



## Genetic identity of the critically endangered Wimmer's shrew *Crocidura wimmeri*

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Coastal primary rainforests have suffered damage in Côte d'Ivoire as a result of a lack of protection and urban pressures. Consequently, the highly endemic and critically endangered Wimmer's shrew, *Crocidura wimmeri*, known only from its type locality, Adiopodoumé, near Abidjan, was considered to have been extinct since 1976. Shrew species assignment is often problematic because of strong phenotypic similarities among many species. The phylogenetic position of *C. wimmeri* within the African *Crocidura* species should thus be clarified. In light of its recent rediscovery in the nearby small Banco National Park (34 km<sup>2</sup>), we investigated the genetic identity of seven specimens of *C. wimmeri*, based on 1020 bp of the mitochondrial DNA *cytochrome b* gene compared to other species sampled in the same region and published sequences from GenBank. *Crocidura wimmeri* formed a well-defined clade, the closest-related species being *Crocidura* sp., with a distance of 9.3%, a yet unknown species from Taï and Ziama forests. These results thus confirmed the validity of this species. This genetic characterization not only contributes to our knowledge of the evolution of West African shrews, but also may help in the discovery of additional populations of this critically endangered species. © 2013 The Linnean Society of London, *Biological Journal of the Linnean Society*, 2014, 111, 224–229.

ADDITIONAL KEYWORDS: Africa – conservation – Crocidurinae – cytochrome *b* – Ivory Coast – Soricidae.

### INTRODUCTION

Correct assignment of shrew species can be problematic because such organisms are often morphologically and karyologically similar and hence lack synapomorphic characters. Consequently, genetic investigations have often revealed systematic inconsistencies, as well as many cryptic species (Vogel, Cosson & Lopez Jurado, 2003; Dubey *et al.*, 2006, 2007a; Yannic *et al.*, 2012; Jacquet *et al.*, 2013; Stanley *et al.*, 2013). For example, in the genus *Crocidura*, the shrews from Sicily (*Crocidura sicula*) and Canary Islands (*Crocidura canariensis*) were considered as conspecific by Sarà (1995, 1996) as a result of the identity of their karyotype (Hutterer,

Lopez-Jurado & Vogel, 1987; Vogel, 1988), whereas molecular dating revealed that these two taxa split 5 Mya [*cytochrome b* gene (*Cytb*) genetic distance: 14%; Vogel *et al.*, 2003; Dubey *et al.*, 2008a]. In addition, local adaptations may also lead to important morphological differences within a single species, as in the African *Crocidura olivieri* (with local morphs varying from 20 to 120 g and fur coloration from beige to black), leading to the description of invalid (sub-)species (see genetic study of Dubey *et al.*, 2007b). These examples show that the validation of a shrew species cannot be based only on morphology and chromosomal analyses and that they should also include genetic studies.

The present study focused on the Wimmer's shrew *Crocidura wimmeri* Heim de Balsac and Aellen, 1958, with the type locality of Adiopodoumé, Côte d'Ivoire. This shrew was first found on the ground of the

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former Office de la Recherche Scientifique et Technique Outre-Mer (ORSTOM), now belonging to the Centre National de Recherche Agronomique. From 1971 to 1973, one of the investigators (PV) collected a total of 15 specimens of this species in this area. These specimens were used for different comparative studies published later (e.g. metabolic rates, defense calls, activity rhythms, and karyotypes) (Vogel, 1976; Hutterer & Vogel, 1977; Vogel, Genoud & Frey, 1981; Meylan & Vogel, 1982). Subsequently, efforts to find new specimens of this species for a genetic study of African shrews failed (Maddalena, 1990). The primary rainforest on the border of the Lagune Ebrié, around the former ORSTOM site, has been degraded by urban pressures (wood-cutting and farming; P. Vogel, pers. observ.) as a result of a lack of protection. This highly endemic shrew has not been observed since 1976 and hence has been considered as extinct (Fisher & Blomberg, 2012), or at least is considered to be one of the most threatened species in Côte d'Ivoire (IUCN, 2010). The question remained as to whether or not this shrew was indeed a highly endemic species restricted to the Adiopodoumé region (and hence likely extinct) or whether it might still occur elsewhere.

A study of a small mammal community in a primary rainforest of the nearby Banco National Park revealed an obviously new population of the Wimmer's shrew (Kadjo *et al.*, 2013). The aim of the present study was to: (1) genetically characterize *C. wimmeri* from the type locality and the new locality; (2) check whether similar genetic material had been published and/or formerly analyzed from misidentified shrews in the GenBank database; (3) ensure the validity of the species from a genetic point of view; and (4) evaluate its phylogenetic position among African *Crocidura* species.

