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# Description of a new pygmy chameleon (Chamaeleonidae: *Brookesia*) from central Madagascar

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## Abstract

We describe a new *Brookesia* species from a forest fragment located 13 km south of Ambalavao in the southern part of Madagascar's central high plateau. *Brookesia brunoi* **sp. nov.** is one of the few arid-adapted *Brookesia* species inhabiting deciduous forests on the western slope of the central high plateau of the island (around 950 m a.s.l.). So far the species has only been observed in the private Anja Reserve. The species belongs to the *Brookesia decaryi* group formed by arid-adapted *Brookesia* species of western Madagascar: *B. bonsi* Ramanantsoa, *B. perarmata* (Angel), *B. brygooi* Raxworthy & Nussbaum and *B. decaryi* Angel. *Brookesia brunoi* differs from the other four species of the group by a genetic divergence of more than 17.6% in the mitochondrial ND2 gene, and by a combination of morphological characters: (1) nine pairs of laterovertebral pointed tubercles, (2) absence of enlarged pointed tubercles around the vent, (3) presence of poorly defined laterovertebral tubercles along the entire tail, (4) by the configuration of its cephalic crest, and (5) hemipenial morphology. Based on our molecular phylogeny this species is sister to a clade containing *B. brygooi*, *B. decaryi*, and probably *B. bonsi* for which no ND2 sequences were available. Our molecular data also confirm the presence of a divergent mitochondrial lineage in the Tsingy de Bemaraha, which might be assigned to either *B. bonsi* or *B. decaryi*, and point to the need of more research on this population.

**Key words:** Squamata, Chamaeleonidae, *Brookesia*, new species, central Madagascar, Ambalavao, Anja Reserve

## Introduction

Madagascar's flora and fauna have been shaped by a long history of isolation and successive colonization events followed by clade diversification, leading to an extraordinary degree of endemism (Goodman & Benstead 2003; Crottini *et al.* 2012). The Old World family Chamaeleonidae is one of the groups with a center of diversity in Madagascar, and the Madagascar-endemic pygmy chameleons of the genus *Brookesia* Gray are considered as the most basal of all extant chameleons (Rieppel 1987; Townsend & Larson 2002; Townsend *et al.* 2009). The genus contains 31 described species and yet undescribed candidate species (Townsend *et al.* 2009; Glaw *et al.* 2012), and thereby is one of the largest genera of Malagasy squamates (Raxworthy & Nussbaum 1995).

*Brookesia* typically are of a dull brownish coloration, and in contrast to other chameleons, their ability to change color is limited. Generally the females have larger body sizes than males, the tail is short and not prehensile but rather used as a walking aid (Boistel *et al.* 2010), and several species bear laterovertebral tubercles on the body (Brygoo 1978). As all other chameleons they have diurnal habits, but in contrast to the mainly arboreal species of the other Malagasy genera *Calumma* and *Furcifer* they mostly inhabit the forest leaf litter and climb up to a roosting position at dusk (Glaw & Vences 2007).

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*Brookesia* are characterized by a high degree of microendemism (Raxworthy & Nussbaum 1995; Glaw *et al.* 1999; Raselimanana & Rakotomalala 2003), and despite the intense herpetological activity that took place on Madagascar during recent decades, many species of the genus are still currently known only from their type localities (Ramanantsoa 1979; Schimmenti & Jesu 1996; Carpenter & Robson 2005). It is probable that *Brookesia* are characterized by a low vagility and might therefore be less prone to disperse over large distances, although given their small size they could possibly float across rivers or be washed away during heavy rains (Townsend *et al.* 2009).

*Brookesia* are currently grouped into three main clades on the basis of both molecular and phenetic datasets (Raxworthy & Nussbaum 1995; Raxworthy *et al.* 2002; Townsend & Larson 2002; Townsend *et al.* 2009): the morphologically and genetically highly divergent *Brookesia nasus* clade; the miniaturized species of the *Brookesia minima* clade; and all the remaining species, which are characterized by more robust bodies. This clade of „typical" *Brookesia* is the most species-rich of the three main clades. Most of the diversification within this clade occurred during the Eocene–Oligocene and might have been tied to the expansion of mesic habitats (Townsend *et al.* 2009).

The "typical" *Brookesia* clade is divided into five major subclades (Townsend *et al.* 2009), the most basal of which contains four species that are restricted to the dry deciduous forests of the west of Madagascar: *Brookesia perarmata*, *B. decaryi*, *B. bonsi* and *B. brygooi* (Townsend *et al.* 2009). From here onwards, we will refer to this species group as the *B. decaryi* group. Species in this group share, among other morphological character states: (1) the absence of a dorsal ridge (keel), (2) the presence of a complete series of laterovertebral pointed tubercles on body, (3) the posteriormost laterovertebral pointed tubercle modified into a diamond-shaped pelvic shield (with the exception of the unique *B. perarmata*), (4) the presence of enlarged pointed tubercles around the vent, and (5) the presence of a cephalic structure, herein in the following simply named crest or cephalic crest (i.e., the term crest herein refers exclusively to structures on the head).

