

Hemipenial differentiation in the closely related congeners *Ablepharus kitaibelii* (BIBRON & BORY DE SAINT-VINCENT, 1833), and *Ablepharus budaki* GÖÇMEN, KUMLUTAŞ & TOSUNOĞLU, 1996 (Squamata: Sauria: Scincidae)

Unterschiede in der Hemipenismorphologie der beiden nahe verwandten Arten
Ablepharus kitaibelii (BIBRON & BORY DE ST-VINCENT, 1833)
und *A. budaki* GÖÇMEN, KUMLUTAŞ & TOSUNOĞLU, 1996
(Squamata: Sauria: Scincidae)

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KURZFASSUNG

Die Hemipenes zweier nahe verwandter *Ablepharus* Arten, *A. kitaibelii* (BIBRON & BORY DE SAINT-VINCENT, 1833) und *A. budaki* GÖÇMEN, KUMLUTAŞ & TOSUNOĞLU, 1996 werden erstmalig beschrieben. Diese Arten haben ähnliches Aussehen und wurden nur durch wenige externe und sehr variable Merkmale unterschieden. In Übereinstimmung zu einer früheren genetischen Studie zeigten die männlichen Geschlechtsorgane jedoch deutliche morphologische Unterschiede. Einige Merkmalsausprägungen der Hemipenes weisen Ähnlichkeiten mit denen der Skink-Subfamilie Lygosominae auf. Darüberhinaus wird eine bisher unbeschriebene Hemipenisstruktur als 'asulcal protrusion' beschrieben. Es wurden ausschließlich epithelial verhornte, unverkalkte Mikrostrukturen beobachtet. Eine neuartige Methode des Ausstülpens kleiner, fragiler Hemipenes von Eidechsen wird vorgeschlagen.

ABSTRACT

The hemipenes of two closely related *Ablepharus* species, *A. kitaibelii* (BIBRON & BORY DE SAINT-VINCENT, 1833), and *A. budaki* GÖÇMEN, KUMLUTAŞ & TOSUNOĞLU, 1996, are described for the first time. These species are of similar general appearance and differ by few external and variable characters only. The genital morphology, however, revealed profound differences, which is in concert with a previous genetic study. Some hemipenial characters resemble those found in lygosomine skinks. Moreover, a previously undescribed hemipenial structure was found: the asulcal protrusion. Only epithelial non-calcified keratinous microstructures were observed. An innovative technique for eversion of small and fragile lizards' hemipenes is proposed.

KEY WORDS

Reptilia: Squamata: Sauria: Scincidae; *Ablepharus kitaibelii*, *Ablepharus budaki*, hemipenis, bulbous lobes, asulcal protrusion, morphology, microstructures, eversion

INTRODUCTION

The genus *Ablepharus* FITZINGER in EVERS-MANN, 1823, currently comprises ten species [*Ablepharus bivittatus* MÉNÉTRIES, 1832; *A. budaki* GÖÇMEN, KUMLUTAŞ & TOSUNOĞLU, 1996; *A. chernovi* DAREVSKY, 1953; *A. darvazi* JEROMČENKO & PANFILOV, 1990; *A. deserti* STRAUCH, 1868; *A. grayanus* (STOLICZKA, 1872), *A. kitaibelii* (BIBRON & BORY DE SAINT-VINCENT, 1833), *A.*

lindbergi WETTSTEIN, 1960; *A. pannonicus* (FITZINGER, 1824), and *A. rueppellii* (GRAY, 1839)] confined mainly to southwestern and southern Asia (SINDACO & JEREMČENKO 2008). A single species, *A. kitaibelii*, is present in Europe. Taxonomic revisions (STEPÁNEK 1937; MERTENS 1952; FUHN 1970; EREMČENKO & ŠČERBAK 1986; GÖÇMEN et al. 1996), resulted in descriptions of

†) The authors Vladislav S. VERGILOV and Boyan P. ZLATKOV dedicate this paper to their good friend and esteemed colleague Nikolay D. TZANKOV, who recently passed away in a tragic car accident.

several subspecies: *A. k. fabichi* STĚPÁNEK, 1937; *A. k. fitzingeri* MERTENS, 1952; *A. k. stepaneki* FUHN, 1970, and *A. k. budaki*. Shortly after its description, the status of the latter taxon was raised to full species by SCHMIDTLER (1997). He also introduced a new subspecies *A. b. anatolicus* from the Anatolian mainland. Based on molecular data, POULAKAKIS et al. (2013) demonstrated that *A. budaki* belongs to a well-supported clade that is further subdivided into three monophyletic lineages. Diagnostic features and arguments for raising *A. budaki* to species level included external morphological traits (GÖÇMEN et al. 1996; SCHMIDTLER 1997) and substantial genetic divergence from *A. kitaibelii* (POULAKAKIS et al. 2005, 2013).

