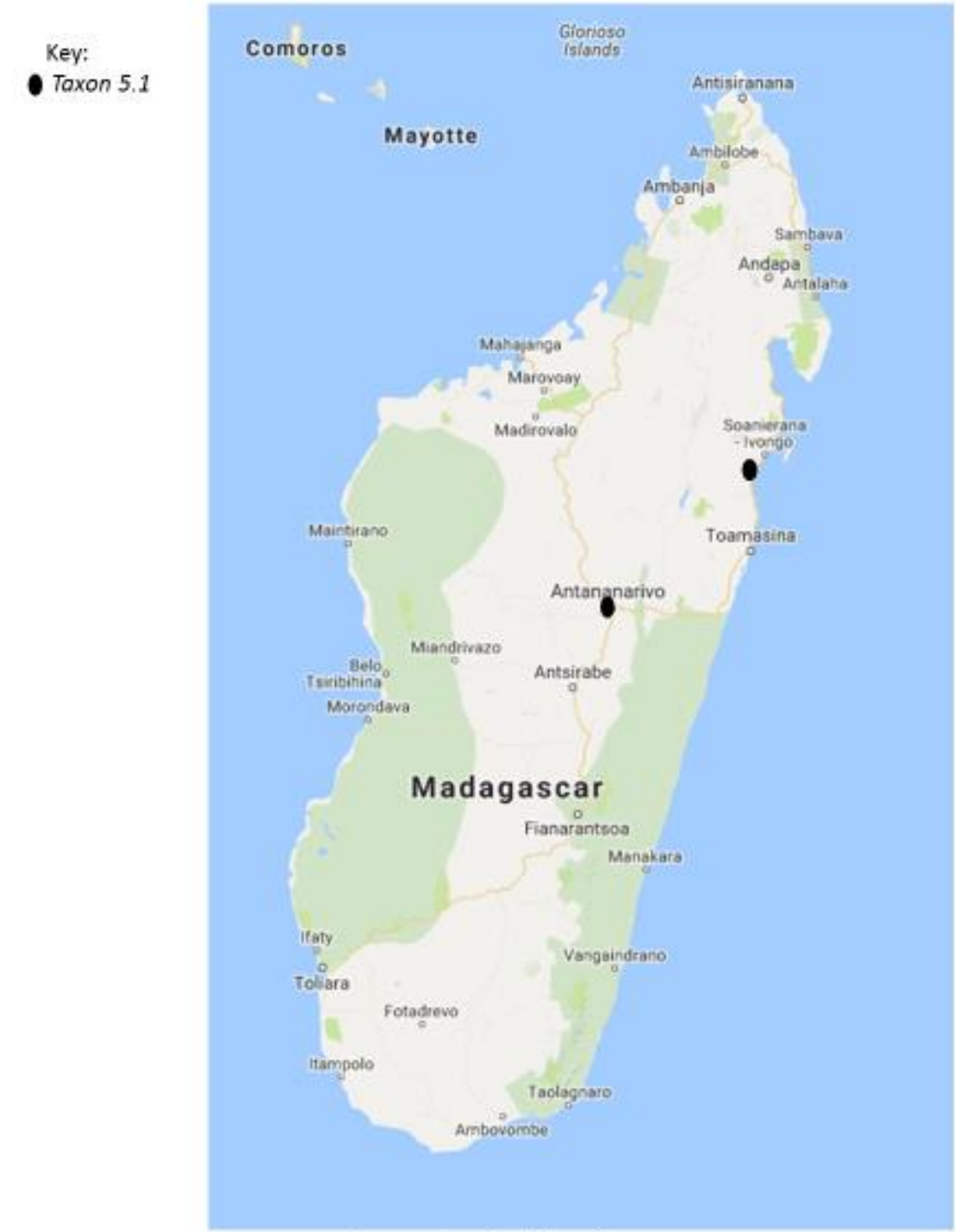

Fig. 15. Distribution data of Clade 5 featuring *Taxa 5.1*.

Key:
● *Marojejy*

Fig. 16. Distribution data of Clade 6 featuring *Marojejy prior to this study.*

Key:
● *Foza ambohitra*

Fig. 17. Distribution data of Clade 7 featuring *Foza ambohitra*.

