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Molecular phylogeny of African hinge-back tortoises (*Kinixys*): implications for phylogeography and taxonomy (Testudines: Testudinidae)

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Abstract

We examine the phylogeography, phylogeny and taxonomy of hinge-back tortoises using a comprehensive sampling of all currently recognized *Kinixys* species and subspecies and sequence data of three mitochondrial DNA fragments (2273 bp: 12S rRNA, ND4 + adjacent DNA coding for tRNAs, cyt*b*) and three nuclear loci (2569 bp: C-mos, ODC, R35). Combined and individual analyses of the two data sets using Bayesian and Maximum Likelihood methods suggest that the savannah species of *Kinixys* are paraphyletic with respect to the rainforest species *K. homeana* and *K. erosa*, and that the rainforest species may be derived from a savannah-living ancestor. The previously recognized savannah species *K. belliana* was a conglomerate of three deeply divergent clades that we treat here as distinct species. We restrict the name *K. belliana* (Gray, 1830) to hinge-back tortoises ranging from Angola to Burundi, while five-clawed hinge-back tortoises from the northernmost part of the formerly recognized range of *K. belliana*, together with four-clawed tortoises from West Africa, are assigned to the species *K. nogueyi* (Lataste, 1886). These two species are allied to *K. spekii*, whereas Southeast African and Malagasy hinge-back tortoises formerly lumped together with *K. belliana* represent the distinct species *K. zombensis* Hewitt, 1931, which is sister to *K. lobatsiana*. The latter two species together constitute the sister group of the rainforest species *K. homeana* and *K. erosa*. Mitochondrial data suggest that *K. natalensis* has a basal phylogenetic position in a clade embracing *K. belliana* sensu stricto, *K. nogueyi* and *K. spekii*, while nuclear data and the two data sets combined favour a sister group relationship of *K. natalensis* to all other hinge-back tortoises. Phylogeographic structure is present in all wide-ranging species and correlates in *K. homeana* and *K. erosa* with the Dahomey Gap and former rainforest refugia. The Malagasy population of *K. zombensis* is weakly differentiated from its South African conspecifics and further sampling is needed to determine whether there is support for the subspecific distinctness of Malagasy tortoises.

Key words: Africa – Madagascar – numt – revision – species delineation – subspecies

Introduction

Hinge-back tortoises (genus *Kinixys* Bell, 1827) are the only extant chelonians with a movable hinge in the carapace, allowing a more or less complete closure of the rear part of their shell. This hinge is positioned between the fourth and fifth costal and the seventh and eighth peripheral bones. *Kinixys* species are confined to sub-Saharan Africa and Madagascar and are true land tortoises occurring in open or forested habitats (Figs S1 and S2). Their carapacial lengths range from 15 to 32 cm (Loveridge and Williams 1957; Ernst and Barbour 1989; Iverson 1992; Ernst et al. 2000; Branch 2008). While species and subspecies delineation have frequently changed over the past 50 years (Loveridge and Williams 1957; Wermuth and Mertens 1961, 1977; Broadley 1981, 1989, 1992, 1993; Ernst and Barbour 1989; Iverson 1992; Ernst et al. 2000; Fritz and Havaš 2007; Iverson et al. 2007; Branch 2008; Rhodin et al. 2010), all authors have agreed explicitly or implicitly that there are two distinct species groups, one comprising rainforest species and the other savannah species. These species groups are thought to represent reciprocally monophyletic clades (Iverson 1992; Iverson et al. 2007). Most recent studies and check-lists recognized two rainforest species and four savannah species (but see McCord et al. 2005 and Rhodin et al. 2008, who treated *K. belliana nogueyi* as a full species), even though subspecific structuring within *K. belliana* has remained controversial (Table 1). One of the contentious issues is the validity of the Malagasy subspecies *K. b. domerguei*. This taxon is restricted to a small region in extreme northwestern Madagascar and was described by Vuillemin (1972) as representing a new genus and new species (*Madakinixys domerguei*). However, its conspecifity with *K. belliana* was soon recognized (Wermuth and Mertens 1977; Obst 1978). Although it is generally assumed that *K. belliana* was introduced to Madagascar (Wermuth and Mertens 1961, 1977; Bour 1978, 1987, 2006; Raselimanana and Vences 2003; Fritz and Havaš 2007; Rhodin et al. 2010), some authors have continued to treat the Malagasy population as a distinct subspecies (Bour 1987, 2006; Rhodin et al. 2010), leaving open the possibility that it originated from an older, natural, colonisation event.

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Table 1. Competing schemes for the taxonomy of *Kinixys* taxa. The rainforest species group embraces *K. erosa* and *K. homeana*, whereas all other species are placed in the savannah species group

	Broadley (1992, 1993), Ernst et al. (2000), Fritz and Havaš (2007), Branch (2008)	Rhodin et al. (2010)
Kinixys belliana belliana (Gray, 1830)	Valid	Valid
Kinixys belliana nogueyi (Lataste, 1886)	Valid	Valid
Kinixys belliana domerguei (Vuillemin, 1972)	Synonym of *K. b. belliana*	Valid
Kinixys belliana zombensis Hewitt, 1931	Synonym of *K. b. belliana*	Valid
Kinixys erosa (Schweigger, 1812)	Valid	Valid
Kinixys homeana Bell, 1827	Valid	Valid
Kinixys lobatsiana Power, 1927	Valid	Valid
Kinixys natalensis Hewitt, 1935	Valid	Valid
Kinixys spekii Gray, 1863	Valid	Valid

Until now, the taxonomy of *Kinixys* species was assessed only by morphological means, although a few publications have used mitochondrial and nuclear DNA sequences of single *Kinixys* species when addressing more general questions. Le et al. (2006) and Fritz and Bininda-Emonds (2007) used sequences of three mitochondrial and two nuclear genes of *K. erosa* and *K. homeana* for unravelling the large-scale phylogeny of land tortoises (Testudinidae), and Sasaki et al. (2006) applied SINE loci of the same species as outgroups in a phylogenetic study of freshwater turtles (Geoemydidae). In a pilot study on barcoding of turtle and tortoise species, Reid et al. (2011) used COI sequences labelled as *K. homeana* and *K. natalensis*.