## MATERIAL AND METHODS

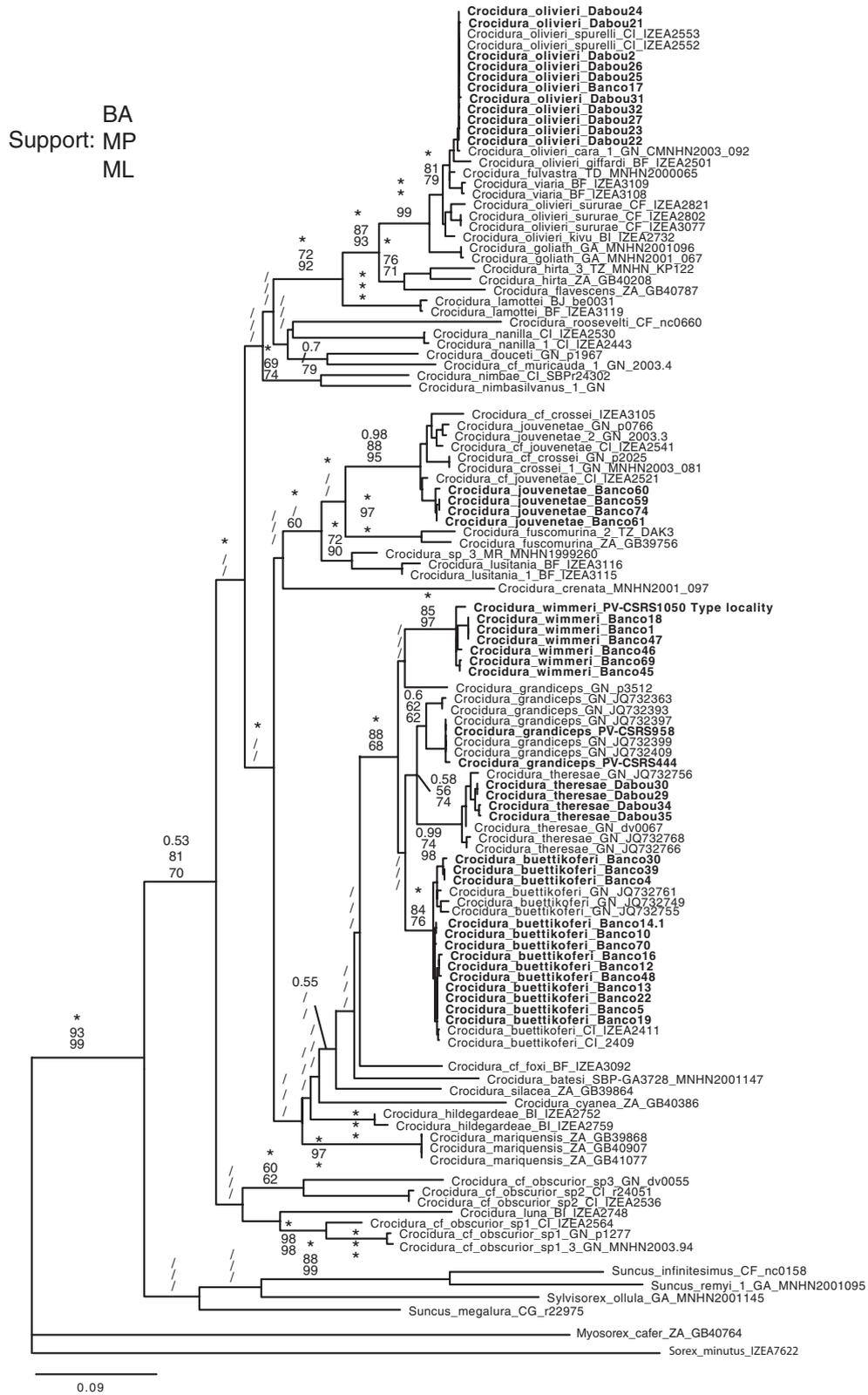
In 2007, an investigation in Banco National Park (5°21' to 5°25'N; 4°01' to 4°05'W) resulted in the trapping of six Wimmer's shrews (Kadjo *et al.*, 2013). Specimens were analyzed with other shrews trapped in the Banco National Park (*Crocidura buettikoferi*, *Crocidura jouvenetae*, *C. olivieri*; Kadjo *et al.*, 2013), as well as shrews from the savannah of Dabou situated 50 km further east, which were sampled during the same period (e.g. *Crocidura theresae*). All tissue samples were stored in the Ethanol collection of the Musée Cantonal de Zoologie, Lausanne. Moreover, one skin of *C. wimmeri* from the type locality Adiopodoumé (PV-CSRS 1050) and two skins of *Crocidura grandiceps* (PV-CSRS 444, PV-CSRS 958) referenced in the original description of this species (all with known karyotypes; Meylan & Vogel, 1982;