*Brookesia brygooi* is one of the most widespread *Brookesia* species in Madagascar, occupying a distribution range spanning from Ankarafantsika in the north-west to Isalo in the south (Glaw & Vences 2007). As seen in other widespread amphibians and reptiles of Madagascar, some significant genetic differentiation has been found in *B. brygooi* from different localities (Townsend *et al.* 2009), warranting a more in-depth study of its variation.

Completing the species inventory of Madagascar is a priority due to the ongoing habitat destruction on the island. During a recent survey of the herpetological diversity of the private Anja reserve, Fianarantsoa province, we found a population of *Brookesia* that cannot be assigned to any currently described species. In this paper we contribute to a better understanding of the systematics of this genus by describing this new species based on its molecular and morphological distinctness, and provide new data on the genetic variability of *B. brygooi* and *B. decaryi*.

## Material and methods

The holotype specimen was anaesthetised and subsequently killed by injection with chlorobutanol, fixed with 90% ethanol, stored in 70% ethanol and deposited in the collection of the Zoologische Staatssammlung München (ZSM 888/2010), Munich, Germany (ZSM). The paratype was found dead in the jaws of a female *Furcifer oustaleti* that predated it a few seconds before the collectors's arrival. The paratype was fixed with 90% ethanol, stored in 70% ethanol and subsequently deposited in the ZSM collection (ZSM 889/2010). Tissue samples were taken by removing a piece of muscle of the hind limb or by tail clipping and stored in 95% ethanol for further genetic analyses. ACZC and ZCMV refer to field numbers of A. Crottini and M. Vences. Locality informations were recorded using a GPS. Other institutional abbreviations used in this manuscript: KUZ, Kyoto University Museum, Japan; MNHN, Muséum National d'Histoire Naturelle, Paris, France; UADBA, Université d'Antananarivo, Département de Biologie Animale, Madagascar; ZFMK, Zoologisches Forschungsmuseum Alexander Koenig, Bonn, Germany.

The holotype was collected at night roosting in low vegetation. Photographs of the living holotype were taken during the afternoon of the day after the capture to record natural coloration (Fig. 1).

All measurements were made on preserved specimens. Morphological measurements were taken with digital callipers to the nearest 0.1 mm by A. Crottini. Snout-vent length (measured from snout tip to vent) is abbreviated to SVL. Other abbreviations used are: TL, total length; a.s.l., above sea level.

The laterovertebral pointed tubercles on the body are numbered with the most anterior pair being the 1st. We distinguish two types of cephalic crest configuration within the *Brookesia decaryi* group (Fig. 1, C). In both types, four tubercles are present on the posterior part of the head. Nevertheless, in the "four-tuberculated" type, four tubercles (i to iv) are present along the posterior portion of the crest, whereas in the "three-tuberculated" crest, only three tubercles (ii to iv) are present, the remaining tubercle (i) being located below the crest.

Total genomic DNA was extracted from the tissue samples using proteinase K digestion (10 mg/ml concentration) followed by a standard salt-extraction protocol (Bruford *et al.* 1992). A mitochondrial fragment of ca. 700 bp comprising a portion of the ND2 gene, the entire $tRNA^{Trp}$ and a portion of the $tRNA^{Ala}$ genes were sequenced for nine individuals: one *Brookesia brygooi* from Isalo (ACZC 2569), one sample from Tsingy de Bemaraha which might be assigned to either *B. bonsi* or *B. decaryi* (ACZC 2998) and seven *Brookesia brunoi* **sp. nov.** from Anja Reserve (holotype: ZSM 888/2010, paratypes: ZSM 889/2010 and ZCMV 12783, and the following samples: ACZC 1938, ACZC 1981, ACZC 1924 and ACZC 1934) using the primers Ala-R2 5'-AAAATRTCTGRGTTGCATTCAG-3' (Macey *et al.* 1997) and ND2_f17 5'-TGACAAAAAATTGCNCC-3' (Macey *et al.* 2000). The thermal profile was as follows: initial denaturation at 94 °C for 90 sec, 35 cycles of denaturation at 94 °C for 30 sec, annealing at 45 °C for 45 sec, elongation at 72 °C for 90 sec, followed by 10 minutes of final elongation. PCR products were resolved on an automated DNA sequencer ABI 3130XL (Applied Biosystems). Sequences were blasted in GenBank and chromatograms were checked by eye and edited, when necessary, using CodonCode Aligner (version 3.7.1; Codon Code Corporation). Additional sequences of *B. brygooi* from Kirindy (ZFMK 66707: AF448774) and from Ankarafantsika (KUZ R61408: FJ975190; ZSM 563/2001: FJ975189), of *B. decaryi* from Ankarafantsika (ZSM 558/2001: FJ975192; UADBA-FGMV 2001.359: FJ975193), of *B. perarmata* from Tsingy de Bemaraha (TMT 50: AF448776), of *B. ebenaui* from Montagne d'Ambre (ZSM 235/2004: FJ975183), of *B. griveaudi* from Marojejy (ZSM 127/2005: FJ975205), of *B. minima* from Manongarivo (FJ975164), of *B. superciliaris* (HDZ-CCR-BM24: AF448778) and of *B. thieli* from Ambohitantely Special Reserve (FM13949: AF448780) were retrieved from GenBank and added to the alignment. The alignment of all sequences required the inclusion of gaps to account for indels in only a few cases. The software GBlocks (Castresana 2000) was used to delete highly divergent regions which could either not being unambiguously aligned or were saturated by multiple substitutions, mostly referring to the $tRNA^{Trp}$ and the portion of the $tRNA^{Ala}$ genes. All newly determined sequences have been deposited in GenBank (JX101752– JX101760). Uncorrected pairwise distances (*p*-distances transformed into percent) within individuals of the same species and between species of the *B. decaryi* group (averaged across individuals belonging to one species) were computed using MEGA, version 4 (Kumar *et al.* 2008) (see Tab. 1).