Our present paper is the first molecular investigation based on a comprehensive sampling of all *Kinixys* species and subspecies. Here we follow tentatively the taxonomy of Rhodin et al. (2010) in that we treat the Malagasy and Southeast African populations of *K. belliana* as the distinct subspecies *K. b. domerguei* and *K. b. zombensis*, respectively. We use sequence data of three mitochondrial genes and three nuclear loci to assess the phylogeny and phylogeography of hinge-back tortoises. By doing so, we re-examine the validity and relationships of the studied taxa, and in particular whether the savannah and rainforest species are reciprocally monophyletic and whether Malagasy hinge-back tortoises are genetically distinct from their continental African conspecifics.

Materials and Methods

Sampling and gene selection

Eighty-six tissue and blood samples of hinge-back tortoises were studied, representing all currently recognized taxa including *Kinixys belliana domerguei* and *K. b. zombensis* (Table S1). Three mitochondrial genes were sequenced that have previously been shown to unravel phylogeny and differentiation of terminal chelonian taxa (e.g. Le et al. 2006; Fritz and Bininda-Emonds 2007; Vargas-Ramírez et al. 2010a,b; Wiens et al. 2010; Fritz et al. 2011), viz. the partial 12S ribosomal RNA (12S rRNA) gene, the partial NADH dehydrogenase subunit 4 (ND4) gene, and the cytochrome *b* (cyt*b*) gene. The DNA sequence containing the partial ND4 gene embraced also the flanking DNA coding for tRNA-His, tRNA-Ser and tRNA-Leu. The DNA sequence containing the cyt*b* gene included also approximately 20 bp of the adjacent DNA coding for tRNA-Thr. In addition to these mtDNA data, up to three nuclear loci were generated for 35 samples being representative for phylogenetic clades revealed by mitochondrial sequences. The nuclear genomic loci were the partial genes coding for the oocyte maturation factor Mos (C-mos) and for ornithine decarboxylase (ODC), and the intron 1 of the RNA fingerprint protein 35 (R35) gene. These three loci are increasingly applied for phylogenetic investigations of turtles and tortoises (e.g. Georges et al. 1998; Fujita et al. 2004; Le et al. 2006; Vargas-Ramírez et al. 2010b; Wiens et al. 2010; Fritz et al. 2011). While the complete mitochondrial data set could be generated for all but one sample, of which only two genes were available, only two nuclear loci of some specimens could be sequenced due to bad DNA quality or small sample size. Remaining samples and DNA are stored at −80°C in the tissue collection of the Museum of Zoology, Dresden.

Laboratory procedures

Total genomic DNA was extracted using either the DTAB method (Gustincich et al. 1991), the innuPREP DNA Mini Kit, the innuPREP Blood DNA Mini Kit (both Analytik Jena AG, Jena, Germany) or the NucleoSpin 8 Tissue Core Kit (Macherey Nagel, Düren, Germany).

The partial 12S rRNA gene was PCR-amplified using the universal primers L1091 and H1478; for the DNA fragment comprising the partial ND4 gene plus flanking DNA coding for tRNAs, the primers ND4 672 and H-Leu were used. The cyt*b* gene was initially amplified in two fragments overlapping by approximately 300 bp using the primer pairs CytbG + mt-E-Rev2 and mt-c2 + mt-f-na. The primers for the ND4 and cyt*b* genes are standard primers for turtles and tortoises. Gel electrophoretic examination of the PCR products of the 12S rRNA gene showed, besides the target amplicon of approximately 400 bp length, the presence of a shorter band of approximately 200 bp length, which is why a preparative gel electrophoresis was applied (see below). While amplification and sequencing of the ND4 gene worked perfectly, the cyt*b* primers yielded in several cases mismatching sequences suggestive of numts, as known to occur in testudinids (Fritz et al. 2010). Therefore, the original approach was abandoned and the cyt*b* gene was amplified in one fragment by combining the primers CytbG and mt-f-na. Since this primer combination produced also regularly the putative numt sequence, the forward primer CytbG was replaced by the specifically designed primer Kinixys_cytb_for. This primer did not work in a few samples of *Kinixys erosa*; then, the forward primer mt-a-neu was used instead. The authenticity of obtained mtDNA sequences was verified as in Fritz et al. (2010). Identification of numts obtained from the primer combination CytbG and mt-f-na was unambiguous because these sequences contained frameshift mutations and stop codons; the numts will be discussed in more detail elsewhere. For amplification of the nuclear genes, the following primers were used: Cmos1 and Cmos3 for the C-mos gene, the chicken primers of Friesen et al. (1999) for ODC, and the primers R35Ex1 and R35Ex2 for the intron 1 of the R35 gene. For all primer sequences and their original references, see Table S2.

PCR was carried out in a total volume of 25 µl containing 0.2 µl *Taq* polymerase (5 u/µl; Bioron, Ludwigshafen, Germany), 1× buffer as recommended by the supplier, 0.4 µM of each primer, and 0.2 mM of each dNTP (Fermentas, St. Leon-Rot, Germany). Alternatively, for challenging samples a total volume of 20 µl containing 0.2 µl GoTaq® Flexi DNA Polymerase (5 u/µl; Promega, Madison, WI, USA) was used according to the recommendations by the supplier. PCR protocols are summarized in Table S3. PCR products were purified using the ExoSAP-IT enzymatic cleanup (USB Europe GmbH, Staufen, Germany) or, for the partial 12S rRNA gene, a preparative gel electrophoresis and the peqGOLD Gel Extraction Kit (peqlab, Erlangen, Germany). Purified PCR products were then sequenced on an ABI 3130xl Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems). Cycle sequencing reactions were purified by ethanol/sodium acetate precipitation or by using Sephadex (GE Healthcare, München, Germany). For sequencing the 12S rRNA and ND4 genes, the same primers as for PCR were applied. For the cyt*b* gene, the internal primers mt-c-For2 and mt-E-Rev2 were used; the

forward primer Kinixys_cytb_for was applied for completing the 5′-end of some samples. For sequencing the nuclear genes, the same primers as for PCR were used. However, for the ODC and R35 of challenging samples the newly designed primers Kinixys_ODC_Seq_F + Kinixys_ODC_Seq_R and Kinixys_R35_Seq_F + Kinixys_R35_Seq_R were used (Table S2).

