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# Integrative taxonomy provides evidence for the species status of the Ibero-Maghrebian grass snake *Natrix astreptophora*

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The grass snake (*Natrix natrix*) is Europe's most widely distributed and, in many regions, most common snake species, with many morphologically defined subspecies. Yet, the taxonomy of grass snakes is relatively little studied and recent work has shown major conflicts between morphologically defined subspecies and phylogeographical differentiation. Using external morphology, osteological characters, and information from 13 microsatellite loci and two mitochondrial markers, we examine differentiation of the subspecies *N. n. astreptophora* from the North African Maghreb region, the Iberian Peninsula and neighbouring France. According to previous studies, *N. n. astreptophora* corresponds to a deeply divergent mitochondrial clade and constitutes the sister taxon of all remaining grass snakes. In the French Pyrenees region, there is a contact zone of *N. n. astreptophora* with another subspecies, *N. n. helvetica*. Our analyses of microsatellites and mitochondrial DNA reveal that the distribution ranges of the two taxa abut there, but both hybridize only exceptionally. Even though many morphological characters are highly variable and homoplastic in grass snakes, *N. n. astreptophora* differs consistently from all other grass snakes by its reddish iris coloration and in having significantly fewer ventral scales and another skull morphology. Considering further the virtual absence of gene flow between *N. n. astreptophora* and *N. n. helvetica*, and acknowledging the morphological distinctiveness of *N. n. astreptophora* and its sister group relationship to all remaining subspecies of grass snakes, we conclude that *Natrix astreptophora* (Seoane, 1884) should be recognized as a distinct species. Further research is needed to explore whether *N. astreptophora* is polytypic because a single sample of *N. astreptophora* from Tunisia turned out to be genetically highly distinct from its European conspecifics. © 2016 The Linnean Society of London, *Biological Journal of the Linnean Society*, 2016, **118**, 873–888.

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## INTRODUCTION

The grass snake, *Natrix natrix* (Linnaeus, 1758), is Europe's most widespread and, in many regions, most common snake species (Kabisch, 1974, 1999). Due to its broad geographical range and considerable morphological diversity, this snake has received considerable scientific attention since the end of the 19th century. With respect to intraspecific variation and taxonomy, conflicting conceptions have been discussed for decades. There are two main concepts, originally proposed by Mertens (1947) and Thorpe (1975a, b, 1979), respectively. Mertens (1947) recognized morphologically defined and parapatrically distributed taxa as distinct subspecies, resembling

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the Rassenkreis concept of Rensch (1929) and the closely related subspecies concept of Mayr (1942). Within this framework, grass snake subspecies are understood as interbreeding groups of populations characterized by differences in total length, body proportions, meristic characters, coloration and pattern. As many as 14 such subspecies have been recognized (Kabisch, 1999; Orlov & Tuniyev, 1999; Kreiner, 2007). Henceforth, this concept will be referred to as the 'multipartite subspecies concept'. It contrasts markedly with the 'four subspecies concept' of Thorpe (1979), which was based on the identification of subspecies with 'incipient species', a concept resembling the semispecies and megasubspecies categories proposed by Mayr (1963) and by Amadon & Short (1976) and Böhme (1982), respectively. Thorpe (1975a, 1979) based his conclusions on multivariate analyses of 160 morphological characters of over 750 grass snakes from 52 regions from the entire distribution range, and suggested that only three (Thorpe, 1975a) – later four (Thorpe, 1979) – subspecies should be recognized, each corresponding to an 'incipient species'. According to this model, the continental subspecies were lumped together as two subspecies, *N. n. helvetica* (Lacepède, 1789), which included all previously recognized subspecies west of the Rhine region, and *N. n. natrix* (Linnaeus, 1758) for all subspecies east of the Rhine region. In addition, *N. n. cetti* Gené, 1838 from Sardinia and *N. n. corsa* (Hecht, 1930) from Corsica were recognized (Table 1).

Besides the taxa listed in Table 1, dark-coloured and large-headed grass snakes from the Caucasus region were later described as a distinct species (*N. megalocephala* Orlov & Tuniyev, 1987), whose validity was debated for decades (Jandzík, 2005; Frotzler, Davitashvili & Mebert, 2011; Göçmen *et al.*, 2011). In particular, Göçmen *et al.* (2011) suggested that the putatively diagnostic characters of *N. megalocephala* are age-related and not taxonomically relevant. Moreover, Vanni & Cimmaruta (2010) elevated Corso-Sardinian grass snakes to species rank (*N. cetti* Gené, 1838).

Until recently, few studies have dealt with genetic differentiation in grass snakes. Using allozyme data, Hille (1997) confirmed Thorpe's (1979) east–west differentiation and found no evidence for the distinctiveness of *N. megalocephala*, despite generally rather inconclusive results. More informative were the studies of Guicking *et al.* (2006), Guicking, Joger & Wink (2008), Fritz, Corti & Päckert (2012) and especially Kindler *et al.* (2013), who progressively increased sample size, ultimately providing a nearly range-wide mitochondrial phylogeography for grass snakes. Important results of these investigations were that neither *N. cetti* nor *N. megalocephala* are distinct species. Furthermore, the revealed complex phylogeographical pattern implied that the four subspecies concept of Thorpe (1979) clearly underestimates taxonomic diversity. Kindler *et al.* (2013) found three well-supported more inclusive clades, which comprised 16 well-supported terminal clades. Most of these terminal clades, however, conflicted with morphologically defined subspecies. Only a few subspecies corresponded to monophyletic clades. One of these, *N. n. astreptophora*, is the well-supported most basal clade, which contains two deeply divergent terminal clades from the Iberian Peninsula plus

**Table 1.** Comparison of the two basic subspecies concepts for grass snakes

| Multipartite subspecies concept | Four subspecies concept |
|---|---|
| *Natrix natrix natrix* (Linnaeus, 1758) | *Natrix natrix natrix* (Linnaeus, 1758) |
| *Natrix natrix cypriaca* (Hecht, 1930) | |
| *Natrix natrix fusca* Cattaneo, 1990 | |
| *Natrix natrix gotlandica* Nilson & Andrén, 1981 | |
| *Natrix natrix persa* (Pallas, 1814) | |
| *Natrix natrix schweizeri* Müller, 1932 | |
| *Natrix natrix scutata* (Pallas, 1771) | |
| *Natrix natrix syriaca* (Hecht, 1930) | |
| *Natrix natrix helvetica* (Lacepède, 1789) | *Natrix natrix helvetica* (Lacepède, 1789) |
| *Natrix natrix astreptophora* (Seoane, 1884) | |
| *Natrix natrix lanzai* Kramer, 1970 | |
| *Natrix natrix sicula* (Cuvier, 1829) | |
| *Natrix natrix cetti* Gené, 1838 | *Natrix natrix cetti* Gené, 1838 |
| *Natrix natrix corsa* (Hecht, 1930) | *Natrix natrix corsa* (Hecht, 1930) |

The taxa below *Natrix natrix natrix* and *N. n. helvetica*, respectively, from the multipartite subspecies model are included in these two taxa in the four subspecies model.

adjacent France and from Tunisia, respectively. This '*astreptophora* clade' is sister to a major clade consisting of two more inclusive clades comprising all other terminal clades from the remaining distribution range of *N. natrix*.

In addition to these studies focusing on external morphology or genetic differentiation of extant grass snakes, there have also been some osteological investigations using fossil and extant material. Based on cranial bones and vertebrae, Szyndlar (1984) described the fossil species *N. longivertebrata* from the late Pliocene of Poland and assumed that it is closely related to *N. natrix*. Later, Rage & Szyndlar (1986) compared four skull characters of *N. longivertebrata* with those of all extant *Natrix* species. They found none of these characters present in *N. maura* and *N. tessellata*, while *N. natrix* shared two characters with its fossil relative. Contradicting basic phylogenetic principles, Szyndlar (1991a) hypothesized then that *N. longivertebrata* was the direct ancestor of *N. natrix*, based on gradual changes in basisphenoid and prootic patterns of the basicranium. With respect to the basisphenoid, there were two distinct character states observed (Szyndlar, 1984, 1991a, b). One of them was thought to be present in virtually all recent grass snakes and many fossil *N. natrix*, and the other corresponded to the fossil species *N. longivertebrata*. Thus, Szyndlar (1991a, b) identified the pattern found in *N. longivertebrata* with 'ancient' or 'primitive' and the pattern of *N. natrix* with 'derived' or 'modern'. Yet, our re-examination of Szyndlar's material revealed that his only three specimens of *N. natrix* sharing the character state of the fossil *N. longivertebrata* (Szyndlar, 1991b) belong to *N. n. astreptophora*. This situation highlights that further research is needed to understand the relationships of extant grass snake taxa and *N. longivertebrata*. However, if the osteological similarity of *N. n. astreptophora* and *N. longivertebrata* is confirmed, it would underline the distinctiveness of *N. n. astreptophora*, which was revealed as the sister group of all remaining grass snake taxa based on analyses of mitochondrial DNA (mtDNA) sequences (Guicking *et al.*, 2006, 2008; Fritz *et al.*, 2012; Kindler *et al.*, 2013). Moreover, in contrast to other morphologically defined subspecies of *N. natrix*, there are no confirmed records of hybrids between *N. n. astreptophora* and *N. n. helvetica*, whose distribution ranges abut in the Pyrenees region, in particular in France. Hence, their subspecies status may reflect traditional taxonomy and established usage of scientific names rather than true conspecificity.