Hutterer, 1983) were also included. DNA was extracted from toe-clippings using a QIA Amp DNA Mini Kit (Qiagen). DNA amplifications of 1020 bp of the mitochondrial *Cytb* gene were performed using the primer pair L14724/H15915 (Irwin, Kocher & Wilson, 1991). In addition, the primer pair L14724/H15149 was used to amplify 295 bp of *Cytb* gene of the skin samples from *C. wimmeri* and *C. grandiceps* (Irwin *et al.*, 1991). Amplification conditions consisted of 35 thermal cycles comprising 60 s denaturation at 94 °C, 60 s annealing at 50 °C and 120 s extension at 72 °C. Polymerase chain reaction (PCR) products were purified with a QIAquick PCR Purification Kit (Qiagen) and reaction sequences were visualized on an ABI3100 genetic analyzer (Applied Biosystems).

To clarify the genetic relationship between *C. wimmeri* and other African crocidurine shrews, additional DNA sequences were obtained from GenBank (Fig. 1). *Sorex minutus*, a member of the tribe Soricini (Dubey *et al.*, 2007c), was used as an outgroup. For maximum likelihood (ML) analyses, the model of DNA substitution was selected using the Bayesian information criterion (Schwarz, 1978) implemented in JMODELTEST, version 0.1.1 (Guindon & Gascuel, 2003; Posada, 2008). The GTR + I + G model best fit the dataset. ML heuristic searches and bootstrap analyses (1000 replicates) were performed using PHYML with the GTR + I + G model (Guindon & Gascuel, 2003). Similarly, Bayesian analyses (BA) were performed with the same model, using MrBayes, version 3.1.2 (Huelsenbeck *et al.*, 2001) and partitioning data by codon position. Finally, we performed maximum parsimony (MP) analyses using PAUP\*4.0b10 (Swofford, 2001) with 100 random additions of sequences followed by tree bisection and reconnection branch swapping, keeping a maximum of 100 trees at each replicate. Branch support was estimated from 1000 bootstrap re-samples using the same heuristic settings. Kimura two-parameter genetic distances (Kimura, 1980) were estimated between close species. In addition, we used BLAST analysis to compare the *Cytb* sequences of captured *C. wimmeri* with sequences in the GenBank database (Benson *et al.*, 2005).

## RESULTS AND DISCUSSION

The results revealed that individuals of the endangered *C. wimmeri* formed a well-defined lineage (support of 1.0, 97, 85 for BA, ML, and MP, respectively; GenBank accession number: KF006297–KF006301, KF006307, and KF417557; for more details, see Supporting information, Table S1) (Fig. 1). In addition, the identity of the *Cytb* sequence of the specimen from the type locality Adiopodoumé confirmed the assignment of the Banco National Park



**Figure 1.** Phylogeny of the 1020-bp *Cytb* fragment analyzed by maximum likelihood analysis. Values in branches are bootstrap indices (1000 replications) of support for maximum parsimony (MP) and maximum likelihood (ML) analyses, and Bayesian posterior probabilities (BA) (\* support of 1.0, 100, 100 and ′ support < 0.5, 50, 50 for BA, ML, and MP, respectively). Haplotypes shown in bold represent samples from the present study.

**Table 1.** Mean corrected pairwise sequence divergences between *Crocidura olivieri*, *Crocidura juvenetae*, *Crocidura buettikoferi*, *Crocidura wimmeri*, and *Crocidura grandiceps* s.s. (Kimura two-parameter distances)

	<i>Crocidura olivieri</i>	<i>Crocidura juvenetae</i>	<i>Crocidura buettikoferi</i>	<i>Crocidura wimmeri</i>	<i>Crocidura grandiceps</i>
<i>Crocidura olivieri</i>	<b>0.014</b>				
<i>Crocidura juvenetae</i>	0.167	<b>0.027</b>			
<i>Crocidura buettikoferi</i>	0.187	0.173	<b>0.009</b>		
<i>Crocidura wimmeri</i>	0.187	0.166	0.09	<b>0.011</b>	
<i>Crocidura grandiceps</i>	0.170	0.185	0.074	0.099	<b>0.013</b>
<i>Crocidura theresae</i>	0.197	0.179	0.081	0.111	0.068