Alignment, partitioning and phylogenetic analyses

All sequences were aligned and inspected using BIOEDIT 7.0.5.2 (Hall 1999) and MEGA 4.0.2 (Tamura et al. 2007); uncorrected *p* distances of the cyt*b* gene were calculated with the latter software. Homologous GenBank sequences of *Manouria emys*, *Stigmochelys pardalis* and *Testudo graeca* were downloaded as outgroups and included in the alignments. Asian tortoises of the genus *Manouria* represent together with the species of the North American genus *Gopherus* a highly distinct clade being sister to all other living testudinids, while *S. pardalis* and *T. graeca* are two species representing clades more closely allied to *Kinixys* (Le et al. 2006; Fritz and Bininda-Emonds 2007). Since no ODC sequences were available for all three outgroups, these were generated for the present study as described above. GenBank accession numbers of newly generated sequences and GenBank sequences of outgroups are listed in Table S1.

Phylogenetic relationships were inferred for three data sets: (1) the concatenated mitochondrial genes of 86 samples, corresponding to a total alignment of 2273 bp; (2) the concatenated nuclear DNA sequences of 35 samples, corresponding to a total alignment of 2569 bp; and (3) a supermatrix of 4842 bp length, in which the mitochondrial data of the same samples were added to their nuclear sequences (for individual gene partitions, see Table S4).

Trees were calculated using the Maximum Likelihood (ML) approach as implemented in RAxML 7.0.3 (Stamatakis 2006); the partition scheme of Table S4 and the GTR + G model across all partitions were applied. Five independent ML searches were conducted using different starting conditions and the fast bootstrap algorithm to examine the robustness of the branching patterns by comparing the best-scored trees. Subsequently, 1000 non-parametric thorough bootstrap replicates were calculated and plotted against the tree with the highest likelihood value. In addition, phylogenetic relationships were inferred by using MRBAYES 3.1.2 (Ronquist and Huelsenbeck 2003) and the implemented Metropolis-coupled Markov chain Monte Carlo algorithm with two parallel runs, each with one cold and three heated chains. The chains run for 10^7 generations, with every 100th generation sampled. The best-fit evolutionary models were determined for each data set and individual genes using the Akaike information criterion of MODELTEST 2.3 (Posada and Crandall 1998). However, using partitioned alignments the two runs did not converge onto a stationary distribution and the estimated evolutionary distances were by far too large. This problem is well known for Bayesian inference (Brown et al. 2010; Marshall 2010). To overcome this problem, analyses were rerun using unpartitioned alignments, with the GTR + I + G model for the mitochondrial data set and the supermatrix, and the HKY + I + G model for the nuclear data. Then, convergence was obtained as evinced by average standard deviations of split frequencies approaching zero. For generating the final 50% majority rule consensus tree, a burn-in of 4×10^4 was used to sample only the most likely trees. *Manouria emys* was used for tree rooting.

To examine the phylogenetic information of individual genes, exploratory RAxML analyses were also run using each mtDNA and nDNA fragment and the same conditions as above.

Results

Phylogeny

The trees obtained from the two methods to infer phylogeny yielded largely congruent results. Differences concerned only the branching patterns of few weakly supported nodes, especially in the poorly resolved trees based on the nuclear data set (Fig. S3).

Based on morphological evidence, previous authors distinguished between two species groups. One species group was thought to comprise the two rainforest species *Kinixys homeana* and *K. erosa*, while the second group should embrace all the remaining *Kinixys* species living mainly in savannah habitats. Accordingly, it has been hypothesized that these two species groups are reciprocally monophyletic (Iverson 1992; Iverson et al. 2007). In the trees based on mtDNA sequences (Fig. 1), the two rainforest species *K. homeana* and *K. erosa* constituted a well-supported clade. Each of the two species corresponded to a clade with maximum support. Within either species, geographic structuring was present, with three weakly to well-supported clades within *K. homeana* and five clades within *K. erosa*. Contrary to expectation, however, the savannah species were not monophyletic and sister to the two rainforest species. Rather, the savannah species were paraphyletic with respect to *K. homeana* and *K. erosa*. Moreover, sequences assigned to *K. belliana* occurred in three deeply divergent, well-supported clades.

All Malagasy tortoises (*K. b. domerguei*) shared the same haplotype. This clade was with high support sister to South African *K. b. zombensis*; their successive sister taxon was with maximum support *K. lobatsiana* (Fig. 1). These three savannah taxa together constituted with high support the sister group of the two rainforest species *K. homeana* and *K. erosa*. The remaining samples of savannah taxa corresponded to a well-supported clade being sister to the mixed savannah-rainforest clade.