The present study aims to clarify this situation using an integrative taxonomic approach (cf. Dayrat, 2005; Padial *et al.*, 2010; Schlick-Steiner *et al.*, 2010), i.e. by combining different lines of evidence corresponding to osteological, external morphological and molecular genetic datasets. We use evidence from maternally inherited mtDNA and information from 13 biparentally inherited microsatellite loci to examine the taxonomic status of *N. n. astreptophora* and *N. n. helvetica* and compare these taxa morphologically with one another and with other grass snake taxa. In doing so, we follow Mayr (1942, 1963) and Coyne & Orr (2004) and understand species as largely distinct genealogical lineages without extensive gene flow ('biological species'), whilst we treat subspecies as distinct lineages that have not reached this stage yet and which are still capable of broad-scale gene flow.

## MATERIAL AND METHODS

### Osteology and morphology

In total, 324 specimens from the collections of the Bayerische Staatssammlung für Paläontologie und Mineralogie (BSPM), the Geographisches Institut der Universität Saarbrücken (GIUS), the Instytut systematyki i ewolucji zwierząt, Polska Akademia Nauk, Kraków (ZZSiD), the Laboratoire de Biogéographie et Ecologie des Vertébrés (BEV), Université Paul-Valéry Montpellier, the Moravské zemské muzeum, Ústav Anthropos (MMUA), Brno, the Museo Nacional de Ciencias Naturales (MNCN), Madrid, the Národní museum (NMP), Prague, the Paleontological Museum, National Museum of Natural History (PM NMNHK), Kiev, and the Zoologisches Forschungsmuseum Alexander Koenig (ZFMK), Bonn, were examined. This material included 189 osteological specimens (fossil and recent material; complete skeletons or disarticulated bones) of *Natrix natrix*, *N. maura*, *N. tessellata* and *N. longivertebrata*, outnumbering by far Szyndlar's (1991a) sample corresponding to only nine complete extant skeletons. Among our osteological material were 46 complete skeletons, including (with one exception) all the specimens used by Szyndlar (1991b). However, not all grass snake subspecies were available as skeletons (Supporting Information, Table S1). All skulls were examined using an Olympus SZX12 stereoscope with DF Plato 1× PF lens and compared with the drawings in Szyndlar (1991a, b).

Vertebrae were examined both visually and by landmark-based geometric morphometric analysis. Compared to traditional morphometrics, geometric morphometrics uses Cartesian coordinates in a two-dimensional or three-dimensional space to quantitatively represent and analyse morphological shape instead of simple measurements (Bookstein, 1991; O'Higgins, 2000; Zelditch, Swiderski & Sheets, 2012). Its major goal is to detect morphological similarity

and dissimilarity in sample data. Following the approach of Szyndlar (1991a, b), one representative mid-trunk vertebra from 26 adult individuals was chosen, and then orientated exactly with respect to the image plane and photographed from three directions (dorsal, lateral and ventral view) using a scale bar. A needle was inserted through the spinal canal to stabilize the position of the vertebra. Vertebrae from *N. maura* and *N. tessellata* were also studied.

The geometry was defined by seven (lateral), six (dorsal) and six (ventral) representative 'type 2 landmarks', which were plotted using TPSDIG2 (Rohlf, 2010; Fig. 1). The relatively low number of landmarks was caused by the paucity of characteristic points in vertebrae but is expected to yield robust results for small datasets (Zelditch *et al.*, 2012). Landmark coordinates were superimposed in MORPHOJ 1.06b (Klingenberg & Gidaszewski, 2010) via Generalized Procrustes Analysis (GPA). GPA aligns differences between specimens caused by size, position and rotation. The resulting corrected landmark coordinates were then tested by regression to infer potential allometric changes between shape and size in the datasets, followed by conversion into covariance matrices. Matrices were analysed using Principal Component Analysis (PCA) and Canonical Variate Analysis (CVA), the latter using either subspecies or genetic clades as classifier variables in groupings.

In addition, 135 grass snakes from the ZFMK and BEV collections were examined for a total of 18 external characters, including the number of preoculars, postoculars, supralabials, sublabials, loreals, nasals, ventrals, dorsals and subcaudals (Supporting Information, Table S2). Additionally, measurements (total length, body length and tail length) were taken, and correlations between these measurements were calculated, based partly on ratios between certain values (tail length/total length, body length/tail length). Snakes from the ZFMK collection were X-rayed to count the number of vertebrae as well as to determine the ratio of the length and width of the centrum of mid-trunk vertebrae (CL/NAW ratio of Szyndlar, 1984, 1991a) using the software IMAGEJ 1.48 (W. Rasband, National Institutes of Health, USA). According to Szyndlar (1991a), a ratio exceeding 1.9 is diagnostic of *N. longivertebrata*, whilst *N. natrix* is characterized by values below 1.9. Depending on the outcome of the examination for normal distribution and variance homogeneity, either *t*-tests or Mann–Whitney *U* tests were calculated to infer potential differences in numerical characters for certain clades. PCAs were run on separate subsets of male and female specimens using

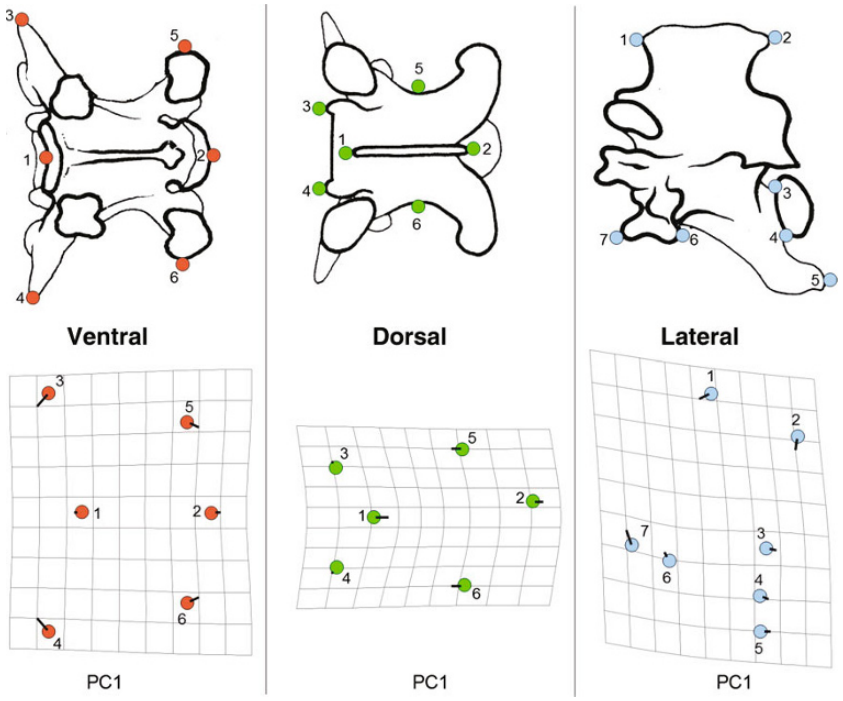


**Figure 1.** Position of landmarks used for geometric morphometric analysis of vertebrae in ventral, dorsal and lateral aspect. At the bottom, the amount of change with respect to the position of the chosen landmarks is shown. Longer lines translate into higher variation. Images of vertebrae modified from Szyndlar (1984).

the R package PCAMETHODS (Stacklies *et al.*, 2007).