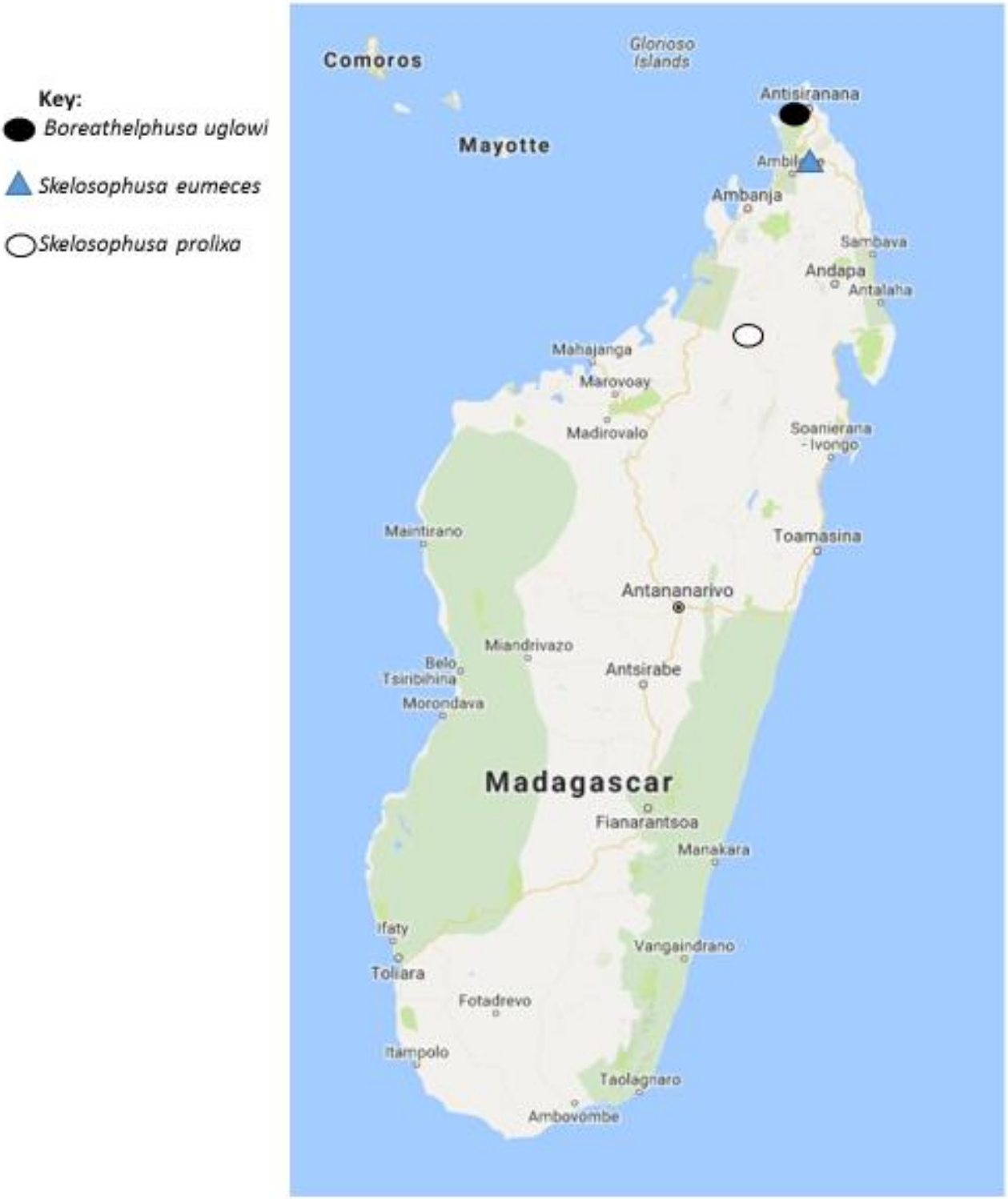

Fig. 18. Distribution data of Clade 8 featuring *Boreathelphusa* and *Skelosophusa eumeces*, and *Skelosophusa prolixa*.