We performed maximum parsimony (MP) and Bayesian inference searches. PAUP* 4.0b10 (Swofford 2002) was used to conduct heuristic searches under the MP optimality criterion, with 100 random addition sequence replicates, equal character weighting, tree bisection and reconnection (TBR) branch swapping, and gaps coded as missing data. Nodal support was calculated by bootstrapping, with 2,000 replicates, ten random addition sequence replicates, and TBR branch swapping. Bayesian analyses were performed in MrBayes 3.1.2 (Ronquist & Huelsenbeck 2003). The HKY+I+G model was determined by AIC in MrModeltest (Nylander 2004) as the best-fitting model of substitution. We performed two runs of 10 million generations (started on random trees) and four incrementally heated Markov chains (using default heating values), sampling the Markov chains at intervals of 1,000 generations. Stabilization and convergence of likelihood values was checked by visualizing the log likelihoods associated with the posterior distribution of trees in the program Tracer (Rambaut & Drummond 2007). The first five millions of generations were conservatively discarded and five thousand trees were retained post burn-in and summed to generate the majority rule consensus tree.

## Results

### *Brookesia brunoi* sp. nov.

(Figs. 1–3)

**Holotype.** ZSM 888/2010 (ZCMV 12784), adult male, collected in Anja Reserve (see Fig. 1 in Crottini *et al.* 2011), Ambalavao 21°51'06.8" S, 46°50'38.5" E, about 950 m a.s.l., Haute Matsiatra Region, Fianarantsoa

province, southern central Madagascar, on 8th December 2010 by Aurélien Miralles and Fanomezana M. Ratsoavina. A 3 mm long piece of muscle of the right hind limb has been cut and preserved in 96% EtOH for genetic analyses.

**Paratypes.** ZSM 889/2010 (ZCMV 13022), adult gravid female, collected in Anja Reserve, Ambalavao 21°51'2.64" S, 46°50'33.80" E, 949 m a.s.l., Haute Matsiatra Region, Fianarantsoa province, southern central Madagascar, on 9th December 2009 by Angelica Crottini, D. James Harris, Iker A. Irisarri, Alexandra Lima, Solohery Rasamison and Emile Rajeriarison. One mm of the tail tip has been cut and preserved in 96% EtOH for genetic analyses. UADBA uncatalogued (ZCMV 12783), adult female, collected at the same locality and dates of the holotype and hosted in the collection of the Université d'Antananarivo, Département de Biologie Animale. A piece of muscle has been cut and preserved in 96% EtOH for genetic analyses.