### Mitochondrial DNA and microsatellite loci

Eighty-five grass snake samples from France, the Iberian Peninsula and northern Africa were studied. For 30 samples, mtDNA data (partial ND4 gene plus adjacent DNA coding for tRNAs = ND4 + tRNAs and/or cytochrome *b* gene = cyt *b*) were available from Guicking *et al.* (2006) and Kindler *et al.* (2013). For additional samples (shed skins, saliva samples, tissues from roadkills or museum specimens) sequence data were generated for the same two mtDNA blocks. ND4 + tRNAs could be sequenced for 49 samples, and cyt *b* for 29 samples. Laboratory procedures followed Kindler *et al.* (2013). The sequences obtained were aligned with the data from Guicking *et al.* (2006) and Kindler *et al.* (2013) using BIOEDIT 7.0.5.2 (Hall, 1999), resulting in an 868-bp-long alignment of 78 ND4 + tRNAs sequences and a 1116-bp-long alignment of 57 cyt *b* sequences. For detailed sample information and accession numbers, see Supporting Information, Table S3. To determine the identity of the mitochondrial haplotypes of new samples, alignments of each mtDNA block were examined by parsimony network analysis using TCS 1.21 (Clement, Posada & Crandall, 2000), with gaps coded as a fifth character state. Using the default 95% connection limit, unconnected haplotype clusters were obtained for the different genetic lineages, which is why the connection limit was arbitrarily set to 100 steps.

Sixty-eight samples were genotyped using 13 polymorphic microsatellite loci in a novel combination (Supporting Information, Table S4). Nine of these loci were either developed for *Natrix natrix* or had already been tested for their applicability for this species (Hille *et al.*, 2002; Meister *et al.*, 2009). Yet, only two loci characterized by Hille *et al.* (2002) could be used because the others failed to amplify, despite repeated efforts. Furthermore, 16 primer pairs developed for related taxa were tested, resulting in the identification of four additional informative loci. The remaining 12 loci were either monomorphic or did not yield PCR products for grass snakes (Supporting Information, Table S5). Three or four of the informative loci were combined in multiplex PCRs; two loci were amplified separately (Supporting Information, Table S4). For PCR, the Type-it Microsatellite PCR Kit (Qiagen) was used following the manufacturer's protocol. However, the total reaction volume was only 10 μL and the amount of chemicals was reduced accordingly: 5 μL of 2× Type-it Multiplex PCR Mastermix, 1 μL of 10× primer mix (2 μM of each primer), 2 μL RNase-free water and 2 μL DNA (~10 ng/μL). Forward primers were fluorescent-labelled. Cycling conditions were: after an initial denaturation at 95 °C for 5 min, 30 cycles were run with denaturation at 95 °C for 30 s, followed by annealing at primer-specific temperatures (51, 55 or 60 °C) for 90 s, extension at 72 °C for 30 s and a final extension step at 60 °C for 30 min. PCR products were diluted with water in a ratio of 1:5–1:120, depending on the multiplex. Fragment lengths were then determined on an ABI 3730 Genetic Analyzer (Applied Biosystems) using the Gene-Scan-600 LIZ Size Standard (Applied Biosystems) and the software PEAK SCANNER 1.0 (Life Technologies). The presence of microsatellites in the amplicons was verified by conventional Sanger sequencing using a homozygote sample, unlabelled primers and the same conditions as described above. PCR products were purified with the ExoSAP-IT enzymatic clean-up (USB Europe GmbH; modified protocol: 30 min at 37 °C, 15 min at 80 °C) and sequenced on an ABI 3730 analyser using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems). This revealed that *N. natrix* shows a triplet instead of a duplex motif between the two simple sequence repeats of the locus Nsμ3, i.e. $(ATCT)_{14}ATC(CA)_{20}$ instead of $(ATCT)_{14}AT(CA)_{20}$. The latter variant has been described before for *Nerodia sipedon*, the snake species for which the locus was originally characterized (Prosser, Gibbs & Weatherhead, 1999).

All microsatellite loci were tested for Hardy–Weinberg equilibrium (HWE) and linkage equilibrium using ARLEQUIN 3.5.1.2 (Excoffier & Lischer, 2010). The presence of null alleles was examined using MICRO-CHECKER 2.2.3 (van Oosterhout *et al.*, 2004). There was no evidence for linkage disequilibrium, null alleles or a deviation from HWE. Thus, all loci were suitable for further calculations. Microsatellite data were then examined using unsupervised Bayesian cluster analyses as implemented in STRUCTURE 2.3.4 (Pritchard, Stephens & Donnelly, 2000; Falush, Stephens & Pritchard, 2003, 2007; Hubisz *et al.*, 2009) with allele frequencies correlated and applying the admixture model. STRUCTURE searches in the dataset for groups which are, as far as possible, in linkage equilibrium and HWE. The Monte Carlo Markov chains ran for 750 000 generations, with a burn-in of 250 000 generations. Calculations were repeated ten times for $K$ ranging from 1 to 10. The optimal number of clusters was determined using the $\Delta K$ method (Evanno, Regnaut & Goudet, 2005) with the software STRUCTURE HARVESTER (Earl & vonHoldt, 2012). STRUCTURE results were visualized using DISTRUCT 1.1 (Rosenberg, 2004). Individuals with proportions of cluster membership below 80% (cf. Randi, 2008) were treated as having mixed ancestries.

The microsatellite data of the inferred STRUCTURE clusters were further examined by calculating population genetic diversity values, pairwise $F_{ST}$ values and analyses of molecular variance (AMOVAs) using CONVERT 1.31 (Glaubitz, 2004), ARLEQUIN 3.5.1.2 and FSTAT 2.9.3.2 (Goudet, 1995).

## RESULTS

### Osteology and external morphology

Generally, skulls of *Natrix natrix* display a characteristic basisphenoid pattern with a distinct sagittal crest between the basipterygoid processes. This crest is lacking or much less pronounced, however, in the basisphenoids of *N. n. astreptophora* and also of *N. longivertebrata*. There are also differences in the course of the vidian canals and adjacent structures. In *N. natrix*, except *N. n. astreptophora*, the posterior margins of the basipterygoid processes are sculpted into pronounced, ventral crests, which are anteromedially orientated, and come into contact at the midline. In ventral aspect, the posterior foramina of the vidian canals are clearly visible, and usually shifted forwards from the posterior margin of the basisphenoid (Szyndlar, 1991a, b). By contrast, in *N. longivertebrata* and *N. n. astreptophora* the posterior margins of the basipterygoid processes are distinctly elongated posteriorly and cover the posterior openings of the vidian canals, which are often positioned close to the posterior margin of the basisphenoid (Fig. 2). Among the material we examined, this basisphenoid morphology occurs exclusively, and invariably, in all specimens of *N. longivertebrata*, *N. n. astreptophora* and *N. maura*. The character state in *N. tessellata*, the sister taxon of *N. natrix* (Guicking *et al.*, 2006), resembles the condition found in *N. natrix* exclusive of *N. n. astreptophora* (Fig. 2).

When present, the cid nerve exit is located on the suture between the parietal and the basisphenoid in most specimens of *N. natrix*, and not directly on the basisphenoid, like in all *N. tessellata*. Where exceptions to that generalization were found, the specimens in question were usually asymmetrical, the nerve exiting on one side directly on the basisphenoid, and on the other side on the suture (Fig. 2). All *N. n. astreptophora* showed at least unilateral cid nerve exits on their basisphenoids, whilst this was otherwise only observed in four specimens of *N. n. helvetica*.

The most common character state regarding the position of the cid nerve foramen in the prootic of *N. natrix* is a separated V4 foramen (Fig. 2). Only a few specimens of *N. n. natrix* and *N. n. scutata* showed the V4 in V2 recess pattern, the normal character state in *N. maura*. It is present on both sides in three *N. n. natrix*, and unilaterally in one *N. n. scutata*. *Natrix maura* has on both sides a V4 foramen in V2 recess (Fig. 2) and *N. tessellata* a distinct V4 foramen. Other taxon-specific differences in skull morphology were not obvious, especially with respect to distinct subspecies of *N. natrix*.

Based on radiographs of 126 grass snakes, the ratios of the length and width of the centrum of midtrunk vertebrae ranged between 0.81 and 2.15, with a strong allometric effect. Adult grass snakes had relatively longer vertebrae than juveniles.