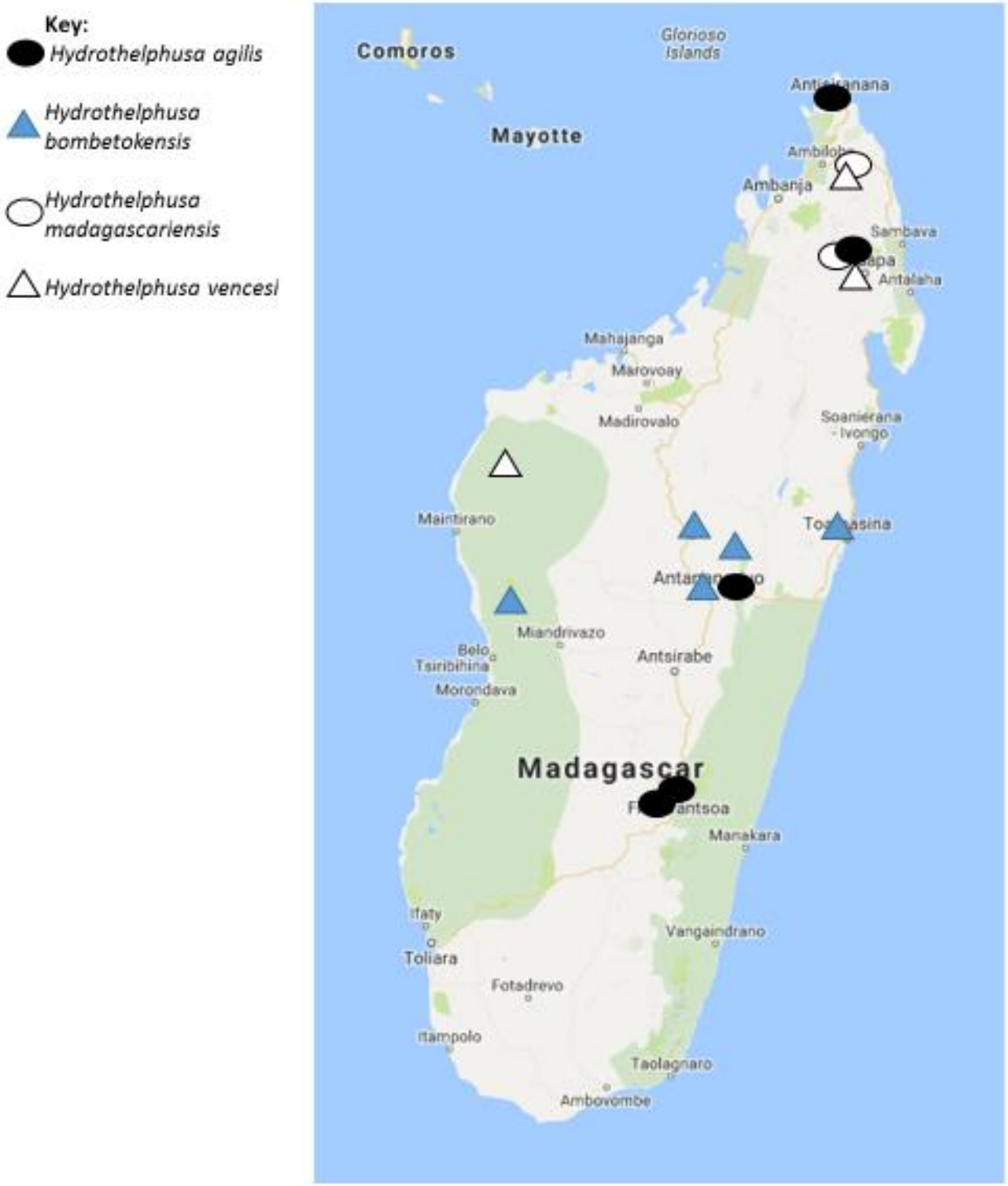

Fig. 19. Distribution data of Clade 9 featuring *Hydrothelphusa*.

3.3.1 Distributional Range Overlap and Sympatry

The distributional ranges of several species were found to overlap, and some records of species collected from the same locality indicate that they are living in sympatry. The distributional ranges of multiple species from Antsiranana Province (*S. eumeces, H. agilis, H. bombetokensis, H. madagascariensis, H. vencesi, F. ambohitra, B. uglowi, Madagapotamon humberti, Malagasya antongilensis,* and *M. goodmani*) plus three unidentified specimens (Taxa 1.1, 4.4, and *Malagasya* sp1).

Antsiranana Province is home to three major national parks and reserves: the Montagne d'Ambre National Park, the Ankarana Reserve, and the Reserve Speciale d'Andrafiamena. There have been a total of 14 different species collected throughout this northern province (Fig. 20). One notable region of species richness in the north centers on the area surrounding Mount Maromokotro in the Tsaratanana Massif. There may also be other species from this part of the island because some of the specimens belonging to *Malagasya* (whose species identification has not yet been resolved) were collected recently from this region but have not been included in this study.

A second locality in the northern Antsiranana Province is the Marojejy National Park where at least eight species of freshwater crabs occur. Freshwater crabs have been collected from the Marojejy Massif in the park where the habitat includes dense lowland rainforests and high altitude peaks with scrub and alpine grass fields. *Marojejy* is endemic to Marojejy National Park, and *H. agilis, H. madagascariensis, H. vencesi, F. ambohitra, S. eumeces,* and Taxon 1.1 are also found there (Fig. 21).

A third area where species distributions overlap is located on the eastern coastal area of Toamasina Province around Tampolo, where a small region of about 50 km^2 is home to *M. antongilensis* and *M. goodmani,* plus two other as yet unidentified specimens that may prove to belong to new species (*Malagasya* sp 1, and Taxon 5.1,). Another significant region of species richness is in Antananarivo Province in the center of the island where six species (*H. agilis, H. bombetokensis, H. madagascariensis, F. goudoti, F. raimundi,* and Taxon 2.1) were all collected close to each other, while *H. madagascariensis* and Taxon 4.3 were collected from the north-west and western river basin ecoregions. (Fig. 22).

To the south and west of Antananarivo Province there are three areas spanning roughly 100 km^2 where the distributions of nine taxa overlap: *H. agilis, H. madagascariensis, H. bombetokensis, F. raimundi, F. goudoti, M. longimerus, Malagasya antongilensis,* Taxa 3.1 and 4.3. These species inhabit an area that includes the Eastern lowlands, and the Eastern Central and Northern Highlands, which have varying topography, altitude, and watershed drainage systems (Fig. 23).