This major clade of savannah taxa only embraced four well-supported minor clades. One of these, corresponding to the two sequences of *K. natalensis*, was sister to a more inclusive clade embracing three well-supported subordinated clades, each of which showed clear geographic substructuring. The basal branching pattern of these three subordinated clades remained poorly resolved, which is why the 50% majority rule consensus of the Bayesian analysis placed them in a polytomy (Fig. 1). One of these three clades contained sequences of *K. b. nogueyi* and of three *K. b. belliana* from the Central African Republic; therein, sequences of *K. b. nogueyi* from Ghana and Senegal clustered in a maximally supported subclade with one of the sequences of *K. b. belliana*. Two other *K. b. belliana* from the Central African Republic were placed with maximum support in another subclade, together with two sequences of *K. b. nogueyi* from Cameroon. The second of the three clades contained sequences of *K. b. belliana* from Angola and Burundi (plus a sequence of a pet tortoise of unknown provenance), and the third clade was formed by sequences of *K. spekii*. Sequences of *K. spekii* from Namibia, South Africa, Zambia and Zimbabwe were only weakly differentiated, whereas a sequence from the Democratic Republic of the Congo was clearly distinct.

Compared to the mitochondrial trees, the trees based on the nuclear data set were only poorly resolved and many branching patterns were only weakly supported (Fig. S3). However, the three nuclear loci also suggested a paraphyly of the savannah taxa with respect to the rainforest species. Here, all savannah taxa except *K. natalensis* were placed together with the two rainforest species *K. homeana* and *K. erosa* in a well-supported major clade, which was sister to *K. natalensis*. Sequences of the two rainforest species were not clearly distinct and appeared, with weak support, to be the sister group of all savannah taxa except *K. natalensis*. Apart from the fact that *K. homeana* and *K. erosa* and *K. b. domerguei* and *K. b. zombensis*,

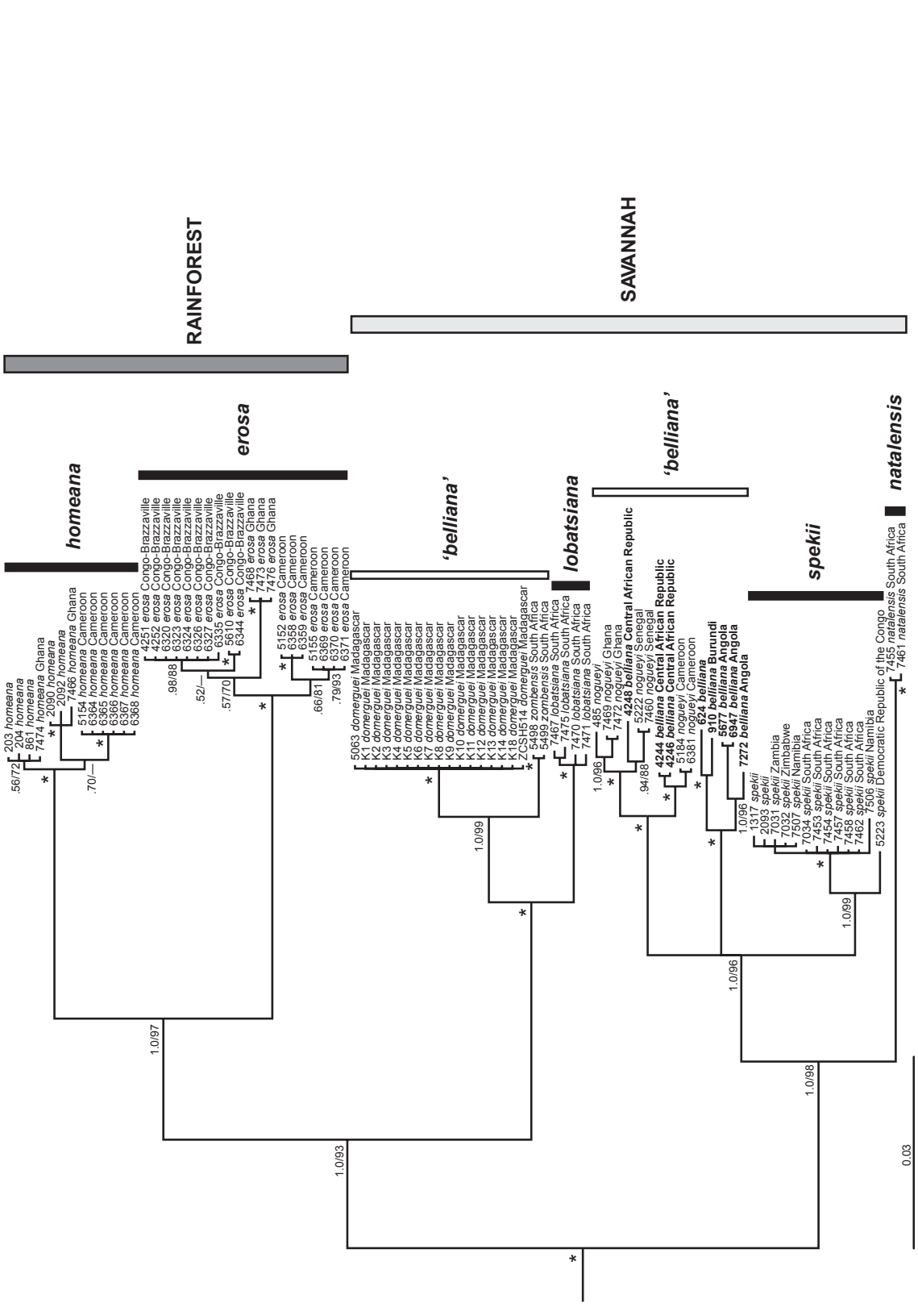

Fig. 1. Phylogeny of *Kinixys* species as inferred from Bayesian analysis with MrBayes 3.1.2, based on a 2273-bp-long alignment of mtDNA (12S rRNA, ND4 + tRNAs, cyt*b* + tRNA-Thr). Outgroups (*Manouria emys*, *Stigmochelys pardalis*, *Testudo graeca*) removed for clarity; tree was rooted with *M. emys*. For explanation of sample codes preceding taxon names, see Table S1. Numbers along branches indicate Bayesian posterior probabilities and thorough bootstrap values as obtained using RAxML 7.0.3. Asterisks indicate maximum support under both methods; dashes, branch not found. Support values are not shown for some terminal clades with short branches. Samples of *K. b. belliana* clustering in distinct clades in boldface. On the right, current species delineation and species groups (rainforest and savannah species groups) indicated

respectively, were not reciprocally monophyletic, all mitochondrial clades were also recovered from the analyses of the nuclear DNA sequences, albeit mostly with weak statistical support.