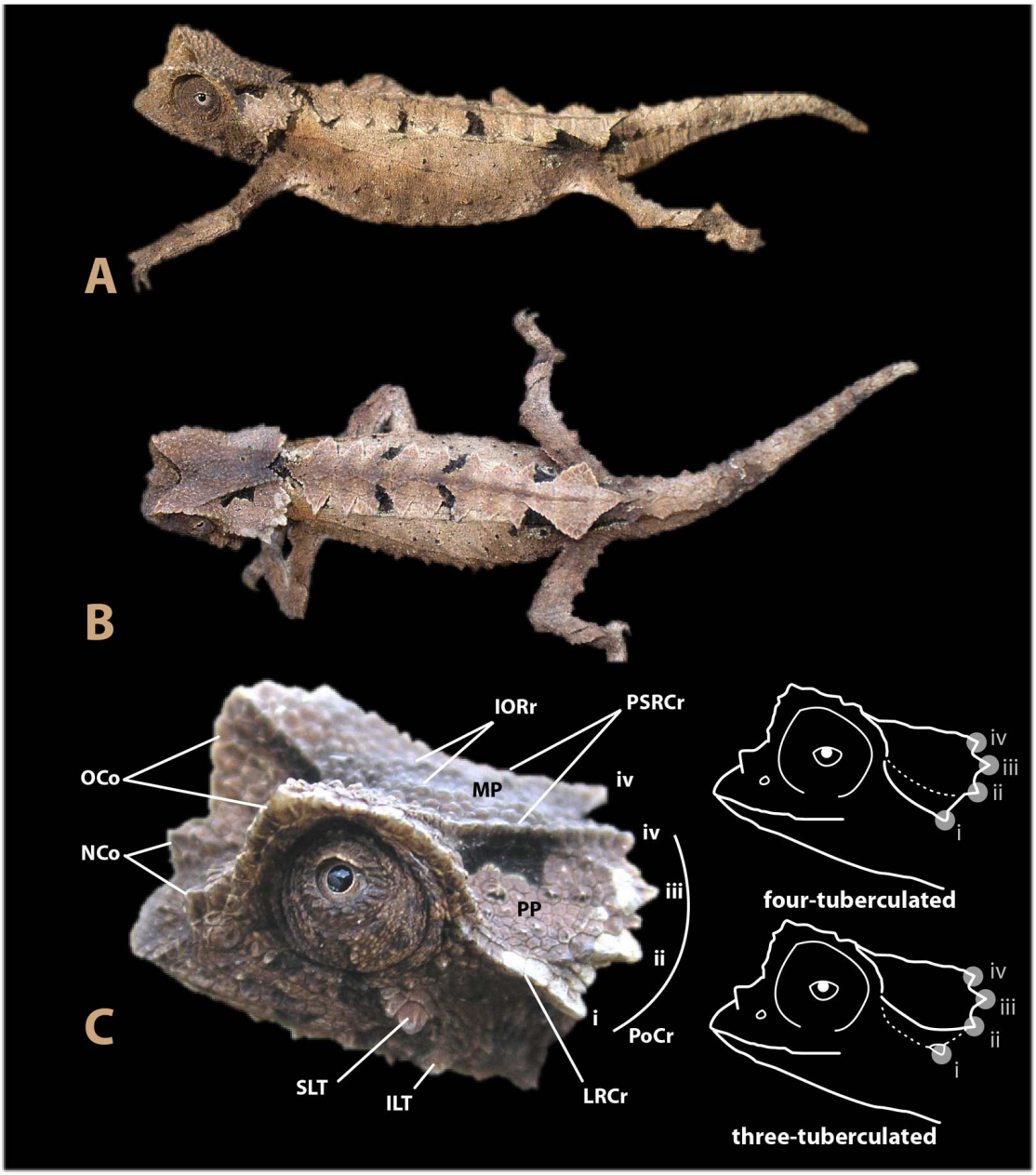


**FIGURE 1.** *Brookesia brunoi* **sp. nov.**, male [holotype (ZSM 888/2010)], in life, in lateral (A) and dorsal (B) views, and close-up view (C) of the lateral side of the head. Schematic drawings represent the two different kinds (three-tuberculated and four-tuberculated) of cephalic crest present in the *B. decaryi* group. i to iv: four pointed tubercles of the posterior portion of the crest; ILT: infralabial tubercle; IORr: interocular ridge; LRCr: lateral ridge of the crest; NCo: nasal cone; OCo: supraocular cone; PoCr: posterior portion of the crest; PSRCr: parasagittal ridge of the crest; SLT: supralabial tubercle; MP: median plain of the crest; PP: parietal plain of the crest. Note that in both the three-tuberculated and four-tuberculated state, a total of four tubercles are present, but in the three-tuberculated state, one of these tubercles is not laterally connected to the crest.

**FIGURE 2.** *Brookesia brunoi* **sp. nov.**, female (not collected) in life.

**Diagnosis.** A medium-sized *Brookesia* species characterized by (1) body without a dorsal ridge (keel) and (2) presence of a complete series of laterovertebral pointed tubercles, with (3) the posteriormost laterovertebral pointed tubercle modified into a diamond-shaped pelvic shield, (4) nine pairs of laterovertebral pointed tubercles, (5) absence of enlarged pointed tubercles around the vent, (6) presence of poorly defined laterovertebral tubercles along the entire tail.

*Brookesia brunoi* differs from all other *Brookesia* species, except for *Brookesia brygooi*, *B. decaryi*, *B. bonsi*, *B. valerieae*, *B. ambreensis*, *B. antakarana*, *B. griveaudi*, and *B. stumpffi* by characters 1–3 as listed in the previous paragraph. *Brookesia brunoi* differs from *B. valerieae* by its smaller size (SVL up to 42.8 vs. 46–53 mm), a clearly defined pelvic shield (vs. poorly defined), and rounded supraocular cone projecting forward to the level of nostril (vs. pointed supraocular cone not projecting forward to the level of nostril). It differs from *B. ambreensis* by the number of laterovertebral pointed tubercles (9 vs. 11–12), presence of poorly defined laterovertebral tubercles on tail (vs. absence), clearly defined pelvic shield (vs. poorly defined); from *B. antakarana* by the number of laterovertebral pointed tubercles (9 vs. 12–13); and from *B. griveaudi* by its smaller size (TL up to 67.8 vs. 86–99 mm), presence of poorly defined laterovertebral tubercles on tail (vs. absence), and rounded supraocular cone (vs. pointed supraocular cone). *Brookesia brunoi* differs from *B. stumpffi* by its smaller size (TL up to 67.8 vs. 81–93 mm) and the presence of poorly defined laterovertebral tubercles on tail (vs. absence).