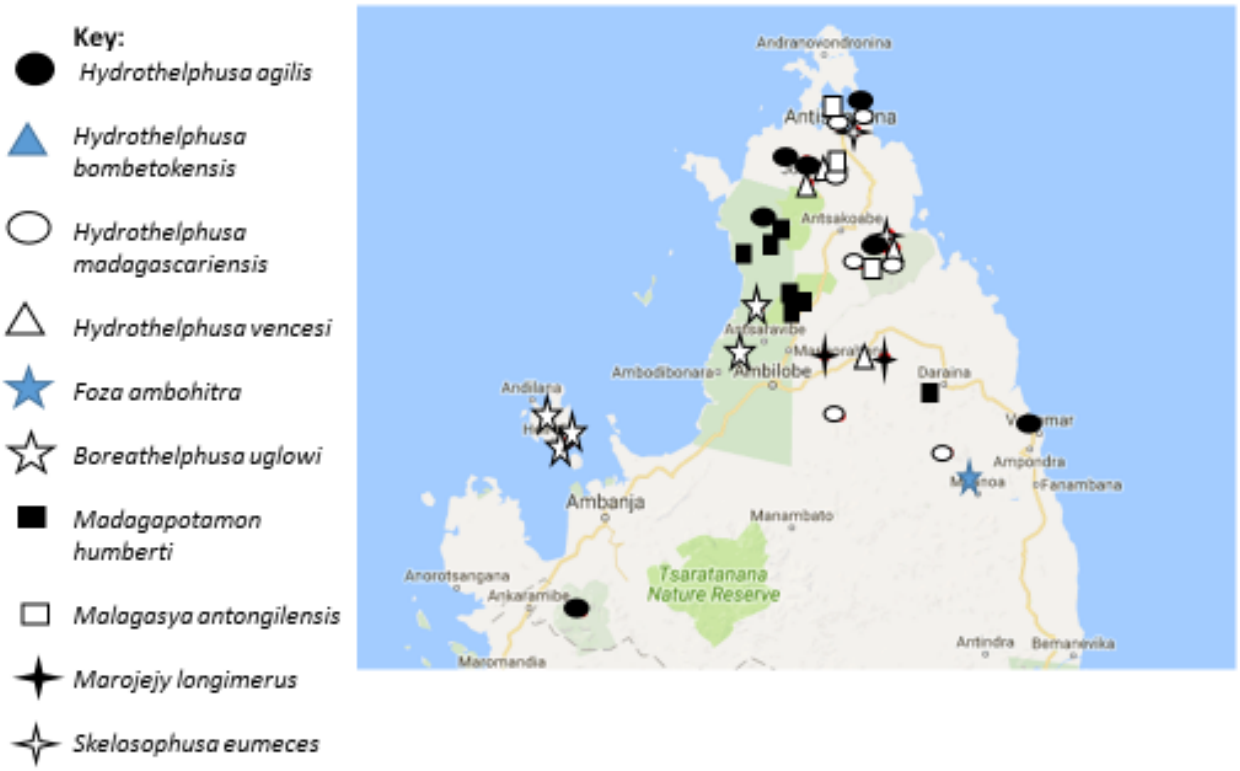

Fig. 20. Overlap distributions in the Antsiranana Province. Each dot represents a different species. Hot spot includes 10 taxa; some FWC were collected with other species so coordinates are the same and cannot be differentiated on the map. Taxa include the following: *S. eumeces*, *H. agilis*, *H. bombetokensis*, *H. madagascariensis*, *H. vencesi*, *F. ambohitra*, *B. uglowi*, *Madagapotamon humberti*, *Malagasya antongilensis*, and *Marojejy longimerus*.

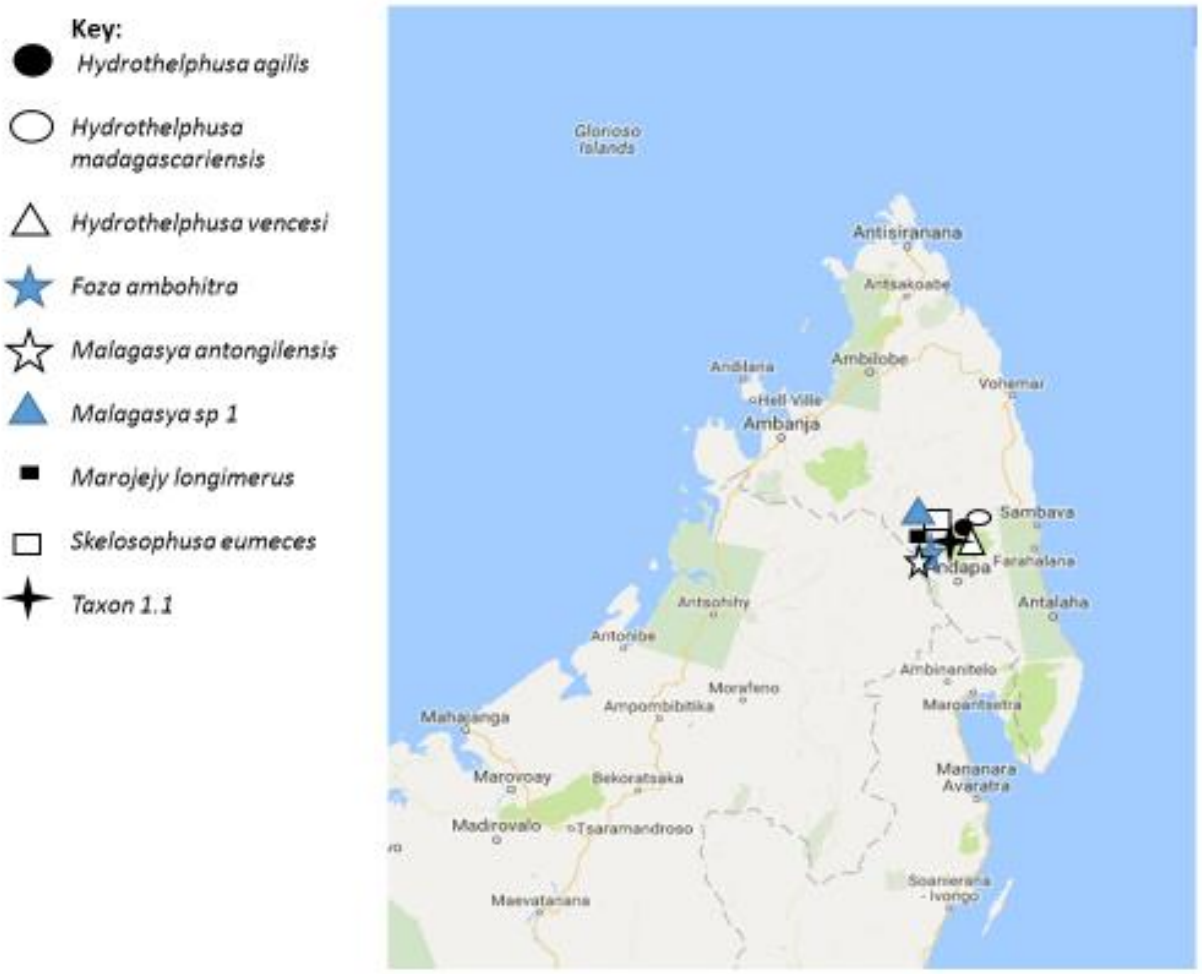

Fig. 21. Overlap distributions in the Antsiranana Province, in the Marojejy National Park. Each dot represents a different species. Hot spot includes 9 taxa; some FWC were collected with other species so coordinates are the same and cannot be differentiated on the map. Taxa include the following: *S. eumeces, H. agilis, H. madagascariensis, H. vencesi, F. ambohitra, M. longimerus, Malagasya antongilensis*, and unidentified *Marojejy sp1,* and *Taxon 1.1*.