The trees based on the supermatrix of concatenated mitochondrial and nuclear DNA sequences (Fig. 2) largely agreed with the trees based on mtDNA only, with one exception. In the mitochondrial trees, *K. natalensis* was placed in the same clade as certain other savannah taxa (*K. b. belliana* from Angola, Burundi and the Central African Republic; *K. b. nogueyi*; *K. spekii*), whereas the trees based on nuclear DNA sequences suggested *K. natalensis* as the most basal species of *Kinixys*. The nuclear signal enforced the latter topology also in the trees based on the supermatrix, but now with decidedly weak support for the monophyly of all of the remaining species. In any case, each data set revealed the savannah taxa as paraphyletic with respect to the rainforest species.

For gaining deeper insight into the conflicting placement of *K. natalensis*, ML trees of individual gene partitions were compared. With respect to the three trees based on each mtDNA fragment, the placement of *K. natalensis* was consistent with the combined analyses of mtDNA, despite a generally weaker resolution in the trees based on single partitions. The individual analyses of the three nuclear partitions suggested that the signal responsible for the conflicting placement of *K. natalensis* in the combined analyses of nuclear DNA and nuclear plus mitochondrial DNA was caused by C-mos and ODC, which delivered, with moderate to weak bootstrap support, a sister group relationship of *K. natalensis* and all other *Kinixys* species. By contrast, an ML tree based on R35 sequences returned *K. natalensis* in a poorly resolved and weakly supported polytomy together with all other *Kinixys*, except *K. erosa*, *K. homeana* and *K. lobatsiana*.

Uncorrected *p* distances of the mitochondrial cyt*b* gene

In several recent studies (Vargas-Ramírez et al. 2010b; Praschag et al. 2011; Stuckas and Fritz 2011; Fritz et al. 2012a,b) uncorrected *p* distances of the mitochondrial cyt*b* gene were used as a tool for species delineation of turtles and tortoises (see Discussion). Therefore, uncorrected *p* distances of a 1058-bp-long alignment (1035 bp cyt*b* gene + 23 bp of the adjacent DNA coding for tRNA-Thr) of the studied *Kinixys* samples were computed using MEGA 4.0.2 (Tamura et al. 2007). Pairwise divergences were calculated within and between the major clades of Figs 1 and 2 (Table 2) and between some finer entities corresponding to subclades (Table S5). Uncorrected *p* distances between the major clades ranged on average between 3.76% (*K. b. domerguei* + *K. b. zombensis* versus *K. lobatsiana*) and 11.59% (*K. erosa* versus *K. natalensis*); divergences within the clades, between 0.25% (*K. spekii*) and 1.93% (*K. b. belliana* from Angola and Burundi plus a pet tortoise of unknown provenance). The haplotypes of the two *K. natalensis* were identical (Table 2).

The divergence between *K. b. domerguei* and *K. b. zombensis* was with 1.33% clearly lower than between each of these two taxa and the closely allied *K. lobatsiana* (3.76% and 3.77%; Table S5). The distinct subclade embracing sequences of *K. b. nogueyi* from Ghana and Senegal and a sequence of a *K. b. belliana* from the Central African Republic differed from its sister group (*K. b. belliana* from the Central African Republic; *K. b. nogueyi* from Cameroon) by 1.47%. The subclade consisting of a sequence of a *K. b. belliana* from Burundi and another pet tortoise differed from its sister group (*K. b. belliana* from Angola) by 2.57%, and the distinct sequence of a *K. spekii* from the Democratic Republic of the Congo differed from all the other *K. spekii* by 1.0% (Table S5). The mean divergences among the corresponding three more inclusive clades, viz. (1) *K. b. belliana* (Central African Republic) + *K. b. nogueyi*, (2) *K. b. belliana* from Angola and Burundi, and (3) *K. spekii*, were 4.08–6.76% (Table 2).

Discussion

The results of our combined and individual analyses of mitochondrial and nuclear DNA were largely consistent. However, contradictory evidence was obtained for the phylogenetic placement of *Kinixys natalensis*. While this species was suggested as the sister taxon of all other *Kinixys* species by concatenated nDNA (Fig. S3) and mtDNA + nDNA (Fig. 2), individual and combined analyses of the mitochondrial partitions (Fig. 1) revealed *K. natalensis* as the sister taxon of a major clade embracing *K. spekii* and two further subordinated clades, one corresponding to *K. b. belliana* from Angola and Burundi and the other to *K. b. belliana* from the Central African Republic plus *K. b. nogueyi*. Individual analyses of the nuclear partitions suggested that the signal responsible for the conflicting position of *K. natalensis* was caused by C-mos and ODC, but not by R35.

This situation is indicative of incongruent phylogenetic information in the individual partitions, which was long considered to be problematic. However, the paradigm that only DNA partitions with congruent phylogenetic signal should be combined has been challenged years ago (Gatesy et al. 1999) and recently been refuted. By combining incongruent individual data sets, even previously undetected phylogenetic signal may be uncovered (Struck 2007; Zanol et al. 2010) – signal which is not revealed by single-gene analyses. Consequently, congruence among data sets is no longer a prerequisite for combining partitions (Cunningham 1997; Gatesy et al. 1999; Zanol et al. 2010). In essence, the conflicting branching patterns of *Kinixys* reflect the expected and well-known differences between gene trees and species trees (e.g. Maddison 1997; Rosenberg 2002; Degnan and Rosenberg 2009), which are exacerbated by the different inheritance modes of mitochondrial and nuclear DNA (Ballard and Whitlock 2004).