Genetic divergence (K2P) within species is indicated in bold.

population. Our BLAST search showed that no similar genetic material had been published and/or formerly analyzed from misidentified shrews in the GenBank database. With regard to its evolution, *C. wimmeri* belongs to a clade that is represented in Côte d'Ivoire (and neighboring countries) by *C. theresae*, *C. buettikoferi*, and two divergent lineages assigned to *C. grandiceps* (support of 1.0, 68, 88 for BA, ML, and MP, respectively) (Fig. 1). All lineages have a high bootstrap support. Interspecific genetic distances within this clade are between 6.8% and 11.1% and, compared to other major clades (with *C. olivieri* and *C. juvenetae*), as high as 16.6% to 19.7% (Table 1).

An important question is which of the two genetically different lineages including samples published earlier under the name *C. grandiceps* Hutterer, 1983 really belongs to this species (Fig. 1). The two dried skins from Côte d'Ivoire (PV-CSRS 444 and PV-CSRS 958; see Material and methods) with known karyotype referenced by Hutterer (1983) in the type description clustered with *C. grandiceps* analyzed by Jacquet *et al.* (2012) (Fig. 1). Consequently, the two specimens considered earlier by the MNHN Paris as *C. grandiceps* (with identical 16S rRNA; Quérouil *et al.*, 2001; Dubey *et al.*, 2008b) have to be assigned to a yet unknown species. One originates from Guinea, Ziama forest (SBP-P3512; GenBank accession numbers: EF524897 and EF524681) and the other one from Côte d'Ivoire, Taï forest (SBP-R24009: AF550877). The validity of this species is confirmed by the genetic distance of 9.3% to its closest relative, *C. wimmeri* from Côte d'Ivoire. This value is indeed well above the lower limit for mammals in terms of the genetic species concept (Bradley & Baker, 2001; Baker & Bradley, 2006).

Our genetic analysis indicates that morphological identification of *C. wimmeri* in the field may be possible, although this method is subject to errors. For example, because of its large size (mean value: 19.3 g, range 15–26 g;  $N = 14$ ), one specimen of the Wimmer's

shrew was misidentified in the field as an African giant shrew. This widespread species found mostly in urbanized areas (Dubey *et al.*, 2007b) has an overlapping body size with *C. wimmeri* in Côte d'Ivoire, (mean value: 31.4 g, range 16–50.5 g;  $N = 181$ ), which can lead in some cases to erroneous species identifications when examining young individuals. In addition, specimens of other species from other regions were assigned by error to *C. wimmeri*, as is the case, for example, for one of our *C. grandiceps* (PV-CSRS 444) from the Nimba region. However, in this case, the misidentification was discovered as a result of karyotypic data inconsistencies (Meylan & Vogel, 1982). Records from Cameroon and Gabon were also based on misidentification (Hutterer, 2005), suggesting that *C. wimmeri* is indeed confined to very few coastal forests of Côte d'Ivoire and thus endemic to a very restricted area.

We present the first published picture of the Wimmer's shrew in Figure 2. Compared to sympatric shrews, this species is of a bright grey, contrasting with the almost black *C. buettikoferi*, and is much heavier than *C. juvenetae*.

In conclusion, the present study has revealed that the Wimmer's shrew is not extinct. However, given its restricted distribution in the Banco National Park (34 km<sup>2</sup>) and the surrounding urban pressures, the future of this endangered species remains uncertain, even if small populations may still exist elsewhere. The genetic characterization of *C. wimmeri* not only contributes to the knowledge of the evolution of West African shrews, but also may help in the location of additional cryptic populations of this critically endangered species.

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**Figure 2.** Wimmer's shrew (*Crocidura wimmeri*) from Banco National Park (Banco 1, male). The shrew is coloured light grey. Mean head–body length = 95.2 mm (range 83–105 mm), tail length = 55.5 mm (range 50–63 mm), hind–foot length = 15.9 mm (range 14–17.5 mm;  $N = 11$ ).

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## SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

**Table S1.** Identification code and GenBank accession numbers of *cytochrome b* sequences used in the present study. Samples from Dubey *et al.* (2008b) have two different GenBank accession numbers because their *cytochrome b* sequences were amplified with two different sets of primers.