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# A New Species of *Callulina* (Anura: Microhylidae) from the West Usambara Mountains, Tanzania

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ABSTRACT.—**The description of the species *Callulina kreffti* was based on specimens collected in the East Usambara Mountains of Tanzania. Successive collecting has shown this species to be widely distributed through the Eastern Arc Mountains. Advertisement calls from populations in the type locality of *Callulina kreffti* were compared with calls from populations in the West Usambara Mountains. Analysis of the calls suggested that these two populations of *Callulina* represent two separate taxa. Subsequent morphological and molecular investigations indicated that these two populations are distinct. Herein, we describe a new *Callulina* species on the basis of call, morphology and molecular sequences.**

The monotypic genus *Callulina* was described by Nieden (1910) to accommodate a brevicipitine microhylid, *Callulina kreffti*, which resembled *Probreviceps* and *Breviceps* but differed from these species with the following characters: the fingers and toes distinctly broadened, inner and outer metatarsal tubercles in contact, and diapophyses of sacral vertebra expanded. A further nine new species (five *Breviceps*, three *Probreviceps*, and one new monotypic genus *Spelaeophryne*) all belonging to the Brevicipitinae were described between Nieden's (1910) description of *Callulina* and Parker's (1934) monograph of the Microhylidae. Parker's monograph (1934:180) distinguished *Callulina* and *Spelaeophryne* from *Probreviceps* and *Breviceps* by the former genera having a double condylar articulation between the coccyx (= urostyle) and the sacral vertebra, whereas the latter two genera exhibit a fused condition. Furthermore, Parker (1934) differentiated *Callulina* from *Spelaeophryne* based on the shape of the terminal phalanges, T-shaped in *Callulina* and simple-shaped in *Spelaeophryne*. However, no other brevicipitine apart from *Callulina* has T-shaped phalanges.

*Callulina* is known from the Usambara, Uluguru, Nguru, Udzungwa, and Pare Mountains (Frost, 2002), which all form part of the Eastern Arc Mountains of Tanzania. The type locality of *C. kreffti* is Amani, Tanga, Tanzania (ZMB 21778); this locality is within the East Usambara.

During an amphibian survey in Tanzania 1999–2000 we realized that the *Callulina* from the West Usambara could be distinguished from the type species in the East Usambara by their advertisement call. Subsequent analyses of external morphology and DNA sequence data support the recognition of these two disjunct populations as distinct taxa. We describe the population from the West Usambara Mountains as a new species.

## MATERIALS AND METHODS

Material was collected from the Mazumbai Forest Reserve in the West Usambara Mountains, Tanzania (Fig. 1). Male specimens were collected while calling from bushes and trees after heavy rains. Calls were recorded using a Sony TCD-5M and directional microphone, or a Marantz model PMD-430 stereo cassette tape recorder and a KE66 Sennheiser directional microphone. Air temperature was recorded using an electronic thermometer. Calling males were collected and deposited as voucher specimens at the USNM. Specimens were fixed in 10% formalin (commercial grade) and subsequently stored in 70% ethanol; sample tissues of muscle and liver were removed and preserved in 95% ethanol. Specimens were cleared and double-stained for bone and cartilage using a modified Dingerkus and Uhler's (1977) technique. Specimens examined are deposited at USNM, BMNH, and MCZ (Leviton et al., 1985).

Standard measurements were taken to the nearest 0.1 mm using digital calipers: SUL (snout–urostyle length), TL (tibiofibula length), ED (horizontal eye diameter), TD (horizontal tympanum diameter), ETD (eye–tympanum distance), ND (nostril diameter), NED (nostril–eye distance), HW (head width at level of jaw articulation), LF3 (length of Finger 3 measured