The most similar species to *B. brunoi* are *Brookesia brygooi*, *B. decaryi* and *B. bonsi*. Together with the morphologically highly distinct *B. perarmata*, these four species form a monophyletic group. Within this clade, *B. brunoi* can be distinguished by a combination of characters, especially by its lower number of laterovertebral pointed tubercles (9 vs. 10 in *B. brygooi*, *B. decaryi*, and *B. bonsi*), absence of enlarged pointed tubercles around the vent (present in the other three species), the configuration of its cephalic crest, the lateral tail tubercles (poorly defined), and hemipenial morphology. A summary of these differences is provided in Table 2.

**Description of the holotype.** Well preserved male with everted hemipenis and extruded tongue. Snout-vent length 40.3 mm; tail length 25.9 mm. Head with lateral, orbital and posterior ridges that form a cephalic crest; crest divided into three plains by a pair of longitudinal parasagittal ridges that start above the eyes converging at the posterior part of the crest resulting in a median plain (MP) on the top of the crest, surrounded by two triangular parietal plains (PP) on each side (see Fig. 1, C). Four pointed tubercles on each side of the posterior part of the crest, one at termination point of lateral ridge, one at termination point of parasagittal ridge, and two between the parasagittal and lateral ridges; two pairs of pointed tubercles on lateral surface of head: one supralabial pair, just above posterior angle of mouth, composed of two adjacent conical scales, the posteriormost the biggest, and one

infralabial pair of tubercules below posterior angle of mouth. Each tubercle is composed by a single conical scale; orbital crest denticulated; two small and poorly marked inter-orbital ridges converging anteriorly, forming a "V-shaped" pattern; supra-ocular cone rounded and projects forward to level of nostril; supra-nasal cone does not reach as far forward as snout tip.

Dorsal surface of body flat (without a dorsal ridge or keel); 9 pairs of laterovertebral pointed tubercles form a complete longitudinal line on body; posterior-most ($9^{th}$) enlarged pair of pointed laterovertebral tubercles project posteriorly and laterally to form a diamond-shaped pelvic shield above insertion point of hind limbs; $1^{st}$-$8^{th}$ anterior pointed tubercles equally spaced and almost equal in size, perpendicular to body; no ventrolateral pointed tubercle just posterior to the vent at the tail base; reduced laterovertebral pointed tubercles on the tail.

Hemipenis short and stout, in general shape similar to that of *B. brygooi* as described by Brygoo & Domergue (1971) under the name *B. ebenaui*. Because only a single male is available and it is uncertain whether the organs are fully everted and turgid, the exact shape of the hemipenis is difficult to discern. No clear differentiation between truncus and apex. The sulcus spermaticus is short and deep, surrounded by a bulging basal lobe ("bourrelet basal" in Brygoo & Domergue 1971) and the lobular truncus. No field of denticulated papillae as in *B. brygooi* (Brygoo & Domergue 1971) is recognizable. A distinct denticulated rotula ("auricule bi-crêté of Brygoo & Domergue 1971; "denticulated lobe" of Raxworthy & Nussbaum 1995) is visible, much larger and more prominent than in *B. brygooi* where this rotula is usually smaller, less prominent and less strongly denticulated in the specimens we examined. In comparison to the hemipenes of *B. brunoi* and *B. brygooi*, the organ of *B. decaryi* (examined in ZSM 558/2001 and 560/2001) differs by a sulcus spermaticus that is continuous to the apex, absence of distinct rotulae or papillary fields, and presence of one very characteristic 1 mm long papilla-like protrusion of the apex, along with two much smaller such apical structures.

*Coloration.* According to the photographs (Fig. 1), the background coloration in life of the holotype specimen is beige, with exception of the flanks, the lateral side of the head, the ocular region, the throat and an indistinctly edged vertebral line that are darker. Several dark marks are present on the body: a median transversal black bar joins the summit of each supraocular cone, a median black mark extending from the inferior side of the posterior crest to the inferior side of the first pair of laterovertebral pointed tubercle, a pair of hourglass-shaped black marks running above the parietal ridge of the crest, one pair of black dots between the third and the fourth pair of laterovertebral pointed tubercles, one pair of black dots between the fifth and the sixth pair of laterovertebral pointed tubercle, large black dots below the eighth and the ninth pair of laterovertebral pointed tubercle (see Fig. 1, B), and a dozen tiny black dots on the flanks. The iris is faded orange with a thin central whitish ring bordering the pupil. Six months after fixation, the coloration was relatively similar to that in life, with the exception of the flanks that were darker, the dorsal side of the head, which was lighter, and the absence of tiny black dots on the flanks.