Based on morphological criteria, two rainforest species (*K. erosa*, *K. homeana*) and four savannah species of hinge-back tortoises (*K. belliana*, *K. lobatsiana*, *K. natalensis*, *K. spekii*) are currently recognized. Each of these ecological groups is thought to represent a reciprocally monophyletic group (Iverson 1992; Iverson et al. 2007). Only within *K. belliana* are two to four subspecies distinguished (Broadley 1993; Fritz and Havaš 2007; Branch 2008; Rhodin et al. 2010; Table 1). However, our molecular data contradict this classification, and according to our results the two ecological groups are also not reciprocally monophyletic. Rather, the savannah species are paraphyletic with respect to the two rainforest species *K. erosa* and *K. homeana*, suggesting that the rainforest species may derive from a savannah-dwelling ancestor. Furthermore, *K. belliana* is not monophyletic, but consists of three highly distinct major clades that were independently revealed by the mitochondrial and nuclear DNA data sets (Figs 1 and S3).

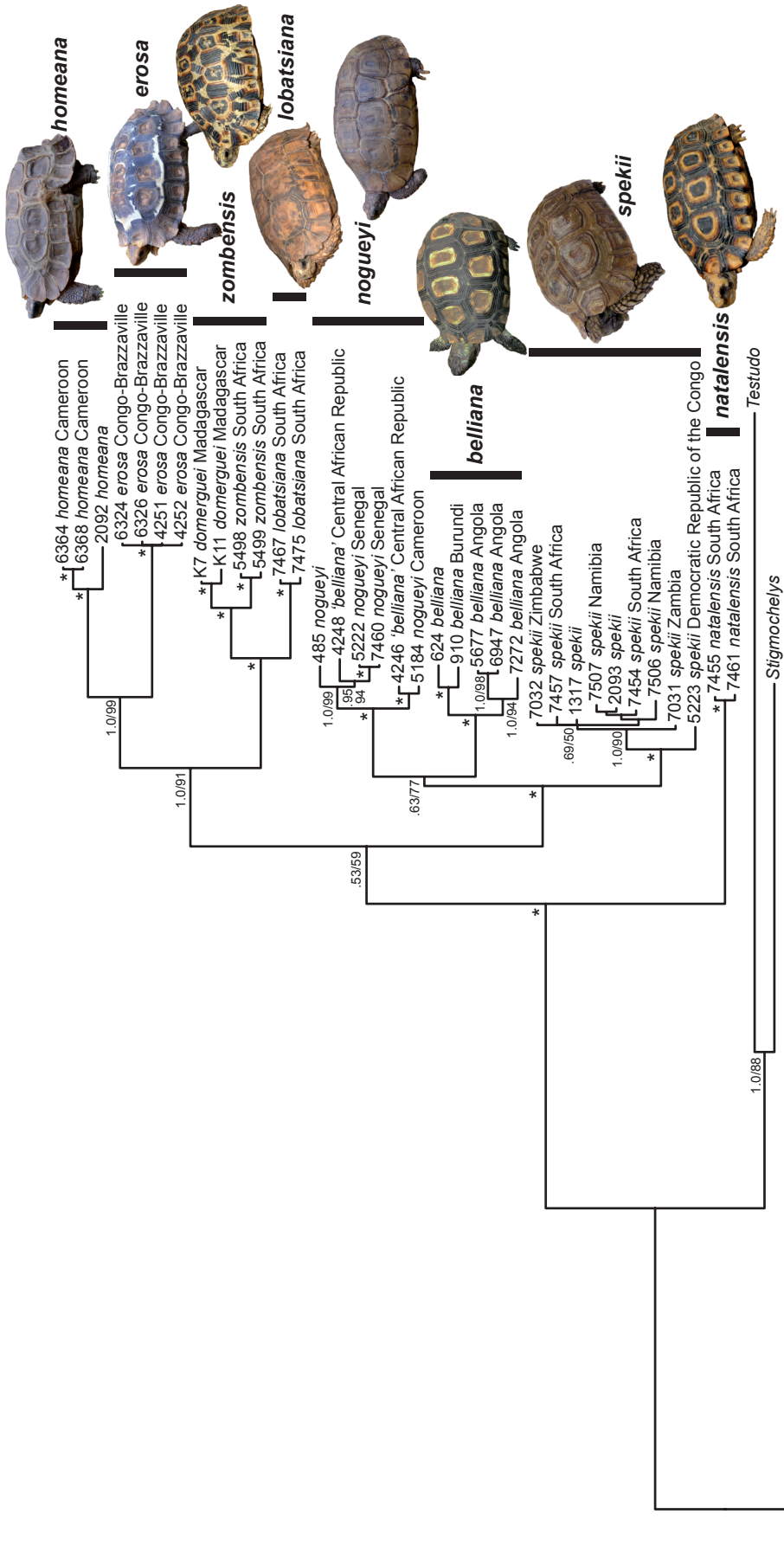

Fig. 2. Phylogeny of *Kinixys* species as inferred from Bayesian analysis, based on the supermatrix (2273 bp of mtDNA: 12S rRNA, ND4 + tRNAs, cyt*b* + tRNA-Thr plus 2569 bp of nDNA: C-mos, ODC, R35). On the right, new species delineation. For further explanations, see Fig. 1 and Discussion