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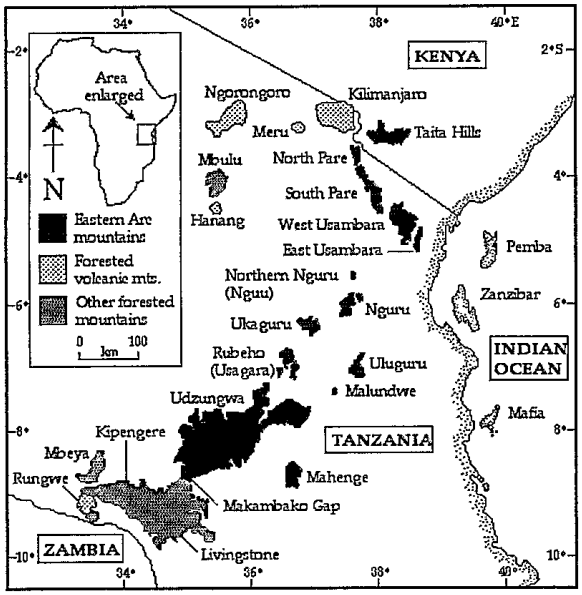


FIG. 1. Map showing the position of the West Usambara Mountains relative to the other highlands of Tanzania. Map courtesy of D. Moyer.

from the distal edge of the basal subarticular tubercle), LT4 (length of Toe 4 measured from the proximal edge of the basal subarticular tubercle), TSL (length of tarsus), HL (humerus length), NLD (nostril–lip distance), WDF3 (width of disc of Finger 3), WDTF3 (width of Finger 3 at level of distal subarticle tubercle), IOD (interorbital distance). Summary statistics (mean ± SD) are provided in Table 1 for 49 specimens of *C. kreffti* (including the holotype) and for 19 specimens of the new species. Specimens examined and locality data are provided in Appendix 1. The calls were analyzed using the software package CANARY 1.2.4 (R. A. Charif, S. Mitchell, and C. W. Clark, Cornell Laboratory of Ornithology, Ithaca, NY, 1995).

Tissue samples were obtained from the specimens listed in Appendix 1. DNA extraction followed Hillis et al. (1996). Two segments of the mitochondrial genome were amplified using the polymerase chain reaction (PCR). A segment of the 12s r RNA of ~350 base pairs and a segment of the 16s r RNA of ~500 bp were amplified. Primers used were: 12Sa (5'-AAACTGGGATTAGATACCCCACTAT-3'), 12Sb (5'-GAGGGTGACGGGCGGTGTGT-3'), 16SaR (5'-CGCCTGTTTACCAAAAACAT-3'), and 16Sd (5'-CTCCGGTCTGAACTCAGATCACGTAG-3'). Double-stranded (DS) PCR amplifications were performed in a final volume of 50 µl containing 0.4 µl of each primer, 1.0 µl of each dNTP, 3.0 µl of 25 mM MgCl, and 1.25 units? of *Taq* (*Thermus aquaticus*) DNA polymerase; the reaction was overlaid with 50 µl of mineral oil. PCR conditions were as follows: 94°C for 60 sec, 57°C for 60 sec, and 72°C for 60 sec, with 25 cycles for the 12S amplification and 30 cycles for the 16S amplification. Purification of DS amplified product was done using Wizard® PCR Preps Kit (Promega). Of the purified DS fragment, 0.5 µl were mixed with 1.5 µl of a single IRD-labeled primer, 7.2 µl of Sequencing Buffer, 1 µl of Sequitherm Excel™ II (Epicentre Technologies Co.) DNA polymerase, and 6.8 µl of $dH_20$. Subsequently, 4.0 µl of this mix was added to each of four tubes containing 2 µl of each nucleotide, respectively. PCR conditions were as follows (30 cycles): 92°C for 30 sec, 55°C for 30 sec, and 70°C for 30 sec. Single-stranded (SS) segments were amplified and

TABLE 1. Comparison of morphometrics in *Callulina* (in millimeters), abbreviations given in Materials and Methods.