The hemipenial morphology has proven its significance as an important trait for interspecific taxonomic differentiation of congeners. This method has been applied for other small representatives of various lizard families, e.g., Chamaeleonidae (KLA-

VER & BÖHME 1986), Lacertidae (BÖHME 1971; ARNOLD 1983, 1986a, 1986b), Gymnophthalmidae (UZZEL 1969, 1973; MYERS et al. 2009; NUNES et al. 2012, 2014), Dactyloidae (KÖHLER 2010; KÖHLER & KREUTZ 1999; KÖHLER & SUNYER 2008; KÖHLER & VESELY 2010; KÖHLER et al. 2007, 2010, 2012). Generally, the members of the Scincidae family have been studied sporadically, with few works concerning this topic (BÖHME 1988; BÖHME et al. 2000; GREER & BISWAS 2004; ZIEGLER & BÖHME 2004; HORNER 2007). In *Ablepharus* this feature received very little attention. Up to this date, a single study dealing with this matter schematically presents the hemipenial morphology of *A. darvazi*, *A. deserti* and *A. pannonicus* (EREMČENKO & PANFILOV 1990).

The purpose of this study is to describe the hemipenial morphology of two closely related representatives of the genus *Ablepharus* and to propose an alternative technique for hemipenial preparation applicable to small lizard species.

MATERIALS AND METHODS

Materials.— *Ablepharus kitaibelii*, three adult males from Bulgaria: Silistra region, near v. Sratsimir, No. NMNHS194; Southern Pirin Mts., near v. Belyovo, No. NMNHS45; Strandzha Mts., near v. Belevren, No. NMNHS170. *Ablepharus budaki*, two adult males from Turkey, Hatay Province, near v. Ylanlı, NMNHS400-401. All specimens are preserved in ethanol in the collection of the National Museum of Natural History, Bulgarian Academy of Sciences (NMNHS) in Sofia, Bulgaria.

Methods.— Early proposed techniques for hemipenial preparation (e.g., ORTENBURGER 1923; BÖHME 1971, 1988; MANZANI & ABE 1988; PESANTES 1994 etc.) appeared to be inapplicable to the studied objects because of their small size and fragility. As small skins possess delicate hemipenes the authors used techniques developed for everting the endophallus of large moths (HARDWICK 1950; DUFAY 1978; FIBIGER et al. 1984) with some modifications. The method is based on manual eversion and dehydration of the soft tissue by injection with absolute ethanol.

The specimens were dissected and both hemipenes together with the retractor muscles and connective tissue were removed carefully with small scissors, forceps and scalpel, and then submerged into 5 % ethanol solution. This ethanol concentration decreases the surface tension without hardening the soft tissues. Then the connective tissue and most of the retractor muscle were extirpated. After that, the hemipenes were everted using fine forceps and syringe with a thin metal needle. Complete eversion was achieved after injecting 5 % ethanol into partially everted hemipenes.

Processing with potassium hydroxide (KOH) solution used for various larger reptiles (PESANTES 1994; ZIEGLER & BÖHME 1997; ZAHER, 1999; HARVEY et al. 2012) and some smaller species (NUNES et al. 2014) proved to be inapplicable, because the tissues became too soft and fragile, and consequent eversion was impossible. The everted hemipenes were stained in 0.5 % Alizarin Red S solution for about 10-15 seconds. This stain is used for contrasting of hemipenial carbonate structures of lizards

(UZZELL 1969, 1973). Both alcohol (70 %) and water solution of Alizarin produced equal results. The staining did not provide the expected result because the stain penetrated in all tissues, hence they were equally stained and no contrasted structures were observed under a stereomicroscope. However, this procedure proved to be useful for subsequent microscopic observation. After staining the hemipenes were inflated with 100 % ethyl alcohol using a syringe with a blunt needle. A continuous flow of alcohol was maintained for a few seconds to harden the hemipenes. To avoid ejecting by pressure, the hemipenes were tied at the pedicel area to the needle by a thin cotton thread. Afterwards, the thread was removed from the dehydrated hemipenes and they were permanently preserved in absolute ethanol. The absolute ethanol dehydrates and hardens the tissue very well and keeps its shape and structures unchanged for an unlimited period.