When only vertebrae of adult specimens were used for geometric morphometric analyses of vertebral shape, no significant allometric changes were evident in any of the three datasets corresponding to different vertebra aspects (dorsal: $P = 0.2194$, ventral: $P = 0.1963$, lateral: $P = 0.1054$). However, the PCAs and CVAs revealed pronounced intraspecific differentiation, in part matching well with morphologically defined subspecies or mitochondrial clades (Fig. 3). Remarkably, often grass snakes from the Iberian Peninsula and North Africa (*N. n. astreptophora*, clades Eu, Tu) do not cluster with the geographically neighbouring taxon (*N. n. helvetica*, clade E), but rather with relatives from the easternmost part of the range, specimens from Corsica and the two outgroup taxa. A two-dimensional morphospace for the lateral dataset (PC1 vs. PC2), coloured by subspecies classification (= external morphology), is shown in Figure 3; additional plots for the dorsal and ventral datasets are presented in the Supporting Information (Fig. S1). The majority of shape variation in all three datasets is described by the first two principal component axes (dorsal: 68.8 and 17.1%, ventral: 63.6 and 22.3%, lateral: 48.1 and 14.2%). PC1 in lateral view mostly accounts for the variation in landmarks 1, 2 (length of neurapophysis) and 7 (most cranially orientated point of the parapophysis), whilst PC2 describes the changes in landmarks 3 (beginning of the condyle) and 5 (caudalmost point of the hypapophysis; Fig. 1C). The dorsal and ventral datasets include symmetrical landmarks. Thus, PC1 shows mainly changes in the landmark pairs 1/2 (length of neurapophysis) and 5/6 (narrowest point of the vertebra) and PC2 in the landmark pairs 3/4 and 5/6 (Fig. 1B) of the dorsal dataset. In the ventral dataset, PC1 summarizes the variation in the landmark pairs 3/5 (distance between terminal point of the prezygapophyseal process and outermost point of the postzygapophyseal articular surface) and 4/6 (mirrored from 3/5), and PC2 accounts for the variation in the landmark pair 1/2 (distance between beginning of the cotyle and caudalmost point of the condyle) and the single landmarks 5 and 6 (outermost points of the postzygapophyseal articular surface; Fig. 1A).

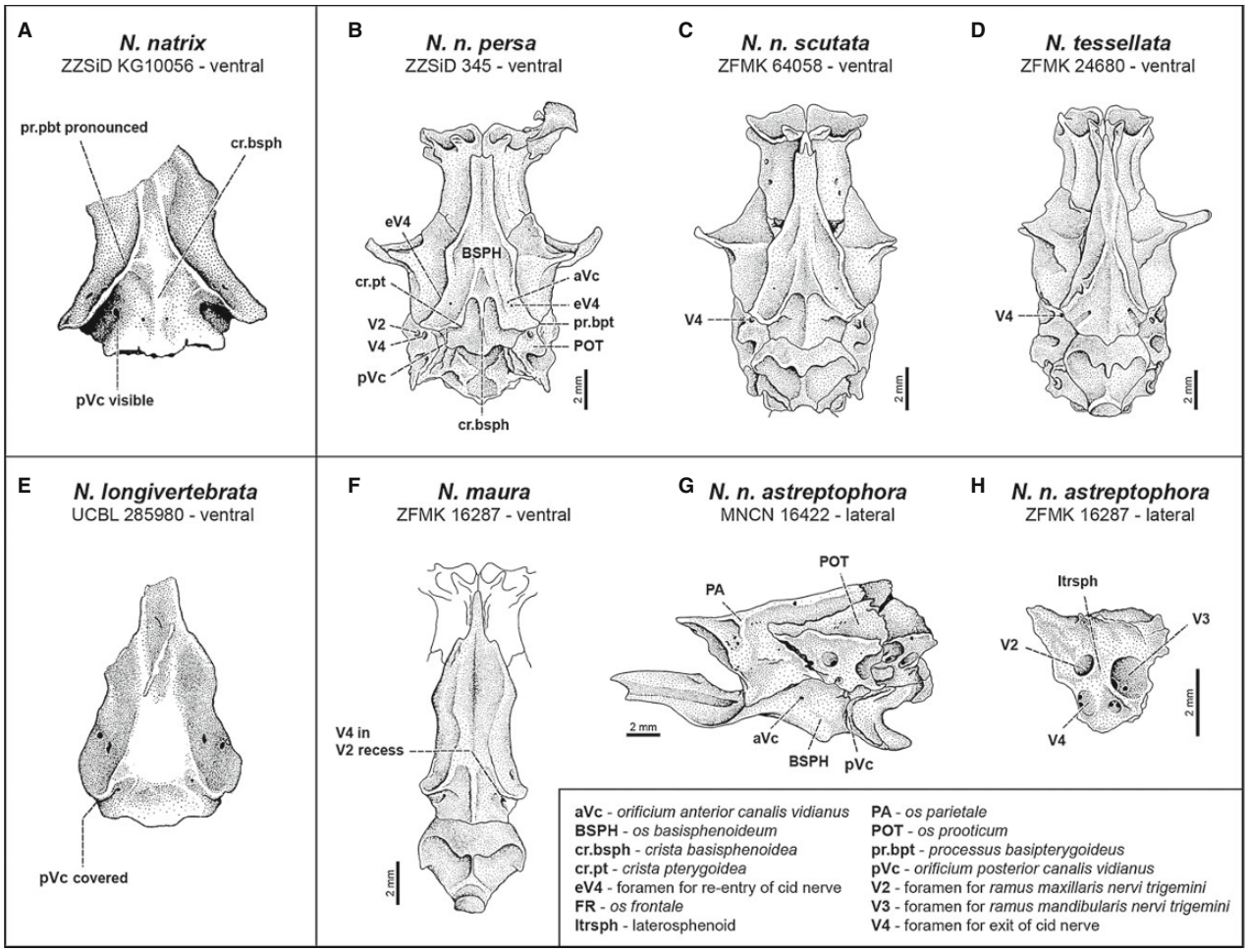


**Figure 2.** Skull details of *Natrix* taxa. A–D, basisphenoids with distinct sagittal crest, characteristic for *N. tessellata* and *N. natrix* except *N. n. astreptophora*. E, F, character state in *N. longivertebrata*, *N. maura* and *N. n. astreptophora*. G, position of the pVc close to the posterior margin of the basisphenoid, which is covered by the basipterygoid processes in ventral view (E, F). H, V4 foramen in prootic, as typical for *N. natrix*. (A, E modified from Szyndlar, 1991a; all other drawings by M. Ivanov).

Two CVAs were run, one using subspecies as a classifier variable, and the other using mitochondrial clades as a classifier. Using subspecies, *astreptophora*, *persa* and *scutata* overlapped strongly and were well separated from *natrix* and *helvetica* in the lateral dataset (Fig. 3: centre). The same discrimination was obtained for the ventral dataset, whereas the dorsal dataset did not resolve any taxa. The first two CV axes describe most of the variation in all datasets (lateral: 51.6 and 22.9%, dorsal: 54.5 and 23.9%, ventral: 55.7 and 33.2%); Mahalanobis distances among groups range from 1.02 to 12.03, depending on the dataset (see Supporting Information, Tables S6–S8 for the lateral, dorsal and ventral datasets). Using mitochondrial clades as classifier variables (Fig. 3: bottom), members of the clades Eu (European *N. n. astreptophora*) and 8 (an eastern genetic lineage comprising snakes morphologically identified with *N. n. natrix*, *N. n. scutata* and *N. n. persa*; Kindler *et al.*, 2013) are virtually indistinguishable in morphospace, whilst clade 4 (corresponding partially to *N. n. natrix* and *N. n. persa*) is highly distinct. Again, most variation is described by the first two CV axes (lateral: 55.0 and 21.0%, dorsal: 52.0 and 22.2%, ventral: 55.7 and 33.2%; Supporting Information, Tables S9–S11).