| | Callulina kreffti (*N* = 47) | | | | *C. kisiwamisitu* n. sp. (*N* = 19) | | | |
|---|---|---|---|---|---|---|---|---|
| Measures | Min. | Max. | Med. | SD | Min. | Max. | Med. | SD |
| SVL | 13.4 | 38.0 | 24.2 | 5.73 | 21.1 | 41.4 | 30.01 | 5.83 |
| TL | 4.7 | 12.8 | 8.55 | 1.91 | 6.8 | 15.2 | 10.62 | 2.26 |
| TD | 0.6 | 1.8 | 1.2 | 0.22 | 0.7 | 1.8 | 1.23 | 0.32 |
| ETD | 0.6 | 2 | 1.05 | 0.30 | 1 | 2.4 | 1.46 | 0.37 |
| ED | 1.6 | 3.8 | 2.8 | 0.43 | 2.4 | 4.4 | 3.15 | 0.55 |
| ND | 1.1 | 2.7 | 1.8 | 0.36 | 1.4 | 2.8 | 1.97 | 0.39 |
| NED | 1.4 | 3.2 | 2.1 | 0.37 | 1.7 | 3.1 | 2.38 | 0.38 |
| HW | 4 | 10.5 | 6.3 | 1.44 | 5 | 11.5 | 7.56 | 1.84 |
| LF3 | 1.6 | 5 | 3.2 | 0.70 | 2.5 | 6 | 4.18 | 0.84 |
| LT4 | 2.3 | 7.4 | 4.75 | 1.00 | 4 | 8.6 | 5.65 | 1.26 |
| TSL | 3.2 | 9.6 | 5.95 | 1.34 | 4.8 | 11.2 | 7.75 | 1.71 |
| HL | 3.9 | 11.7 | 7.2 | 1.62 | 5.4 | 13.4 | 9.04 | 2.13 |
| NLD | 0.7 | 1.6 | 1.1 | 0.22 | 0.9 | 1.6 | 1.21 | 0.19 |
| WDF3 | 0.7 | 1.9 | 1.2 | 0.29 | 0.7 | 1.5 | 1.04 | 0.22 |
| WDTF3 | 0.5 | 1.2 | 0.8 | 0.18 | 0.7 | 1.3 | 0.94 | 0.18 |
| IOD | 3.3 | 7.5 | 5 | 0.89 | 3.8 | 7.3 | 5.41 | 0.99 |
| WDF3/WDTF3 | 0.58 | 0.78 | | | 0.83 | 1.00 | | |

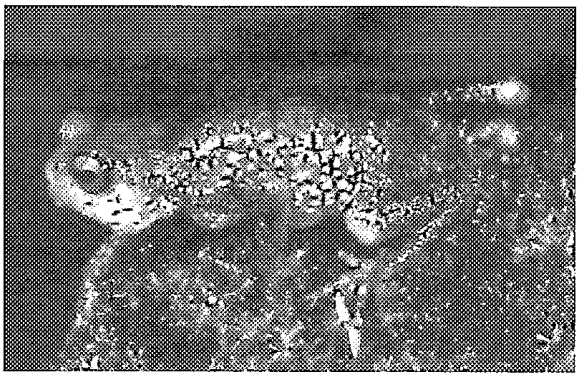

FIG. 2. Photograph of *Callulina kisiwamistu* in life.

infrared labeled fragments were sequenced in a LI-COR 4200 IR DNA Sequencer on 6% acrylamide gels. Phylogenetic analyses were performed using PAUP* (Swofford, 1998).

*Callulina kisiwamsitu* sp. n.
Figure 2

*Holotype.*—USNM 556132(originally field number RdS 930), adult male, collected by R.O. de Sá and A. Channing on 14 March 2000, at Mazumbai, West Usambaras Mts. (04°48'46.5"S, 38°30'12"E), Tanzania.

*Paratypes.*—USNM 556133, adult male, same data as holotype; USNM 556134 adult female, USNM 556139 adult male, collected by R. O. de Sá and A. Channing on 15 March 2000, at Mazumbai, West Usambara Mtns.; MCZ A-13632, and MCZ A-13633, adult females, collected by A. Loveridge on 24 December 1926, at Phillipshof, Usambara Mtns, Tanga Territory (these specimens were originally identified as *C. kreffti*); BMNH 1982.592, immature juvenile, collected by Kim Howell on 13 February 1981, at Shume-Mugambo FR, West Usambara Mtns.; BM 2002-45, adult male, collected by Simon Loader on 28 October 2001, at Mazumbai FR, West Usambara Mtns.; BMNH 2002-46, adult female with eggs, collected by Simon Loader on 28 October 2001, at Mazumbi FR, West Usambara Mtns.