**Variation.** Based on the female paratype ZSM 889/2010: SVL 42.8 mm; tail length 24.9 mm + 1 mm (cut for molecular analyses). The color of the paratype after one year and a half in alcohol was similar to that at the time of collection. Ground color of head, body, tail and dorsal parts of limbs dorsally and laterally beige or brown, often becoming darker dorsally. The ground color is contrasting, with a mosaic of whitish, beige, and brown areas, which can be interpreted as leaf mimesis (or more in general as a mimesis to plant debris of the dry forest floor including also sticks and dried bark) (Fig. 3). The blotches on the back break up the background color providing discontinuity thus enhancing the overall cryptic appearance. No measurements are available for the female paratype specimen in the UADBA collection (ZCMV 12783).

**Distribution, conservation and IUCN Red List status.** The new species is currently known only from the type locality within the Anja Reserve (see Fig. 1 in Crottini *et al.* 2011), although a recent *Brookesia* record from Ambovombé in the far south (Hofmann 2012), ca. 375 km air distance from the type locality, seems to refer to *B. brunoi.* Furthermore, it is possible that some literature records of *B. decaryi, B. brygooi, B. ebenaui* and *B. stumpffi* from the central areas of Madagascar might actually refer to *B. brunoi* indicating that further investigations are required to better understand its actual distribution.

In the Ambalavao area we did not observe any mineral or precious stone extractions or collecting for the pet-trade, but deforestation for agriculture, logging and cattle grazing is widely afflicting the area. Although relatively abundant in the Anja reserve (several individuals were observed in a few hours of active searching) it is possible that *B. brunoi* will qualify for inclusion in one of the threatened categories, depending on the actual size of its distribution area. However, due to the limited knowledge on this species we suggest to consider its conservation status as "Data Deficient" according to IUCN criteria (IUCN 2001).


**FIGURE 3.** (A–B) Photographs of *B. brunoi* **sp. nov.** (not collected) in its natural habitat in Anja Reserve during the day. The picture in (A) highlights the mosaic of whitish, beige and brown areas on the body of the specimens that characterise the life coloration of the species. This coloration can be interpreted as plant, stick and dried bark debris mimesis.

**Habitat and habits.** The holotype of *B. brunoi* and the UADBA paratype (ZCMV 12783) were found around 21:00 at a roosting height of about 30 cm from the leaf litter on small bushes in the forest of Anja Reserve. With the exception of the paratype specimen ZSM 889/2010, found dead in the jaws of a female *Furcifer oustaleti*, all other individuals sampled for the molecular analyses were found between 13:00 and 14:00 in the leaf litter (ACZC 1938, ACZC 1981, ACZC 1924) (Fig. 3), in shaded spots inside the forest fragment with closed canopy. Another individual was found at night around 21:00 at a roosting height of about 40 cm on small herbal bushes (ACZC 1934). No other *Brookesia* species were found around the type locality. Although inhabiting the humid leaf litter this new species is one of the few *Brookesia* species adapted to the semi-arid deciduous forest of Madagascar. The other species found in arid habitats of the west are *B. bonsi*, *B. exarmata*, *B. decaryi*, *B. perarmata*, *B. brygooi* and *B. stumpffi*. Other reptiles found in the forest of the Anja Reserve during our two visits (December 2009, December 2010) were: *Paragehyra* sp. aff. *petiti*, *Paroedura* sp. aff. *bastardi*, *Phelsuma gouldi*, *Thamnosophis lateralis*, *Furcifer lateralis*, *Furcifer oustaleti*, *Oplurus quadrimaculatus*, *Madagascarophis meridionalis*, *Trachylepis vato*, and *Hemidactylus frenatus.*

**Etymology.** A. Crottini dedicates this new species to Bruno Grassi in recognition of his love and support during these years. The specific name is thus a patronym, but in addition recalls the cryptic brownish coloration of the newly described species in Italian language.

**Mitochondrial variation, differentiation and phylogenetic relationships.** The molecular data confirms the attribution of *B. brunoi* to the *B. decaryi* group (Townsend *et al*. 2009). The analyzed specimens of *B. brunoi* are genetically very uniform and show an intraspecific uncorrected divergence of 0.3%, while the analyzed specimens of *B. brygooi* and *B. decaryi* (including the sample from Tsingy the Bemaraha) are more heterogeneous and show intraspecific uncorrected divergence of 4.8% and 7.7% respectively, in the ND2 gene fragment sequences. The genetic distance between *B. brunoi* and the three other species of the *B. decaryi* group (molecular data of *B. bonsi* are not available for this gene) ranges between 17.6% (comparison between *B. brunoi* and *B. brygooi*) and 19.7% (comparison between *B. brunoi* and *B. perarmata*), and the genetic distance between *B. brunoi* and *B. decaryi* is 19.1%. Among the species of the analyzed species group the smallest genetic distance is observed between *B. brygooi* and *B. decaryi* (16.2%) and the highest value between *B. perarmata* and *B. decaryi* (20.8%). More details are provided in table 1.