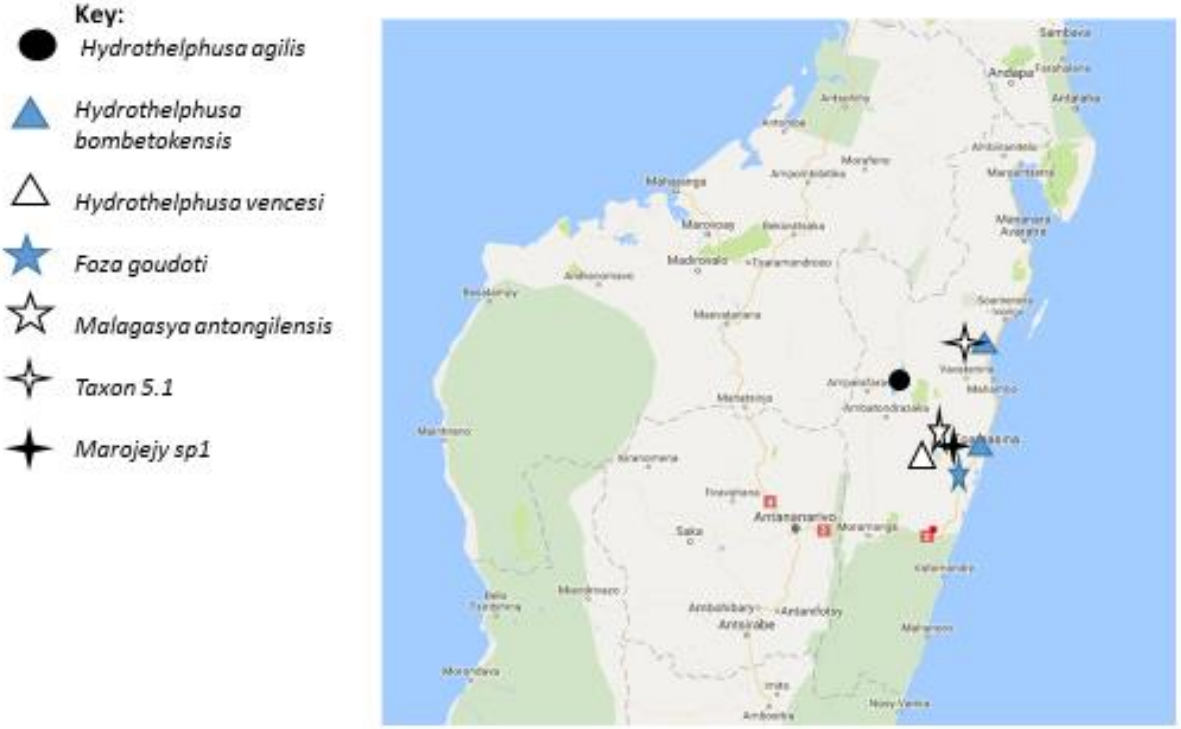

Fig. 22. Overlap distributions in the Toamasina Province. Each dot represents a different species. Hot spot includes 7 taxa; some FWC were collected with other species so coordinates are the same and cannot be differentiated on the map. Taxa include the following: *H. agilis, H. bombetokensis, H. vencesi, Foza goudoti, Malagasya antongilensis,* and unidentified *Marojejy sp1,* and *Taxa 5.1.*

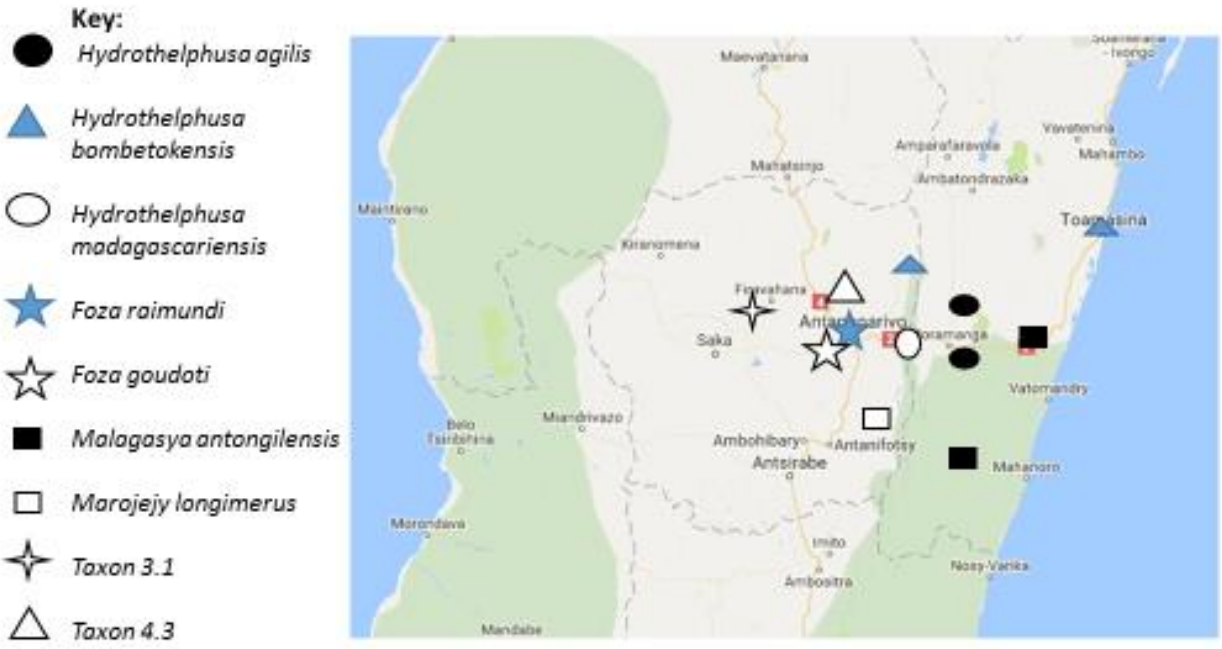

Fig. 23. Overlap distributions in the south central Toamasina province and westward into Antananarivo Province. Each dot represents a different species. Hot spot includes 8 taxa; some FWC were collected with other species so coordinates are the same and cannot be differentiated on the map. Taxa include the following: *H. agilis, H. madagascariensis, H. bombetokensis, F. raimundi, F. goudoti M. longimerus, Malagasya antongilensis*, and unidentified *Taxon 3.1 and* Taxon 4.3.