*Referred Specimens.*—USNM 556135, USNM 556136-138, 556141, USNM 556140, BMNH 1982.591, BMNH 1986.595-96, BMNH 1986.597, BM 2002-47 (specifically not designated as types; see Appendix 1 for details).

*Diagnosis.*—The new species is assigned to the genus *Callulina* based on the following characteristics: (1) triangular-shaped terminal digits (simple, not expanded, in *Balebreviceps, Breviceps, Probreviceps,* and *Spelaeophyrne*); (2) expanded terminal phalanges (simple in *Spelaeophyrne, Probreviceps, Breviceps,* and *Balebreviceps*); and (3) a double condylar articulation between the urostyle and the sacral vertebra (fused in *Balebreviceps, Breviceps,* and *Probreviceps*).

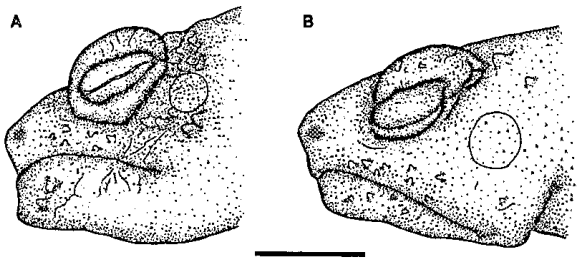


FIG. 3. Lateral view of the head region of (A) *Callulina kreffti* (MCZ A-13625) and (B) *Callulina kisiwamsitu* (USNM 556134). Bar = 5 mm.

The new species is overall morphologically similar to *C. kreffti* (Table 1). However, the two taxa are distinguished by the following characters: (1) *Callulina kisiwamsitu* has a rounded canthus rostralis and a distinctly truncated snout (the latter is less pronounced in *C. kreffti*, Fig. 3); (2) In *C. kisiwamsitu*, there is no contact between inner and outer tubercles on either the hand and the foot (tubercles very close or in contact in *C. kreffti*, Figs. 4 and 5); (3) Inner metatarsal tubercle larger than outer metatarsal tubercle in *C. kisiwamsitu* (metatarsals tubercles are about equal in size in *C. kreffti*, Fig. 5); (4) Dorsal and lateral body surfaces of *C. kisiwamsitu* have uniformly small warts (*C. kreffti* have large, broad-based warts as well as small warts); (5) The ratio between the widths of Finger 3 at the level of the distal subarticle tubercle relative to the width of its toe tip is always more than three-fourths in *C. kisiwamsitu* (three-fourths or less in *C. kreffti*, see Table 1); (6) Cleared-and-stained specimens show Y-shaped expanded terminal phalanges in *C. kisiwamsitu* (T-shape in *C. kreffti*, Fig. 6); (7) In *C. kisiwamsitu*, the distance between the tympanum and the posterior corner of the eye is equal to or greater than the tympanum diameter (Fig. 3; usually less than the tympanum diameter in *C. kreffti*, in a few cases it is slightly larger); and (8)

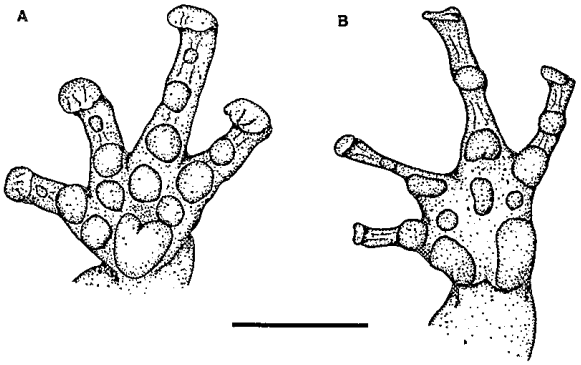


FIG. 4. Ventral view of right hand of (A) *Callulina kreffti* (MCZ A-13625) and (B) *Callulina kisiwamsitu* (USNM 556134). Bar = 5 mm.