With respect to external morphology, *N. n. astreptophora* (European and north-west African snakes combined) has the lowest numbers of ventrals ($P = 0.0019$), whilst the numbers of subcaudals are high, compared to the other clades, although not significantly different ($P = 0.8359$; Fig. 4). Also, if *N. n. astreptophora* is compared only to *N. n. helvetica*, the geographically neighbouring taxon, *N. n. astrep-*

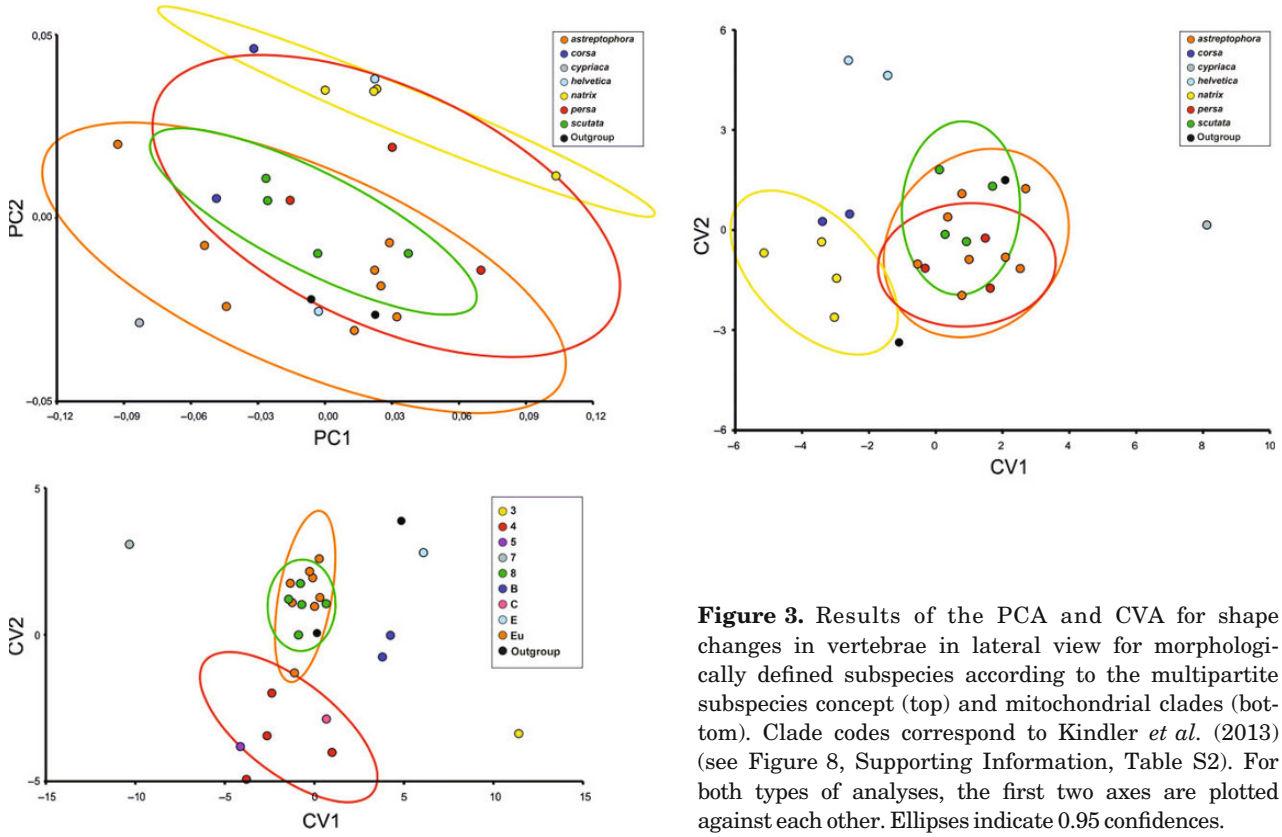


**Figure 3.** Results of the PCA and CVA for shape changes in vertebrae in lateral view for morphologically defined subspecies according to the multipartite subspecies concept (top) and mitochondrial clades (bottom). Clade codes correspond to Kindler *et al.* (2013) (see Figure 8, Supporting Information, Table S2). For both types of analyses, the first two axes are plotted against each other. Ellipses indicate 0.95 confidences.

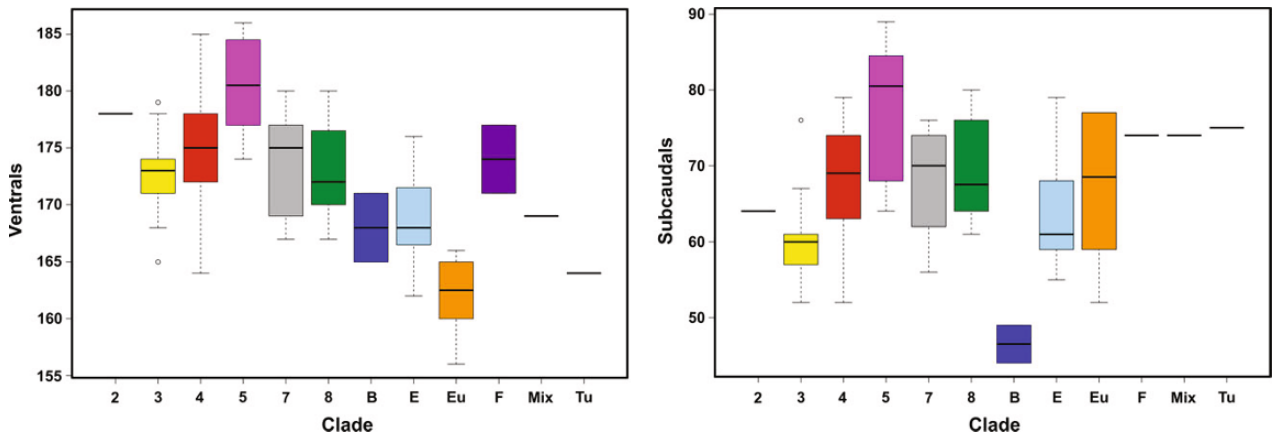


**Figure 4.** Variation of numbers of ventralia and subcaudalia in *Natrix natrix*. Clade codes correspond to Kindler *et al.* (2013) (see Figure 8, Supporting Information, Table S2). *Natrix n. astreptophora* (European clade = Eu; Tunisian clade = Tu) has on average significantly fewer ventrals than other grass snakes (top), whereas the number of subcaudals is not lower (bottom). 'Mix' refers to one *N. n. helvetica* with slight evidence for admixture with *N. n. astreptophora*.

*tophora* has significantly lower ventral numbers ($P = 0.0102$), and higher, but not significantly higher, numbers of subcaudals ($P = 0.1919$). Corso-Sardinian grass snakes have the lowest number of subcaudals ($P = 0.0161$) and thus the shortest relative tail length ($P = 0.0320$). Tail length and number of subcaudals are strongly correlated in grass snakes, independent of sex. All other scale counts have very little or no variation at all between the clades or subspecies, respectively, with the PCA showing inconclusive results. Loreal and nasal numbers were excluded from analysis, because these two

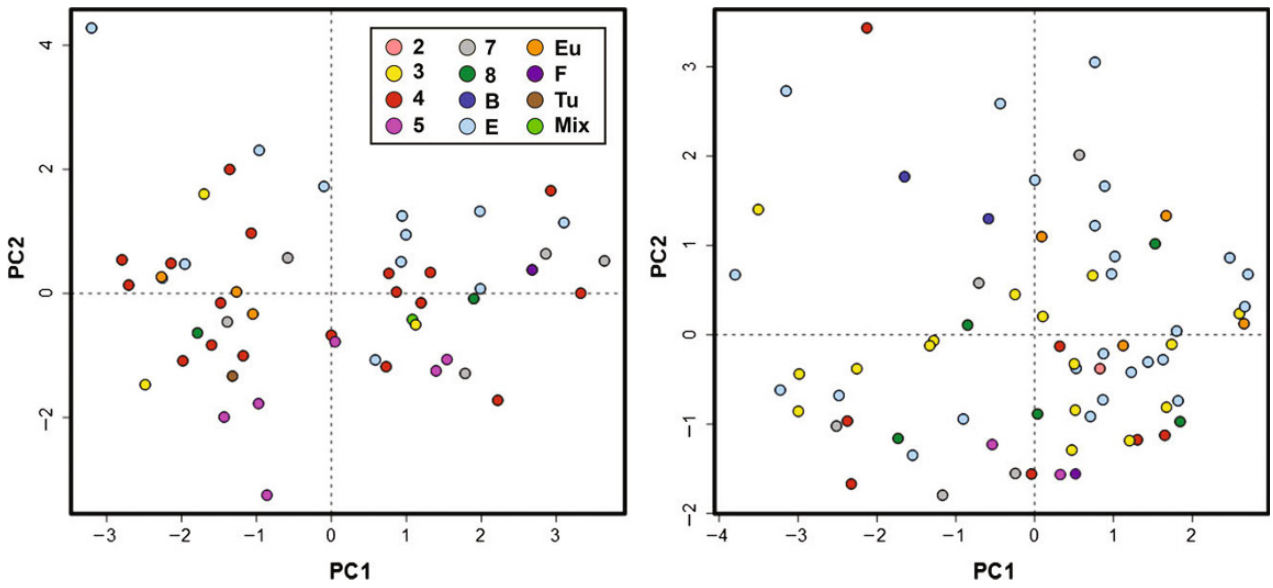


**Figure 5.** Results of the PCAs for male (left) and female (right) grass snakes using metric and meristic characters and mitochondrial clades as classifier variables. Clade codes correspond to Kindler *et al.* (2013) (see Figure 8, Supporting Information, Table S2). No obvious taxonomic groupings are revealed.

characters showed no variation. The first two PC axes reveal little variation and there are consequently no clear clusters (Fig. 5).