Six species of freshwater crabs are found in Ranomafana National Park and elsewhere in Fianarantsoa Province: *H. agilis*, *H. madagascariensis*, *H. bombetokensis Malagasya antongilensis, M. antongilensis*, and Taxon 3.2. (Fig. 24). There is a second hotspot about 100km south of the Ranomafana National Park in the Central Highland ecoregion with 6 taxa: *H. madagascariensis, H. vencesi, Malagasya antongilensis, Marojejy longimerus*, and Taxa 3.2 and 4.3 (Fig. 25).

Two other areas of interest where species distributional ranges overlap are in southern Toliara Province and western Mahajanga Province. Until the present study there were almost no records of freshwater crabs from either of these provinces, so these distributional range extensions are of great interest. Toliara Province has a unique community of freshwater crabs with four species. The extension of the distributional range of *M. antongilensis* to the above provinces is important because this species was previously known only from the northern region of the island. Finally, the distributional range of *H. bombetokensis*, *H. madagascariensis, M. antongilensis*, and Taxon 4.3 overlap in Toliara Province, while the ranges of *S. eumeces* and *H. vencesi* overlap in the west of Mahajanga Province. (Fig. 26).

Fig. 24. Overlap distributions in Fianarantsoa province. Each dot represents a different species. Hot spot includes 5 taxa; some FWC were collected with other species so coordinates are the same and cannot be differentiated on the map. Taxa include the following: *H. agilis*, *H. madagascariensis*, *H. bombetokensis*, *Malagasya antongilensis*, and unidentified *Taxa 3.2*.

Fig. 25. Sympatric distributions in southern Fianarantsoa province. Each dot represents a different species. Hot spot includes 7 taxa; some FWC were collected with other species so coordinates are the same and cannot be differentiated on the map. Taxa include the following: *H. madagascariensis, H. vencesi, Malagasya antongilensis, Marojejy longimerus, Foza goudoti* and unidentified *Taxa 3.1,* and T*axa* 4.3.

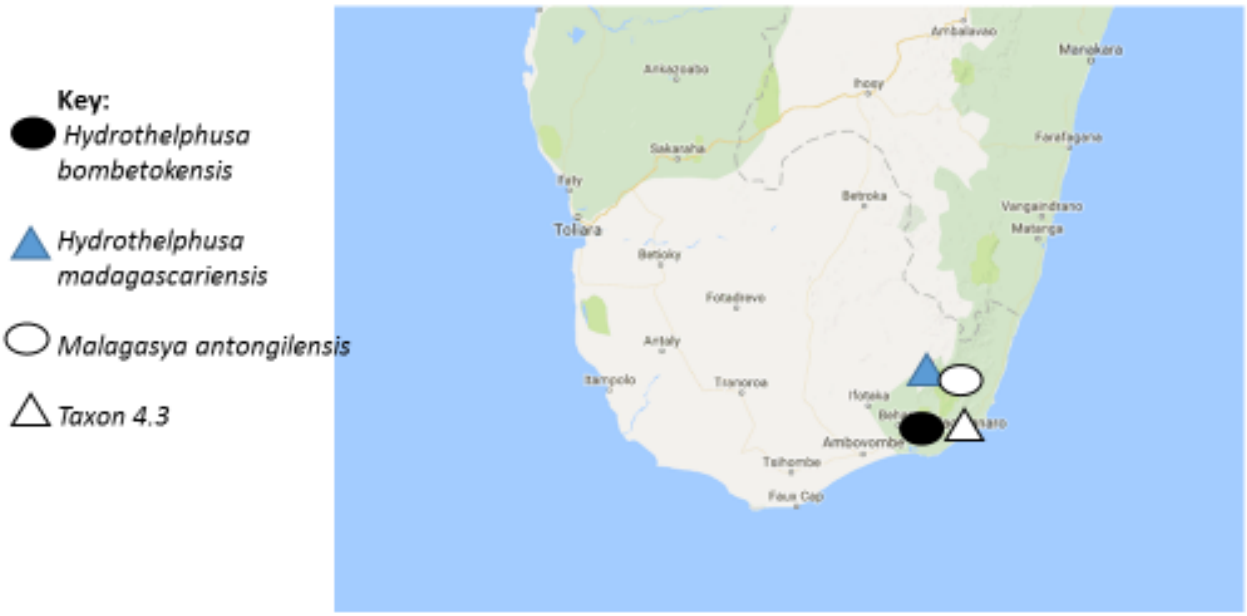

Fig. 26. Sympatric distributions in southern Toliara province. Each dot represents a different species. Hot spot includes 4 taxa; some FWC were collected with other species so coordinates are the same and cannot be differentiated on the map. Taxa include the following: *H. madagascariensis,* H. bombetokensis, *Malagasya antongilensis,* and unidentified *Taxa* 4.3.