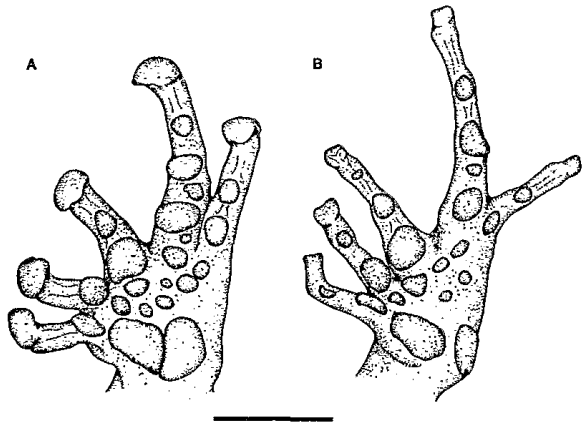


FIG. 5. Ventral view of right foot of (A) *Callulina kreffti* (MCZ A-13625) and (B) *Callulina kisiwamsitu* (USNM 556134). Bar = 5 mm.

Peak dominant frequency of advertisement calls of *C. kisiwamsitu* is always below 2 Khz, and peak dominant frequency in *C. kreffti* is always above 2 Khz, usually around 2.5–2.6 KHz (Fig. 7).

*Description of the Holotype.*—Body stout; head small, as wide as the body; head width about equal to head length; snout truncate in lateral view (Fig. 3); snout tip extending slightly anteriorly to the jaws; lower and upper jaw with small warts, less dense on upper jaw; canthus rostralis rounded; loreal region sloping at a shallow angle, without warts; nostril openings rounded, directed laterally, nearer to the tip of snout than the eye. Tongue rounded. Tympanum slightly ovoid, distinct, defined by smooth, light colored skin, with warts around edge of the disc. Warts on dorsal surface of head small, rounded. Pupil horizontal. Forelimbs slender, short, covered with small warts, more densely concentrated on the ventral surfaces. Hind limbs stout, also covered with small warts, concentrated ventrally. Digits of hands and feet moderately long, relatively slender; tip of digits truncate, slightly triangularly expanded, more pronounced on hand than on foot. A white spot defines the dorsal junction between the penultimate and ultimate phalanges in hands and feet; this spot is

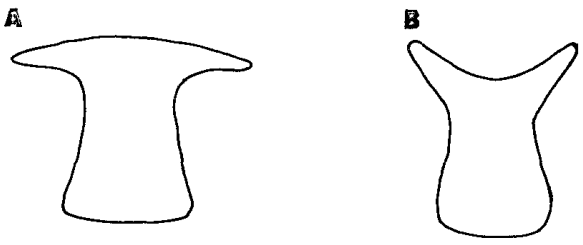


FIG. 6. Distal phalanges of (A) *Callulina kreffti* (MCZ A-105871) and (B) *Callulina kisiwamsitu* (USNM 556141).

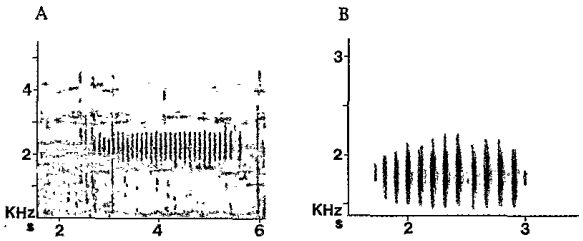


FIG. 7. Calls of (A) *Callulina kisiwamsitu* and (B) *Callulina kreffti.*

a fold of skin. Subarticular tubercles are distinct and rounded. Palmar tubercles are distinct and not contacting; inner and outer metatarsal tubercles distinct, not contacting each other, inner tubercle larger. Dorsal and ventral surfaces of body glandular, with rounded shallow warts; laterally the warts are slightly larger and white.

*Measurements.*—SUL = 30.4; TL = 10.5; ED = 3.3; TD = 1.1; ETD = 1.3; ND = 1.8; NED =2.3; HW = 7.0; LF3 = 4.1; LT4 = 5.3, TSL = 7.8; HL = 8.7; NLD =1.1; WDF3 = 1.1; WDTF3 = 1.0; IOD = 5.4.