Table 2. Mean uncorrected *p* distances (percentages ± standard errors) within and between major mitochondrial clades of *Kinixys* using a 1058-bp-long alignment of mtDNA (1035 bp cyt*b* gene + 23 bp of the adjacent DNA coding for tRNA-Thr). Below the diagonal, divergences between clades are given; on the diagonal, divergences within clades in boldface. *Kinixys b. belliana* (1) refers to tortoises from the Central African Republic; *K. b. belliana* (2), Angola and Burundi (plus a pet tortoise of unknown provenance)

	n	*homeana*	*erosa*	*domerguei* + *zombensis*	*lobatsiana*	*belliana* (1) + *nogueyi*	*belliana* (2)	*spekii*	*natalensis*
K. homeana	13	**0.55 ± 0.14**							
K. erosa	19	7.09 ± 0.75	**0.62 ± 0.13**						
K. b. domerguei + *K. b. zombensis*	19	8.52 ± 0.84	8.70 ± 0.78	**0.26 ± 0.06**					
K. lobatsiana	4	8.55 ± 0.78	8.78 ± 0.77	3.76 ± 0.56	**0.30 ± 0.11**				
K. b. belliana (1) + *K. b. nogueyi*	10	9.75 ± 0.82	11.35 ± 0.95	9.43 ± 0.83	10.23 ± 0.91	**1.05 ± 0.20**			
K. b. belliana (2)	5	9.99 ± 0.81	11.20 ± 0.89	9.58 ± 0.85	10.37 ± 0.92	4.86 ± 0.55	**1.93 ± 0.31**		
K. spekii	13	8.88 ± 0.84	10.54 ± 0.91	8.72 ± 0.87	9.48 ± 0.91	6.76 ± 0.51	4.08 ± 0.58	**0.25 ± 0.06**	
K. natalensis	2	10.54 ± 0.90	11.59 ± 0.94	9.97 ± 0.95	10.36 ± 0.89	8.42 ± 0.81	8.36 ± 0.83	7.15 ± 0.78	**0**

One of the three clades within what is currently identified with *K. belliana* is, together with *K. lobatsiana*, the sister group of the two rainforest species; the other two clades are most closely allied to *K. spekii*. This situation argues for the revision of the current species delineation of *K. belliana*.

Moreover, our analyses (Figs 1, 2 and S3) confirm the evolutionary distinctiveness of *K. lobatsiana*, *K. natalensis* and *K. spekii*; three species formerly lumped together with *K. belliana* (Loveridge and Williams 1957), but whose taxonomic disparity had been revealed by Broadley (1981, 1989, 1992, 1993). *Kinixys belliana* is the only species currently thought to be polytypic. Accordingly, several parapatric subspecies are regarded as valid (Table 1; Fig. S1), and our findings necessitate examining whether some of these subspecies, or unrecognized taxa within *K. belliana*, may represent distinct species. The phylogenetic placement of *K. belliana* sequences alone suggests that two or three distinct species are involved, one being sister to *K. lobatsiana* and allied to the rainforest species, and one or two further species being related to *K. spekii*. One of the clades allied to *K. spekii* comprises sequences of *K. b. belliana* from Angola and Burundi, and the other sequences from the northern part of the range of *K. b. belliana* (in our tree represented by samples from the Central African Republic) and of *K. b. nogueyi*. The question is whether these two clades could be conspecific with *K. spekii*. This possibility can be excluded with respect to the clade comprising sequences of *K. b. belliana* from Angola and Burundi, because these tortoises occur sympatrically with *K. spekii* (Fig. S1). By contrast, *K. b. nogueyi* is allopatric from all other savannah taxa, and the same is true for tortoises from the northern part of the range of *K. b. belliana*.

In absence of direct evidence, such as sympatric occurrence, several recent studies have used uncorrected *p* distances of the mitochondrial cyt*b* gene as a proxy for assessing the taxonomic status of turtles and tortoises. However, congeneric chelonian species differ by uncorrected *p* distances of 1.5–18.3% (Vargas-Ramírez et al. 2010b; Praschag et al. 2011; Stuckas and Fritz 2011; Fritz et al. 2012a,b), and this wide range implies that a rigid threshold is not helpful for species delineation. Rather, the threshold value needs to be adjusted by comparison with closely related species. Among congeneric tortoise species (family Testudinidae), uncorrected *p* distances range from 3.7% to 12.7% (Fritz et al. 2012a), and these values resemble the ones of *Kinixys* (Table 2). Particularly helpful is to use the closest related sympatric species as a yardstick for allopatric or parapatric taxa. Among the taxa that occur sympatrically with *K. spekii* (Fig. S1) is the clade corresponding to sequences of *K. b. belliana* from Angola and Burundi. Consequently, there is direct evidence that *K. spekii* and the hinge-back tortoise from Angola and Burundi represent distinct species under the strict Biological Species Concept (Mayr 1942; Coyne and Orr 2004). When the uncorrected *p* distances among *K. spekii* and the two allied *K. belliana* clades are compared (Table 2), it is obvious that the mean divergences between *K. spekii* and the clade consisting of *K. b. belliana* from the Central African Republic plus *K. b. nogueyi* (6.76%) and between the latter clade and the *K. b. belliana* from Angola and Burundi (4.86%) exceed the value between the sympatric *K. spekii* and *K. b. belliana* from Angola and Burundi (4.08%). This suggests that each of the three clades of *K. belliana* represents a distinct species. This is corroborated by the mean divergence of 3.76% between *K. b. domerguei* + *K. b. zombensis* and their sister species *K. lobatsiana*, resembling the value of 4.08% between *K. spekii* and *K. belliana* from Angola and Burundi. By contrast, the divergences within each of the three clades hitherto lumped together under *K. belliana* are decidedly lower (Table S5). Between *K. b. domerguei* and *K. b. zombensis* a value of 1.33% occurs; between the two subclades of *K. b. belliana* from the Central African Republic plus *K. b. nogueyi*, a value of 1.47%; and between the two subclades of *K. b. belliana* from Angola and Burundi, 2.57%, so that we suggest that this level of divergence represents phylogeographic variation within one and the same species, as does the value of 1.0% between a *K. spekii* from the Democratic Republic of the Congo and the samples of *K. spekii* from Namibia, South Africa, Zambia, and Zimbabwe.