Fig. 27. Sympatric distributions in southern Mahajanga province. Each dot represents a different species. Hot spot includes 2 taxa. Taxa include the following: *H. vencesi*, and S. eumeces.

4. Discussion

4.1 Taxonomic Conclusions

The fact that six identified genera of Madagascan freshwater crabs included in the study were recovered as well-supported clades (*Malagasya* (Clade 2), *Marojejy* (Clade 6), *Foza* (Clade 7), *Boreathelphusa* and *Skelosophusa* (Clade 8), *Hydrothelphusa* (Clade 9) provides molecular support for the continued use of these taxonomic groups. The earlier molecular studies included six genera and six molecular markers (Daniels et al., 2006), and seven genera and four molecular markers (Daniels et al., 2015) to build the trees of relationships. Both of those studies provided support for the recognition of the current view of Madagascan freshwater crab taxonomy and classification. Although the Malagasy freshwater crab genera *Madagapotamon* and *Glabrithelphusa* were not included in the molecular part of this study the morphological comparisons of these species with the unknown specimens in the present study indicated that there were no representatives of either of these genera in the specimens sequenced here, including among the unidentified specimens. Phylogenetic evidence for the validity of *Madagapotamon* comes from the study by Daniels et al. (2015) where it was recovered on their well-supported ML tree as the most basal lineage.

The specimen currently identified as *'Foza goudoti'* (FMNH 4652) was recovered on Clade 5 (together with AMNH 17531 and NMU 1987) and not on the same clade as other specimens assigned to *Foza*. For example, the four specimens of *F. ambohitra* grouped closely together on Clade 7 as sister to *Skelosophusa* and *Boreathelphusa*. The

same grouping of these three genera was recovered by Daniels et al. (2015) for *Foza raimundi*, *Skelosophusa*, and *Boreathelphusa*. The clade for *F. ambohitra* (Clade 7) and for *F. raimundi* (Daniels et al., 2015) are considered here to represent the genus *Foza* because the specimen of *F. raimundi* in Daniels et al. (2015) (FMNH 7438) is the type species of the genus *Foza*. The study of Daniels et al. (2015) included '*F. goudoti'* (FMNH 4652) and *F. raimundi* (FMNH 7438) but flagged a problem because these taxa were recovered on different lineages (Daniels et al., 2015: fig. 2, table 1). There is therefore reason to believe that the specimens identified as '*F. goudoti'* may belong to a previously described taxon or may even be new to science. Further studies are needed to settle this taxonomic problem arising from a conflict between morphological and molecular findings, that involve examination of the type specimen of *Thelphusa goudoti* and intensified DNA analyses that use more genetic markers.

Many unidentified specimens included in the present study must remain unidentified at this point but are all worthy of further taxonomic investigation because the molecular analysis recognizes that they occupy well-supported phylogenetic lineages. The MP tree (Fig. 4) does nevertheless allow limited and tentative conclusions to be drawn about the probable taxonomic status of these specimens. For example, Taxon 2.1 most likely belongs to the genus *Malagasya*, Taxon 6.1 most likely belongs to the genus *Marojejy*, and Taxon 9.1 most likely belongs to the genus *Hydrothelphusa*. The placement of several other unidentified specimens in an already described genus is not possible here because they are not positioned in lineages that include any of the already identified genera (Fig. 4). The resolution of the precise taxonomic status of these specimens is of great scientific

interest, but requires a great deal of additional study that would include running molecular assays including a wider range of genes above using only a single gene, like COI. However, due to resource constraints this became well beyond the scope of this thesis.

The in-group taxon sampling here (48 previously unstudied specimens of freshwater crabs from all parts of the island) compares well with the taxon sampling in previous studies and is the first molecular study of the Madagascan freshwater crab fauna to include more than 13 individuals. The improved sampling used here, although only a preliminary study, has revealed an unexpectedly high amount of hidden diversity in the Malagasy freshwater crab fauna. The findings of Daniels et al. (2006; 2015) that the Madagascan freshwater crabs are monophyletic and are part of the wider Afrotropical freshwater crab fauna (resulting in their inclusion in the African family Potamonautidae) is also supported in the present work. The diversification within the phylogenetic tree represented by nine well-supported clades represents the best-supported summary of Malagasy freshwater crab relationships that have been recovered to date.

Table 8. List of the unidentified Malagasy specimens included in the present study. These specimen need further taxonomic investigation because of the molecular analysis placing them in their well-supported lineage with no other already indemnified taxa.