*Coloration.*—In life, the holotype was brown with irregular dark brown marbling and a thin cream middorsal line; warts on the lateral surfaces of body were clearly white. Ventral surface was cream with brown marbling on edges. In preservative, the overall coloration is similar to that in life; however the warts on side of body are less pronounced, both relative to their color and their size.

*Variation.*—Females are larger than males, but otherwise all specimens examined are morphologically very similar to the holotype. However, in some individuals, the first and second toes are subequal in size instead of having a smaller first toe. Tympanum diameter is always at least equal to the distance from the tympanum to posterior edge of the eye; however, this distance may be larger than the tympanum diameter itself. Tympanum is partially hidden in some individuals. Warts on the edge of the eyelid tend to be more pronounced in larger specimens; however, this is difficult to detect in old museum specimens. Coloration is mainly uniform, however some specimens may also have three to four dark bands across the back. The middorsal stripe may be poorly defined. The extent of dark marbling varies slightly on the ventral regions.

*Advertisement Call.*—Calls of *C. kisiwamsitu* were recorded between 12 and 16 March 2000, at Mazumbai Reserve, Tanzania, by RdS and AC, between 2000 and 2300 h, with air temperatures ranging from 17–20°C. Call rate was determined for a three-minute recording period of individual USNM 556136 recorded on 15 March 2000, 2300 h, air temperature 20°C. Other call characteristics are based on analyses of 59 calls (six individuals).

The call is a long trill (Fig. 7), with average 13.3 notes per call (range 8–18), average call duration is 126 msec. There is an average of 5.44 pulses per note. The intensity of the dominant frequency averages 1.84 KHz; sometimes a second and third harmonic are present at about 3 and 5.5 KHz.

*Phylogenetic Analyses.*—Alignment of nine DNA-sequences resulted in a matrix of 760 unambiguously aligned characters, of which 541 were constant and 219 variable; of which 136 were informative under parsimony (only 1 gap present, alternative coding of this gap made no difference in resulting trees). Two non-brevicipitine microhylids were used as outgroups, *Hoplophryne* and *Phrynomantis*. An exhaustive search option using parsimony yielded five best trees (377 steps), a strict consensus tree is shown in Figure 8. Maximum Likelihood analyses (heuristic search using 10 random addition sequence replicates and TBR swapping method under a GTR + G model as suggested by Modeltest 3.04 [Posada and Crandall, 1998]), also agrees with the MP analysis in that *Callulina* forms a clade. Support for clades was measured with bootstrap proportions (Felsenstein, 1985; 1000 pseudoreplicates) and decay indices (Bremer, 1988) determined by enforcing a converse topological constraint. In summary, the tree demonstrates that *C. kisiwamistu* forms a clade with *C. kreffti*, and furthermore that the two samples of *C. kisiwamistu* form a well-supported clade (bootstrap proportions of 100% and a decay index 10). For further details of analyses, see Loader et al. (2004).

*Etymology.*—The specific name derives from the Swahili *kisiwa* (island) and *msitu* (forest) and refers to the habitat of this species that is now just a remnant forest that once covered the West Usambara Mountains. The word is a noun in apposition.

*Natural History.*—Observations of calling behavior were noted. As the rainy season starts, males climb into low bushes and other vegetation where they call. It was often observed that calling males were positioned vertically on small trunks, from 0.5–2 m off the ground, and initially were mistaken as notches in the trunks. Sometimes they were also found calling at the junction of branches. Gut contents of one specimen consisted of relatively large arthropods (Hemiptera, Orthoptera, Diplopoda), and nematodes; parasitic nematodes were found in the lungs. Nothing else is known of the natural history of this species.

*Distribution.*—The new species is presently known from remnant forest patches on the West Usambara Mountains: Mazumbai FR, Ambangula FR, Shume-Mugambo FR, and Philipshof.

*Remarks.*—Molecular analyses of sequence data demonstrate that the two populations of the new species sampled are sister taxa to *C. kreffti*.