### Mitochondrial DNA

Data for *Natrix natrix astreptophora* and *N. n. helvetica* from previous studies (Guicking *et al.*, 2006; Kindler *et al.*, 2013) were combined with our newly generated mtDNA sequences for parsimony network analyses, revealing that 29 new samples corresponded mitochondrially to *N. n. helvetica* (lineage E of Kindler *et al.*, 2013) and the remaining 20 samples to European representatives of *N. n. astreptophora* (lineage Eu of Kindler *et al.*, 2013). In total, 34 genetically verified samples represented *N. n. helvetica* (lineage E), 44 samples the European lineage (Eu) of *N. n. astreptophora* and one sample the Tunisian lineage (Tu) of *N. n. astreptophora* (Fig. 6, top). The distribution ranges of the two European lineages abut in the eastern Pyrenees region of Catalonia and adjacent France.

In parsimony network calculations, each genetic lineage (E, Eu, Tu) corresponded under the default 95% connection limit to an unconnected haplotype cluster for each mtDNA block. Here we show networks with a manually enforced connection (Fig. 7). For the mtDNA block comprising ND4 + tRNAs, European *N. n. astreptophora* were represented by 12 distinct haplotypes differing by a maximum of three mutation steps. The haplotype of the Tunisian sample of this subspecies was highly distinct and differed by a minimum of 22 steps from the haplotypes of European *N. n. astreptophora*. With only four haplotypes differing by maximally two mutations, there was distinctly less variation in *N. n. helvetica*; the haplotype cluster of *N. n. helvetica* was connected by a minimum of 47 mutational steps with the haplotype cluster of European *N. n. astreptophora* and by a minimum of 43 steps with the Tunisian haplotype of the latter taxon. A similar pattern was also revealed for the cyt *b* gene, with a diverse cluster of 19 distinct haplotypes for European *N. n. astreptophora*, a highly distinct haplotype of the only Tunisian sample of this taxon and another cluster of only three haplotypes for *N. n. helvetica*. The individual haplotypes of European *N. n. astreptophora* differed by up to nine mutation steps, and the Tunisian haplotype differed from the European ones by a minimum of 42 steps. Among the three haplotypes of *N. n. helvetica* a loop occurred, resulting in a maximum number of three mutation steps. The cluster of haplotypes of *N. n. helvetica* was connected by another loop with the network. It differed from the cluster of European *N. n. astreptophora* by minimally 78 steps, and by 66 steps from the Tunisian haplotype of *N. n. astreptophora*.

### Microsatellite data

Microsatellite data were available for 29 European *Natrix natrix astreptophora* and 39 *N. n. helvetica*. Allele numbers of the 13 studied loci ranged from four to 23 per locus, with a total allele number of 158 (Supporting Information, Table S4).

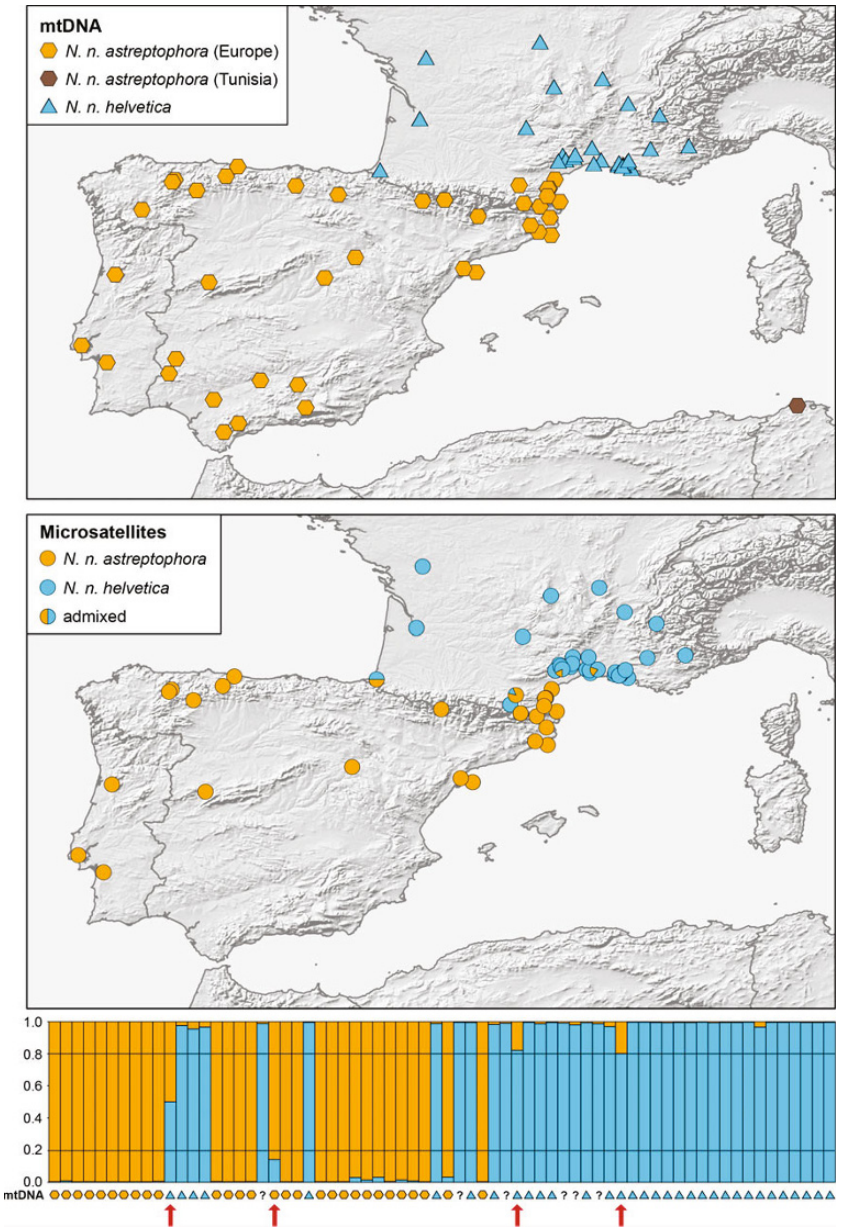


**Figure 6.** Top: sampling sites and mitochondrial identity of studied grass snakes ($N$ = 79). Centre: genotypic assignment of 68 grass snakes using STRUCTURE analyses of 13 microsatellite loci. Symbol colours as in STRUCTURE bar plot (bottom). Mixed ancestries are indicated by differently coloured sectors corresponding to inferred genetic percentages of the respective cluster. Bottom: STRUCTURE bar plot of the run ($N$ = 10) with best probability value for $K$ = 2. Columns corresponding to individual grass snakes are arranged according to collection sites from west to east. Horizontal black lines indicate 80% thresholds. Symbols below the bar plot correspond to mitochondrial lineages. Arrows highlight hybrids or potential hybrids.