	Specimen	GROUP & CLADE	Identification	Province	Genus
		GROUP I: CLADE 1			
1	FMNH 7579	Clade 1	*Taxon 1.1*	Antsiranana	Unidentified
2	FMNH 7589.2	Clade 1	*Taxon 1.1*	Antsiranana	Unidentified
3	FMNH 7589.3	Clade 1	*Taxon 1.1*	Antsiranana	Unidentified
3		**GROUP II: CLADES 2-5**			
4	FMNH 12645	Clade 2	*Taxon 2.1*	Antsiranana	*Malagasya*
5	FMNH 12646	Clade 2	*Taxon 2.1*	Antsiranana	*Malagasya*
6	FMNH 12648	Clade 2	*Taxon 2.1*	Antsiranana	*Malagasya*
7	FMNH 5464	Clade 3	*Taxon 3.1*	Fianarantsoa	Unidentified
8	FMNH 5769	Clade 3	*Taxon 3.2*	Antananarivo	Unidentified
9	FMNH 5727.1	Clade 3	*Taxon 3.2*	Fianarantsoa	Unidentified
10	FMNH 5727.2	Clade 3	*Taxon 3.2*	Fianarantsoa	Unidentified
11	FMNH 5731.2	Clade 3	*Taxon 3.2*	Fianarantsoa	Unidentified
12	FMNH 6643	Clade 4	*Taxon 4.1*	Fianarantsoa	Unidentified
13	FMNH 5745	Clade 4	*Taxon 4.2*	Toliara	Unidentified
14	FMNH 5712	Clade 4	*Taxon 4.3*	Antananarivo	Unidentified
15	FMNH 5758	Clade 4	*Taxon 4.3*	Toliara	Unidentified
16	FMNH 5759.1	Clade 4	*Taxon 4.3*	Toliara	Unidentified
17	FMNH 5759.2	Clade 4	*Taxon 4.3*	Toliara	Unidentified
18	MNHN B27556	Clade 4	*Taxon 4.4*	N/A	Unidentified
19	FMNH 12526	Clade 4	*Taxon 4.4*	Fianarantsoa	Unidentified
20	FMNH 13939	Clade 4	*Taxon 4.4*	Toliara	Unidentified
21	FMNH 5732.1	Clade 4	*Taxon 4.4*	Antsiranana	Unidentified
22	FMNH 5748	Clade 4	*Taxon 4.4*	Fianarantsoa	Unidentified
23	AMNH 17531	Clade 5	*Taxon 5.1*		Unidentified
24	FMNH 4652	Clade 5	*Taxon 5.1*	"Foza goudoti'	Unidentified
25	NMU 1987	Clade 5	*Taxon 5.1*		Unidentified
		GROUP III: CLADE 6			
26	FMNH 7581	Clade 6	*Taxon 6.1*	Antsiranana	*Marojejy*
27	FMNH 7598	Clade 6	*Taxon 6.1*	Antsiranana	*Marojejy*
28	FMNH 7599	Clade 6	*Taxon 6.1*	Antsiranana	*Marojejy*
		GROUP IV: CLADE 9			
29	FMNH 6637	Clade 9	*Taxon 9.1*	Antsiranana	*Hydrothelphusa*

4.2 Biogeographical Conclusions

The new field collections analyzed in this study have expanded our knowledge of Malagasy freshwater crab relationships and distributional ranges, and has confirmed their presence in new parts of the island such as the southern and western river basins of Toliara Province. This expanded distribution highlights areas of the island that were previously thought to be unsuitable habitats for freshwater crabs. For example, freshwater crabs were found to occur in the south-west arid regions where the southern limit of the distribution of freshwater crabs in the eastern highland and eastern lowland ecoregions parallels the boundary between the year-round freshwater habitats (Cumberlidge et al., 2003). Previous species distribution studies have been limited by the specimen label information provided by collectors. Fortunately, most of the specimens included in this study were collected recently by Dr. S. M. Goodman, a field biologist for The Field Museum of Natural History in Chicago, Illinois, who has provided detailed locality and habitat data for all specimens that he collected. Madagascan freshwater crabs are presumably found in most freshwater habitats throughout the island, but exploration for freshwater crabs is far from comprehensive and thus there are still a number of gaps in the distribution maps for the species in many parts of the island. Past studies have established that there is a distinct species richness hotspot in Antsiranana Province, followed by high diversities in the forested eastern mountain range from north to south.

Daniels et al. (2006; 2015) concluded by using substitution models with a molecular clock that freshwater crabs reached Madagascar by transoceanic dispersal 55 mya and present day patterns of distribution are the result of subsequent dispersal

throughout the island. The largest freshwater faunal survey on Madagascar was the nine-year island wide study conducted by Jean-Marc Elouard and colleagues from the Institut de Recherche pour le Developpement. The freshwater crab data from that study were analyzed and reported on by Cumberlidge et al. (2003), who created the first-ever comprehensive distribution maps for the island's freshwater crabs. What was learned from that survey was that Malagasy freshwater crabs were found in most, but not all parts of the island where the team collected. However, there were also large areas of the island (about one third) that has not been surveyed at all, and for which there is simply an absence of data for freshwater crabs. This situation has improved with subsequent increases in island-wide collecting in recent years with the result that some of the gaps have now been filled, including specimens of freshwater crabs in this study.

4.3 Overall Conclusions

The distribution maps presented here (Figs. 7-10) are based on comprehensive data from 500 identified individuals, 74 of which represent new localities that are all part of the Field Museum of Natural History, Chicago, and Northern Michigan University freshwater crab collections that were studied for morphological characters. Of these, 48 individual specimens were sampled for DNA analysis and were added to the molecular phylogenetic records making this study the most comprehensive Malagasy freshwater crab study of its kind. Improved taxonomic sampling for this study supports the validity of the current taxonomic assignment of seven of the eight currently recognized genera, based on morphological and molecular studies (Cumberlidge and Sternberg, 2002;

Cumberlidge et al., 2003; Cumberlidge et al., 2002; Daniels et al., 2006; Reed and Cumberlidge, 2006; Cumberlidge and Meyer, 2009; Meyer et al., 2014; Daniels et al., 2015, Cumberlidge et al., 2015).

The broadened molecular analyses presented here point to a great deal of undiscovered diversity in Madagascar's freshwater crab fauna at both the genus and species levels that is worthy of intensified collection efforts and intensified molecular studies. Using the partial COI gene to investigate the current distribution and evolutionary history of the Madagascan freshwater crab has proved to be quite successful. However, to better understand the true diversity and the evolutionary relationships between the many highly diversified lineages of freshwater crabs in Madagascar, it would be necessary to use more than a single gene. This approach has been demonstrated in the works of Daniels et al. (2006; 2015) that have provided powerful insights into the history and origins of the freshwater crabs of this island that supported a new classification system for the entire Afrotropical faunal assemblage of freshwater crabs.

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