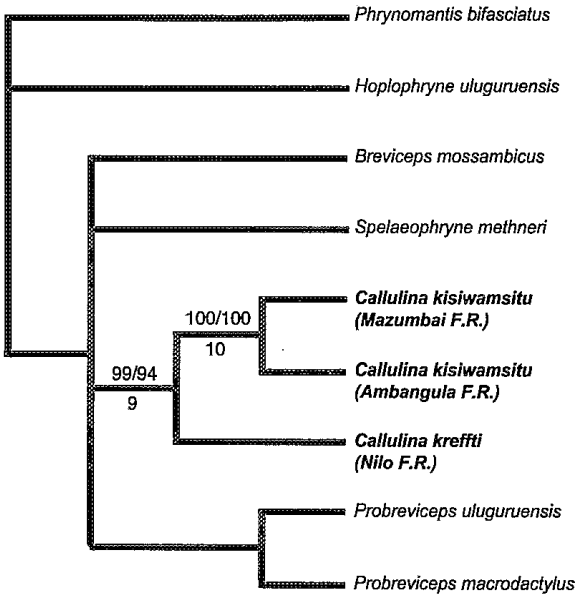


FIG. 8. Strict consensus tree of 760 base pairs of 12S and 16S mtDNA sequence data, using MP. Bootstrap proportions for both MP and ML analyses are shown above branches and decay index values are below.

Furthermore, the two samples of *C. kisiwamsitu* in the West Usambara Mountains (Ambangula FR and Mazumbai FR) are geographically more distant from each other (31.13 km apart), than samples from Mazumbai are to *C. kreffti* from Nilo, East Usambara (19.73 km apart). This suggests that the phylogenetic relationships do not appear to be the result of clinal variation among separated populations but perhaps could be indicative of the populations of the East and West Usambara mountains being specifically distinct. This assessment is consistent with the known differences in the amphibian assemblages of the East and West Usambara Mountains (Howell, 1993). The recognition of another distinct species in this area is therefore not unsurprising.

Overall the current morphological, behavioral (calls) and molecular data support the recognition of the disjunct populations of *Callulina* in the East and West Usambaras as separate species. We anticipate that other disjunct populations of *Callulina* throughout the Eastern Arc Mountains may also prove to be distinct species.

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## Appendix 1

*Material Examined.—Callulina kisiwamsitu*: BM 1982.591-2, Shume-Mugambo FR, 7000 ft. 4°40'S, 38°15'E. collected on 13 February 1981 by Kim Howell; BM 2002-45-46, Mazumbi FR, 4°48'45.4"S, 38°30'12.9"E, collected by Simon Loader and Jean Mariaux on 28 October 2001; BM 2002-47, Ambangula FR, 5°03'97.0"S, 38°24'63.0"E, collected by Simon Loader, Wilirk Ngalason and David Gower on 15 May 2002; USNM 556132-141, Tanga, Lushoto, Mazumbi FR, 4°48'45.4"S, 38°30'12.9"E, collected by Rafael O. de Sá and Allan Channing on 12–16 March 2000; MCZ A12632-33 Philipshof, West Usambara Mountains; *Callulina kreffti*: ZMB 21777–78 and 23341 Amani, East Usambara Mountains; MCZ A 13623–27, Amani, East Usambara Mountains; MCZ A 13628–29, Kizeru (Nilo FR), East Usambara Mountains; MCZ A 25490–93, Magrotto Hill, East Usambara Mountains; MCZ A 105871 and MCZ 107068, Magrotto Hill, East Usambara Mountains; USNM 200072, Magrotto Hill, East Usambara Mountains and USNM 226754 Amani, East Usambara Mountains; BM 2000-185, 187–188, Mtai FR, East Usambara Mountains; BM 2000-186, Kwamgumi FR, East Usambara Mountains, collected by Frontier; BM2000-189, 193–194, Segoma FR, East Usambara Mountains, collected by Frontier; BM 2000-190–192, 195–196, Amani, East Usambara Mountains, collected by Frontier.