**TABLE 1.** Within- (bold) and among-species genetic divergence of the analysed ND2 mitochondrial fragment based on the pairwise distance calculation for *B. perarmata*, *B. brunoi*, *B. brygooi* and *B. decaryi.* nc: not calculated.

| | *B. perarmata* | *B. brunoi* | *B. brygooi* | *B. decaryi* |
|---|---|---|---|---|
| *B. perarmata* | **nc** | | | |
| *B. brunoi* | 19.7% | **0.3%** | | |
| *B. brygooi* | 19.0% | 17.6% | **4.8%** | |
| *B. decaryi* | 20.8% | 19.1% | 16.2% | **7.7%** |

The phylogenetic analyses resulted in a tree with largely unresolved basal relationships (Fig. 4) but with good support for the monophyly of the samples of the *B. decaryi* group, and for each species of this species group, hence clearly supporting the distinctness of *B. brunoi* from the other three analysed species. In the analysis of Townsend *et al*. (2009) *B. bonsi* was sister to *B. decaryi* based on DNA sequences of the ND4 gene available from the study of Raxworthy *et al.* (2002), and these two species were sister to *B. brygooi*. We could not include *B. bonsi* in our analysis due to the lack of samples and ND2 sequences for this species, but the combined evidence from this study and the tree of Townsend *et al.* (2009) suggests these species are related as follows: (*perarmata*(*brunoi*(*brygooi*(*bonsi*,*decaryi*)))).

In our analysis, samples of *B. brygooi* are organised in two mitochondrial lineages. Samples from Ankarafantsika belong to one lineage, while the samples of *B. brygooi* from Isalo and Kirindy belong to a second lineage, with an uncorrected average inter-lineage divergence of 5.9%. Furthermore, our analysis includes one sample from the Tsingy de Bemaraha that in our tree is sister to *B. decaryi* from the type locality Ankarafantsika, albeit with a high average divergence (11.3%). Because our sampling does not include *B. bonsi,* the sister species of *B. decaryi* (Townsend *et al*. 2009), we cannot decide whether the Tsingy de Bemaraha sample is to be assigned to *B. decaryi*, to *B. bonsi*, or possibly to a third, undescribed species. Neither *B. bonsi* nor *B. decaryi* have so far been reported from the Tsingy de Bemaraha, and a more detailed molecular and morphological study of this population is therefore necessary.

**TABLE 2.** Diagnostic character states of *B. brunoi* compared to those of its closest relatives *B. brygooi*, *B. decaryi*, and *B. bonsi*. The table also includes *B. stumpffi* which is the only other superficially similar *Brookesia* occurring in parts of western Madagascar. Data for *B. bonsi* were taken from Ramanantsoa (1979).

| | *B. brunoi* | *B. brygooi* | *B. decaryi* | *B. bonsi* | *B. stumpffi* |
|---|---|---|---|---|---|
| Laterovertebral pointed tubercles | 9 | 10 | 10 | 10 | 9–10 |
| Enlarged pointed tubercles around the vent | absent | present | present | present | absent |
| Laterovertebral tubercles along the entire tail | usually present but poorly defined | often only up to 2 tubercles anteriorly | typically distinct and strongly expressed on anterior two thirds | as far as known present along almost entire tail length | absent |
| Posterior part of the cephalic crest | four-tuberculated | typically three-tuberculated | four-tuberculated | possibly three-tuberculated | three-tuberculated |
| Hemipenis | Short and stout. Large and prominent denticulated rotula. No apical field of denticulate papillae. No apical projection. | Short and stout. Smaller and usually less prominent rotula, sometimes only weakly denticulate. Often with apical field of denticulate papillae. No apical projection. | Short and stout. No rotula. No field of papillae. Distinct and long papilla-like apical projection. | Rather elongated. Small slightly denticulate rotula. No field of papillae. Distinct but short papilla-like apical projection. | Elongated. Large central denticulate rotula. Two lateral lobes with denticulate papillae. No apical projection. |
| SVL [mm] | 40.3–42.8 | 42–52 | 43–53 | 37–40 | 46–55 |
| TL [mm] | 66.2–67.7 | 68–81 | 63–80 | 59–67 | 81–93 |

## Discussion

The description of *B. brunoi* **sp. nov.** adds a distinctive new species to the most basal subclade of the „typical“ *Brookesia* clade that contains *B. decaryi*, *B. brygooi*, *B. bonsi* and *B. perarmata*. All these four species inhabit dry deciduous forest areas of the west. On the contrary, *B. brunoi* is so far known to inhabit only the dry forest of the Anja Reserve, in the central high plateau of Madagascar, at an elevation of ca. 950 m a.s.l.. This small area (ca. 30 hectares) close to the town of Ambalavao has been protected since 1999, and is managed by the local community. Recent herpetological surveys in this area found evidence for the existence of several local endemics (Crottini *et al.* 2011) and the present study represents a step further towards the knowledge and description of its endemic fauna.

Based on its morphological characteristics, *B. brunoi* in addition to its peculiar distribution, has at least two morphological characters that seem to be unique when compared with the other *Brookesia* species of the *B. decaryi* group: 1) the number of the laterovertebral pointed tubercles; and 2) the absence of enlarged pointed tubercles around the vent.