Microsatellite data were subjected to unsupervised Bayesian cluster analyses using STRUCTURE 2.3.4. The $\Delta K$ method suggested two as the optimal number of clusters (Fig. 6, centre and bottom). One of these clusters represented European *N. n. astreptophora* and the other *N. n. helvetica*. The clusters matched well with mitochondrial clades, without evidence for pronounced cytonuclear discordance. Only one individual harbouring a mitochondrial haplotype of *N. n. helvetica* showed unambiguous evidence for hybridization. According to microsatellite data, this grass snake from the western Pyrenees was perfectly intermediate between *N. n. astreptophora* and *N. n. helvetica*, suggestive of an F1 cross

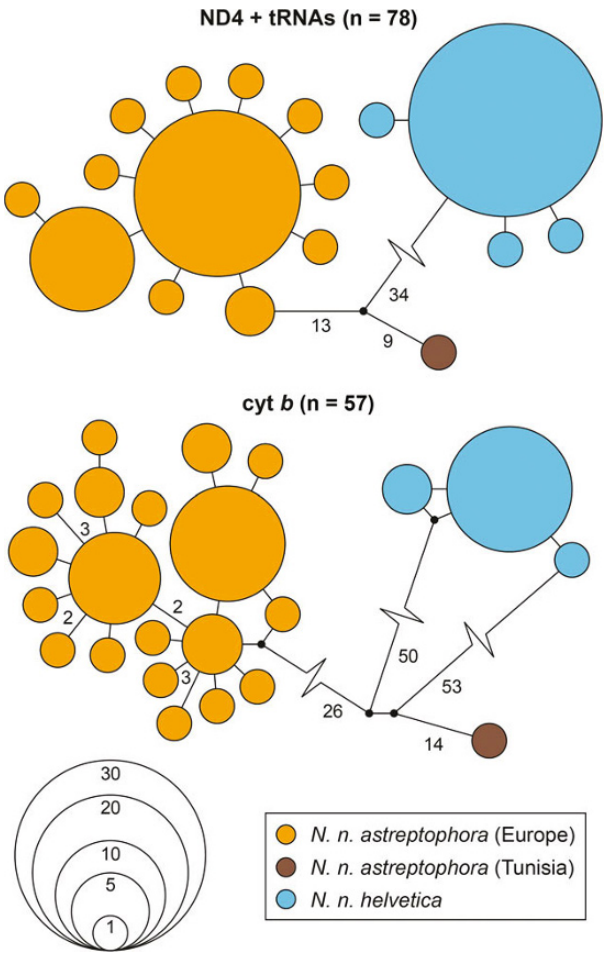


**Figure 7.** Parsimony networks of mtDNA sequences. Symbol sizes reflect haplotype frequencies. Small black circles are missing node haplotypes; each line connecting two haplotypes corresponds to one mutation step, if not otherwise indicated by numbers.

between a female *N. n. helvetica* and a male *N. n. astreptophora*. Another individual from the Montpellier region (Lansargues, Hérault), also with a mitochondrial haplotype of *N. n. helvetica*, had an assignment proportion of 79.8% for the *helvetica* cluster. Thus, this snake was by definition also a hybrid, even though it clearly represents a borderline case. Two further grass snakes from southern France, one with a mitochondrial haplotype of *N. n. astreptophora* and the other with a haplotype of *N. n. helvetica*, had membership proportions just beyond the threshold (81.4 and 86.0%). We cannot exclude that the genotypes of these three grass snakes indicate limited gene flow, because their collection sites are close to the putative contact zone of *N. n. astreptophora* and *N. n. helvetica*. These potentially admixed snakes and the unambiguous hybrid were excluded from the calculations presented below.

**Table 2.** Genetic diversity of STRUCTURE clusters based on 13 microsatellite loci

| Cluster ($K$ = 2) | $N$ | $N_A$ | $N_{\bar{A}}$ | $N_p$ | AR | $H_O$ | $H_E$ |
|---|---|---|---|---|---|---|---|
| *N. n. astreptophora* | 28 | 105 | 8.08 | 60 | 2.42 | 0.50 | 0.57 |
| *N. n. helvetica* | 36 | 96 | 7.39 | 51 | 2.51 | 0.57 | 0.67 |

Individuals with mixed ancestries were not considered.
$N$, number of individuals; $N_A$, number of alleles; $N_{\bar{A}}$, average number of alleles; $N_p$, number of private alleles; AR, allelic richness; $H_O$, average observed heterozygosity; $H_E$, average expected heterozygosity.

An AMOVA indicated for the microsatellite data that 73.1% of the molecular variance occurred within and 26.9% between the two clusters, equalling an $F_{ST}$ value of 0.27 between the two groups.

When diversity indices of the two clusters were compared, the cluster corresponding to *N. n. astreptophora* showed slightly higher values with respect to the number of alleles and private alleles, whilst for allelic richness and observed and expected heterozygosity the relationship was the reverse (Table 2).

## DISCUSSION

Using 18 metric and meristic characters, the present study confirmed that external morphology in *Natrix natrix* provides few reliable diagnostic characters. This was ultimately responsible for the inability of Thorpe (1975a, 1979) to discriminate more than four morphological groupings based on multivariate analyses of 160 morphological characters of over 750 grass snakes, leading to his 'four subspecies concept' (Thorpe, 1979). Like the 'multipartite subspecies concept' of other authors (e.g. Mertens, 1947; Kabisch, 1999; Kreiner, 2007), the 'four subspecies concept' conflicts greatly with the 16 identified mitochondrial lineages within grass snakes (Kindler *et al.*, 2013). The latter authors highlighted that this is also true for coloration and pattern characters, for instance, with striped morphotypes thought to be diagnostic of the subspecies *N. n. persa* occurring in no fewer than seven deeply divergent mitochondrial clades, often together or even syntopically with unstriped morphotypes. Due to this taxonomic unreliability of coloration and pattern, we did not try to evaluate such characters in the present study. Yet, it is noteworthy that *N. n. astreptophora* differs consistently from all other grass snake taxa by its reddish (instead of brown or yellowish) iris coloration. Moreover, juvenile *N. n. astreptophora* display a unique bicoloured (instead of uniformly coloured) pileus region, being deep

black posteriorly behind the eyes with a contrasting yellow collar of lunar spots that tend to fuse on the dorsal midline. These yellow markings fade during growth and become indistinct or invisible, as in old *N. n. helvetica*. However, unlike in *N. n. helvetica*, the collar disappears in *N. n. astreptophora* much earlier, already in snakes only 2 or 3 years old (W. Böhme & P. Geniez, unpubl. data).

In the present study, PCAs of external characters could reliably discriminate neither morphologically defined subspecies nor genetic lineages, and, not surprisingly, clades represented by greater sample sizes were generally more variable than clades represented by fewer individuals. Yet, one external character turned out to be promising. The very low number of ventrals in both males and females of European *N. n. astreptophora* constitutes a marked and statistically significant difference compared to the geographically neighbouring *helvetica* subspecies and all other eastern taxa. In the light of our data, a clinal gradient of increasing scale numbers from west to east, as proposed by Mertens (1947), does not account well for this difference. In more eastern taxa, ventral numbers do not increase from west to east and, moreover, no other scale characters are affected. In addition, the studied representatives of *N. n. astreptophora* originate from all over the Iberian Peninsula, so that population-specific abnormalities cannot explain this result. The only other significant difference in the dataset is the very low number of subcaudals in Sardinian grass snakes (*N. n. cetti*), but this observation is based on only two specimens.

Only a single grass snake having weak genetic evidence for admixture was available for morphological analyses. It originates from the vicinity of the contact zone of *N. n. astreptophora* and *N. n. helvetica* (BEV.9029, Lansargues, Hérault; Supporting Information, Table S2) and is a *N. n. helvetica* showing slight evidence for introgression from *N. n. astreptophora*. Morphologically, it falls into the variation range of *N. n. helvetica* (Figs 4, 5).

With respect to osteological characters, geometric morphometric analyses of vertebrae provided evidence for taxon-specific shape differences. In particular in lateral view, there are differences in the length of the neurapophysis, the distance between the terminal points of the prezygapophyseal processes and the position of the most cranially orientated point of the parapophysis. However, the evolutionary understanding of these characters is not straightforward. When morphologically defined subspecies are used for describing the observed variation, it is clear that taxa with abutting ranges, in particular *N. n. astreptophora* and *N. n. helvetica*, can be rather easily distinguished using vertebral shape, whilst *N. n. astreptophora* and geographically distant taxa, such as *N. n. persa* and *N. n. scutata*, are difficult to tell apart (Fig. 3). Nevertheless, our study underlines the notion that vertebral shape is a promising character for elucidating taxonomic variation of grass snakes and it would be worthwhile to study larger sample sizes from the whole distribution range.

Regarding the length/width ratio of the centra of mid-trunk vertebrae of the 126 X-rayed snakes, our results indicate a strong allometric shift from lower to higher ratios during growth, as already noted by Szyndlar (1991b). Ratios greater than 2.0 are common in adult *N. natrix*, thus contradicting Szyndlar's (1984, 1991a, b) belief that such ratios are diagnostic of *N. longivertebrata*. It seems likely that Szyndlar's sample of extant grass snakes was at least mainly comprised of subadult or juvenile specimens. Consequently, this character cannot be used for diagnosing *N. longivertebrata*. In addition, we found no obvious correlation between a high or low length/width ratio and any morphologically defined taxa or mitochondrial clades in *N. natrix*.