These findings do have taxonomic implications (Fig. 2). Firstly, the name *Kinixys belliana* (Gray, 1830) has to be restricted to only one species-level clade. This name is currently identified with five-clawed hinge-back tortoises from the western part of the species' range (Loveridge and Williams 1957; Fritz and Havaš 2007; Branch 2008; Rhodin et al. 2010). Therefore, we restrict this name to the taxon represented in our sampling by tortoises from Angola and Burundi. Secondly, fore-foot claw number seems to be of only limited taxonomic use in the north-central part of the range of the *K. belliana* complex. Until now, four-clawed West African hinge-back tortoises were identified with the allopatric subspecies *K. b. nogueyi* (Lataste, 1886). Our data imply that this taxon is conspecific with five-clawed tortoises from the Central

African Republic, which are combined here with the four-clawed West African tortoises as the distinct species *K. nogueyi* (Lataste, 1886). Thirdly, the formerly recognized subspecies *K. b. domerguei* (Vuillemin, 1972) and *K. b. zombensis* Hewitt, 1931 together represent another distinct species. Unlike *K. belliana* and *K. nogueyi*, this species is not allied to *K. spekii*, but rather to *K. lobatsiana* and the rainforest species *K. erosa* and *K. homeana*. The valid name for this Malagasy and Southeast African savannah species is *K. zombensis* Hewitt, 1931 because Bour (1978) gave precedence to this name over the simultaneously published name *K. zuluensis* Hewitt, 1931 (ICZN 1999: Article 24, First Reviser Principle). Further investigations are warranted to delineate the exact distribution ranges of *K. zombensis* and in particular of *K. belliana* sensu stricto and *K. nogueyi*.

We studied from the putative continental African range of *K. zombensis* only two samples from the southernmost tip of its distribution (Fig. S1). These samples differed in the cyt*b* gene by an uncorrected *p* distance of 1.33% from 17 Malagasy samples, a value that resembles the phylogeographic variation within other *Kinixys* species (Tables 2 and S5). Malagasy hinge-back tortoises are thought to represent a distinct taxon by some authors (Bour 1987, 2006; Rhodin et al. 2010), while others believe that Malagasy tortoises are introduced and not taxonomically distinct (e.g. Ernst et al. 2000; Raselimanana and Vences 2003; Fritz and Havaš 2007; Branch 2008). The low molecular divergence between Malagasy and South African samples of *K. zombensis* renders the validity of the Malagasy taxon problematic. It is well known that the fauna of Madagascar has been severely altered since the first human settlers arrived approximately 2300 years ago (Goodman and Benstead 2003; Burney et al. 2004), and Raselimanana and Vences (2003) assume that *K. zombensis* was introduced 1000–1500 years ago. We believe that the mitochondrial uniformity of our 17 Malagasy samples, together with the tiny distribution range on Madagascar, supports the hypothesis that the species was introduced. In all other *Kinixys* taxa with comparable sample sizes greater variation is evident (Fig. 1; Tables 2 and S5), suggestive of a founder effect in the Malagasy population, as expected for an introduced population. We predict that the same genetic lineage will be discovered in the more northern continental distribution range of *K. zombensis*. The populations of several other chelonians from Madagascar (*Pelomedusa subrufa* and *Pelusios castanoides*), the Seychelles (*Pelusios castanoides* and *P. subniger*), and even Guadeloupe (Lesser Antilles, *P. castaneus*) have also been shown to represent recent, probably anthropogenic, colonisations (Silva et al. 2010; Vargas-Ramírez et al. 2010b; Fritz et al. 2011).

Phylogeographic structure was evident not only in each savannah species having a wide distribution range, but also in the two rainforest species *K. homeana* and *K. erosa* (Figs 1 and 2). In *K. homeana*, two weakly to well-supported mitochondrial clades from Ghana were discovered and another one from Cameroon; in *K. erosa*, two clades from Cameroon, a clade from Ghana, and two further clades from Congo-Brazzaville. Pronounced phylogeographic structure was also reported in a South American tortoise species occupying savannah habitats (*Chelonoidis carbonaria*), whereas the South American rainforest species *C. denticulata* is phylogeographically weakly structured, despite an extensive range (Vargas-Ramírez et al. 2010a). This difference could be related to distinct histories of the rainforests in South America and Africa. For South America, the formerly widely accepted Pleistocene forest refugia hypothesis has been challenged in recent years. By contrast, it is still widely accepted that African rainforests repeatedly contracted during the Pleistocene (Hamilton and Taylor 1991; Maley 1996; Kastner and Goñi 2003; Pennington et al. 2004; Primack and Corlett 2005; Penner et al. 2011). The distinct clades of *K. homeana* and *K. erosa* correlate with the Dahomey Gap, a 300-km-wide aisle separating until today the West African and Central African rainforests (Primack and Corlett 2005), and with the location of forest refugia during the last major arid phase approximately 18 000 years ago (compare our Fig. S2 with Fig. 5 of Maley 1996). This suggests that, as with Gabon vipers (*Bitis gabonica* and *B. rhinoceros*; Lenk et al. 2001) and frogs (Penner et al. 2011), vicariance has shaped the observed extant structure.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Distribution ranges of the savannah taxa of *Kinixys* and sampling sites.

Figure S2. Distribution ranges of the rainforest species of *Kinixys* and sampling sites.

Figure S3. Phylogeny of *Kinixys* species as inferred from Bayesian analysis, based on a 2569-bp-long alignment of nDNA (C-mos, ODC, R35).

Table S1. *Kinixys* samples and outgroups used in the present study.

Table S2. Primers used for PCR and sequencing.

Table S3. PCR protocols for mitochondrial and nuclear genes.

Table S4. Partitions in the mitochondrial and nuclear alignments used for phylogenetic inference.

Table S5. Mean uncorrected *p* distances (percentages ± standard errors) within and between major mitochondrial clades and subclades using a 1058-bp-long alignment (1035 bp cyt*b* gene + 23 bp of the adjacent DNA coding for tRNA-Thr) of *Kinixys* sequences.

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