The molecular analyses suggest that this species group forms a well-supported monophyletic lineage with high genetic divergences observed between all four species (more than 16% uncorrected pairwise sequence divergence in the ND2), indicating a long divergent evolutionary history. It has been suspected that the genetic variability observed between the different populations of *B. brygooi* across the island reflects the existence of a species complex (Townsend *et al.* 2009). However, the comparatively high genetic variability within *B. brygooi*, coupled with the lack of obvious morphological differences among the individuals analyzed in this study (see Appendix) suggests that this divergence might more appropriately be considered as intra-specific variability.

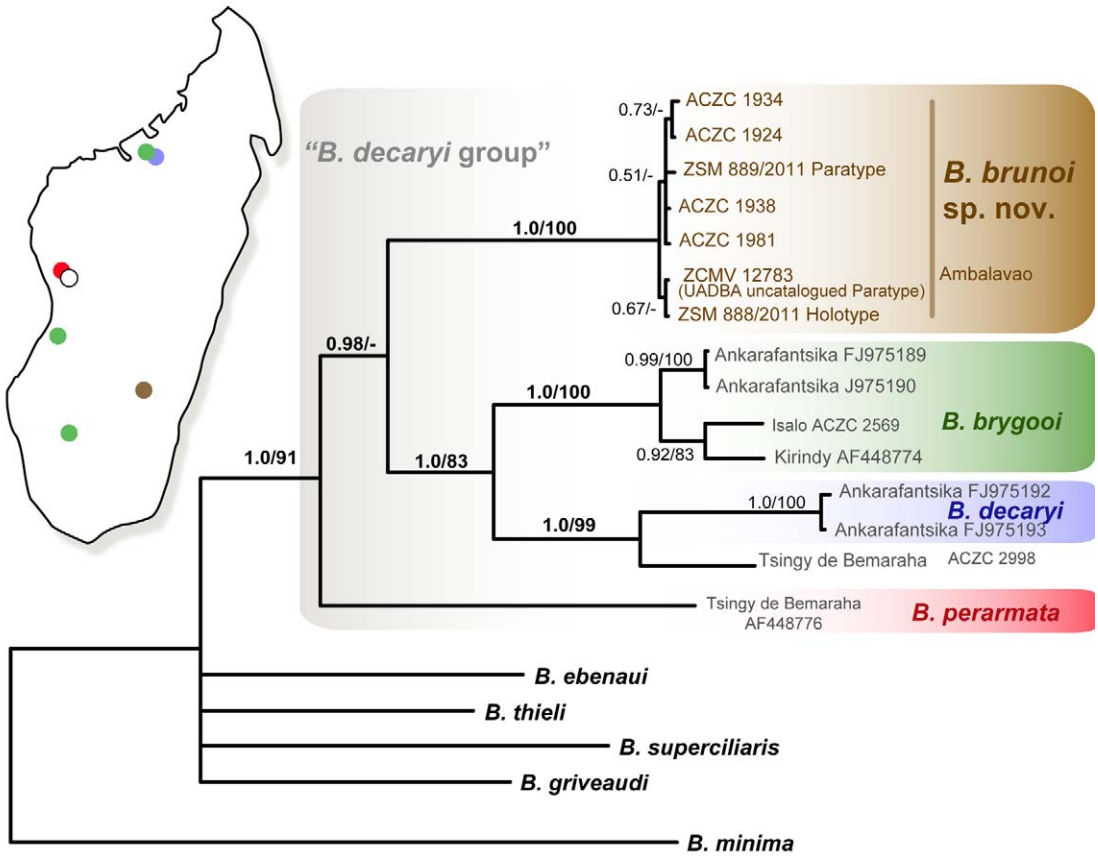


**FIGURE 4.** 50%-majority consensus tree derived from a Bayesian inference analysis of 590 bp of the mitochondrial ND2 gene. *Brookesia minima* was used as outgroup. Bayesian posterior probabilities are followed by the bootstrap support values >50 from Maximum Parsimony analysis. Sequences of the *B. decaryi* group retrieved from Genbank are marked with their accession numbers. The map indicates the sampling localities of the specimens of the *B. decaryi* group.


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**APPENDIX.** List of analysed specimens.

*Brookesia brunoi*: Holotype (ZSM 888/2010) and paratype (ZSM 889/2010) collected in Anja Reserve near Ambalavao.

*Brookesia brygooi*: Holotype MNHN 8219 collected in Analavelona; ZSM 563/2001 collected in Ankarafantsika, Ampijoroa; ZSM 11/2006, ZSM 12/2006 and ZSM 35/2006 collected in Tsingy de Bemaraha, Andranopasazy; ZSM 80/2006 and ZSM 119/2006 collected in Tsingy de Bemaraha, Bendrao Forest; ZSM 138/2006 collected in Tsingy de Bemaraha, Andafiabe.

*Brookesia decaryi*: ZSM 640/2000, ZSM 863/2000, ZSM 864/2000, and ZSM 427/2002 without locality data; ZSM 558/2001, ZSM 559/2001 and ZSM 560/2001 collected in Ankarafantsika, Lac Ravelobe.