In contrast, skulls of *N. longivertebrata* consistently differ in basisphenoid morphology from those of extant grass snakes, except *N. n. astreptophora*, in that a distinct sagittal crest between the basipterygoid processes is lacking or only weakly developed. In addition, in *N. longivertebrata* and *N. n. astreptophora*, unlike other *N. natrix* taxa, the posterior foramina of the vidian canals are covered by the elongated basipterygoid processes in ventral view. The same character state as in *N. longivertebrata* and *N. n. astreptophora* is also found in *N. maura*, whilst *N. tessellata* shares the character state found in *N. natrix* exclusive of *N. n. astreptophora*. Szyndlar (1991a, b) proposed that the basisphenoid morphology of *N. longivertebrata* represents the ancestral character state, whereas the character state of *N. natrix* is derived. According to the mitochondrial phylogeny of the genus *Natrix* (Fig. 8), *N. maura* is the sister taxon of *N. natrix* + *N. tessellata*. If Szyndlar's hypothesis is translated into a phylogenetic framework, the character states of *N. longivertebrata*, *N. maura* and *N. n. astreptophora* were plesiomorphic, whilst the basisphenoid pattern of all other *N. natrix* and *N. tessellata* would then represent a synapomorphy, implying that *N. n. astreptophora* branched off from the last common ancestor of *N. natrix* and *N. tessellata* before these two species diverged. In light of the unambiguous molecular evidence for the monophyly of *N. natrix* (inclusive of *N. n. astreptophora*) and its sister group relationship to *N. tessellata*, this seems unlikely, suggesting rather homoplasy as an explanation for the occurrence of

identical basisphenoid patterns in certain *Natrix* taxa.

In summary, examination of the studied osteological and external morphological characters of *N. natrix* reveals much individual variation, but little taxon-specific differentiation. Only grass snakes from the Iberian Peninsula and north-western Africa (*N. n. astreptophora*) were consistently differentiated and were characterized by a significantly lower number of ventral scales (156–166 compared to 162–182 ventralia in other grass snakes) and differences in skull characters.

The morphological distinctiveness of *N. n. astreptophora* corresponds well to its sister group relationship to the remaining genetic lineages of grass snakes, revealed by analyses of mtDNA (Guicking *et al.*, 2006, 2008; Fritz *et al.*, 2012; Kindler *et al.*, 2013). According to molecular clock calculations, *N. n. astreptophora* diverged from other *N. natrix* 9.6–10.6 million years ago (Mya), suggesting that the Pyrenean uplift as part of the Alpine orogenesis played an important role in the origin of the Ibero-Maghrebian taxon. The split between the two more inclusive clades embracing all terminal mitochondrial clades of *N. natrix* except *N. n. astreptophora* was dated to 7.3–8.2 Mya, whereas divergence times of the remaining terminal clades were distinctly younger (up to 4.4 Mya; Fritz *et al.*, 2012).

Using 13 microsatellite loci and two mitochondrial markers, we inferred only limited gene flow across the contact zone of *N. n. astreptophora* and *N. n. helvetica*. We identified only one unequivocal hybrid and three additional grass snakes with questionable evidence for introgression, whilst the majority of individuals from the contact zone represented either pure *N. n. astreptophora* or pure *N. n. helvetica* (Fig. 6). This pattern is also reflected in the completely parapatric distribution of mitochondrial haplotypes corresponding to each taxon. For other genetic lineages of *N. natrix*, in particular in eastern Central Europe and the Balkans, Kindler *et al.* (2013) found a completely different situation with broadly overlapping distribution ranges of haplotypes of distinct lineages across wide contact zones.

One might argue that our results have been biased by small sample size from the contact zone of *N. n. astreptophora* and *N. n. helvetica*. However, in spite of years-long fieldwork in the contact zone, two of us (M. Cheylan, P. Geniez) were unable to obtain more extensive sampling due to the unusual rarity of grass snakes there. Yet, the genetic pattern in the contact zone and adjacent regions in southern France indicates a lack of large-scale gene flow between *N. n. astreptophora* and *N. n. helvetica*, as would be expected between two conspecific taxa. For instance, Vamberger *et al.* (2015) found extensive and wide-ranging gene flow between two subspecies of *Emys orbicularis* in eastern Italy, with genetic admixture reaching from the Padan Plain down to southern Italy. Thus, the revealed genetic pattern for *N. n. astreptophora* and *N. n. helvetica* corresponds clearly to what is expected for two distinct species. The sister group relationship of *N. n. astreptophora* to all other grass snakes (Guicking *et al.*, 2006, 2008; Fritz *et al.*, 2012; Kindler *et al.*, 2013; cf. Fig. 8) and its morphological divergence provide additional lines of evidence for the distinctiveness of *N. n. astreptophora*. Thus, we conclude that this taxon should be elevated to species status, *Natrix astreptophora* (Seoane, 1884).

Our Tunisian sample of *N. astreptophora* is genetically clearly divergent from its European conspecifics (Fig. 7). Unfortunately, there are no genetic data available for Moroccan grass snakes. A common phylogeographical pattern in many Maghrebian amphibians and reptiles is a deep genetic break, with a Moroccan lineage and a distinct Algerian–Tunisian lineage. Often the Moroccan lineage is not or only weakly differentiated from its Iberian conspecifics, whilst the Algerian–Tunisian lineage is highly distinct (cf. the recent reviews by Husemann *et al.*, 2014; Stuckas *et al.*, 2014). We do not know whether North African grass snakes conform to this paradigm as well, but the genetic divergence of our Tunisian sample makes this at least possible. Considering that genetic data are only available for a single individual from Tunisia, we abstain from taxonomic conclusions, pending further study. However, we wish to

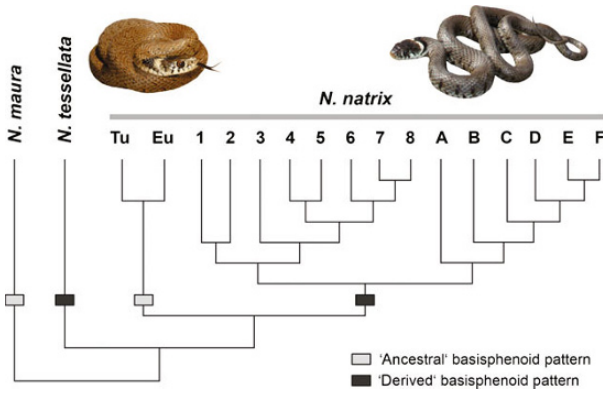


**Figure 8.** Schematic phylogeny of the genus *Natrix* according to Guicking *et al.* (2006, 2008), Fritz *et al.* (2012) and Kindler *et al.* (2013). Clade codes for *N. natrix* correspond to Kindler *et al.* (2013). Boxes indicate basisphenoid patterns (cf. Fig. 2). On the top of the tree are shown *Natrix natrix astreptophora* (left, Cangas de Onís, Asturias, Spain) and *N. n. helvetica* (right, Gloucestershire, South West England). Photos: David Nixon (Midlands Reptiles).

point out that the name *Tropidonotus natrix* var. *algericus* Hecht, 1930 (type locality: southern Algeria) could refer to the Tunisian lineage, if its distribution matches the general Maghrebian pattern.

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## SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article:

**Figure S1.** Results of the PCA and CVA for shape changes in vertebrae in ventral and dorsal view.
**Table S1.** List of specimens used for osteological analyses in this study.
**Table S2.** List of specimens used for external morphological analysis and determination of ratio of the length and width of the centrum of mid-trunk vertebrae using radiographs.
**Table S3.** Genetically studied grass snake samples.
**Table S4.** Microsatellite loci and multiplex sets.
**Table S5.** Additional microsatellite loci tested.
**Table S6.** Mahalanobis distances among *Natrix natrix* subspecies and the two outgroup taxa resulting from the CVA using lateral views of the vertebrae.
**Table S7.** Mahalanobis distances among *Natrix natrix* subspecies and the two outgroup taxa resulting from the CVA using dorsal views of the vertebrae.
**Table S8.** Mahalanobis distances among *Natrix natrix* subspecies and the two outgroup taxa resulting from the CVA using ventral views of the vertebrae.
**Table S9.** Mahalanobis distances among clades of *Natrix natrix* and the two outgroup taxa resulting from the CVA using lateral views of the vertebrae.
**Table S10.** Mahalanobis distances among clades of *Natrix natrix* and the two outgroup taxa resulting from the CVA using dorsal views of the vertebrae.
**Table S11.** Mahalanobis distances among clades of *Natrix natrix* and the two outgroup taxa resulting from the CVA using ventral views of the vertebrae.