Permanent microscopic slides of three Alizarin stained hemipenes of *A. kitaibelii*

and two of *A. budaki* were made. For the purpose, the hemipenes were slit laterally to produce sulcal and asulcal halves, then compressed between two slides and dehydrated with absolute ethanol for five min, after that removed from the slides and cleared with Euparal Essence for five min and mounted permanently on a slide with Euparal embedding agent. To test the presence of carbonate structures, two hemipenes of *A. kitaibelii* and one of *A. budaki* were treated with 10 % HNO₃ for a minute (under direct microscope observation on temporary slide mounts), then washed with distilled water (2 x 5 min) and eventually, dehydrated and mounted permanently on slides. Additionally, the slides were observed through a compound microscope Amplival (Carl Zeiss Jena) with polarizing device. The slides were photographed using a compound microscope (Olympus BX41) with mounted camera (Olympus ColorView 1).

The terminology of basic hemipenial structures follows DOWLING & SAVAGE (1960) and KLAVER & BÖHME (1986).

RESULTS

In *A. kitaibelii* the hemipenis has no visible macrostructures and ornaments (Figs. 1, 3). The hemipenis is deeply bilobed, with two equally long and symmetrical branches. Sulcus spermaticus bifurcation point is close to that of the branching. Length to width branches ratio is ca. 3:1. Thick, well developed labia surround the sulcus. Small v-shaped folds are visible inside each sulcus in the distal area of the branches. The sulci open on the tip of the branches of the hemipenis. On each side of the labium, at the end of the sulci, there is an elongated fold – the terminal awl. The branches are as long as the body of the hemipenis. The truncus of the hemipenis bears two weakly pronounced, asymmetrical lobes. The total length of the hemipenes is 4-4.5 mm. Observation of the hemipenis surface under a microscope demonstrated that there are microscopic structures, non-degradable after nitric acid treatment (Fig. 2). The microstructures were observed also through a polarizing microscope and did not show

birefringence, which is a typical feature of calcium carbonate crystals. Evidently, these structures are not calcified but keratinous, polygonally shaped. The keratin structures are densely distributed on the tip of the two parts of the hemipenis and are absent in the sulci region and on the base of the hemipenis. On the middle part of the hemipenis, around the branching, the keratin structures are more sparsely distributed than on the tip. Shed epithelium was observed in some specimens during eversion of the hemipenes.

The general shape of the *A. budaki* hemipenis looks similar to that of *A. kitaibelii* but some major differences were observed (Figs. 1, 3). Two distinct outpocketing, leaf-shaped folds are located on both sides of the truncus of the hemipenis, placed at the same position as the bulbous lobes of the *A. kitaibelii* hemipenis. A well-pronounced structure was observed on the asulcal surface of the hemipenial truncus. Basal of the branching, where the epithelial tissue is protruding, it appears as a large

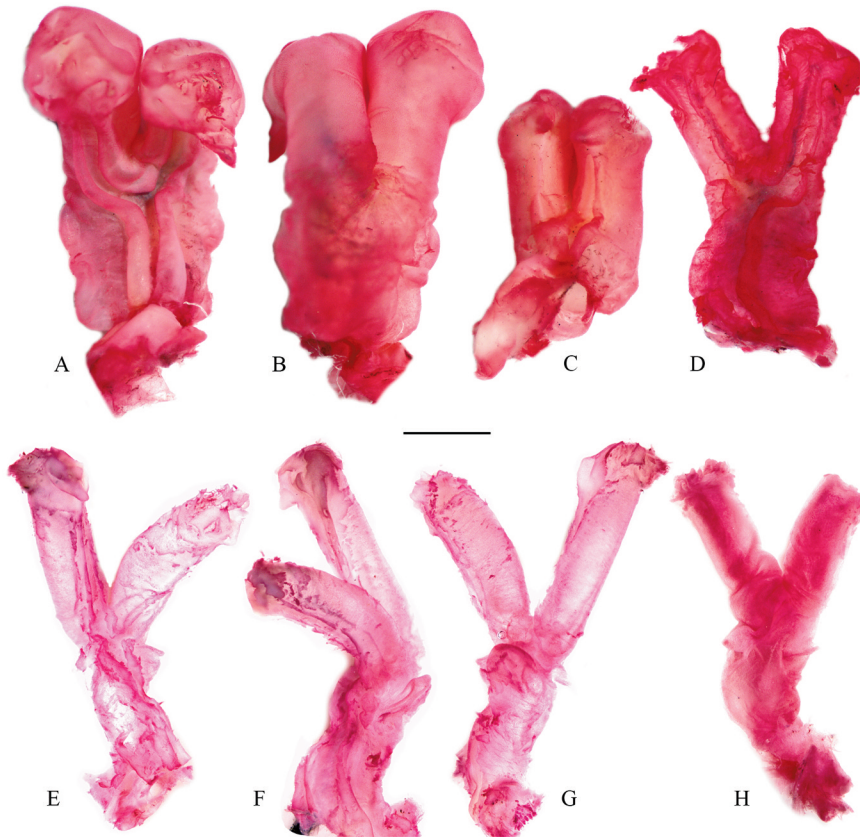


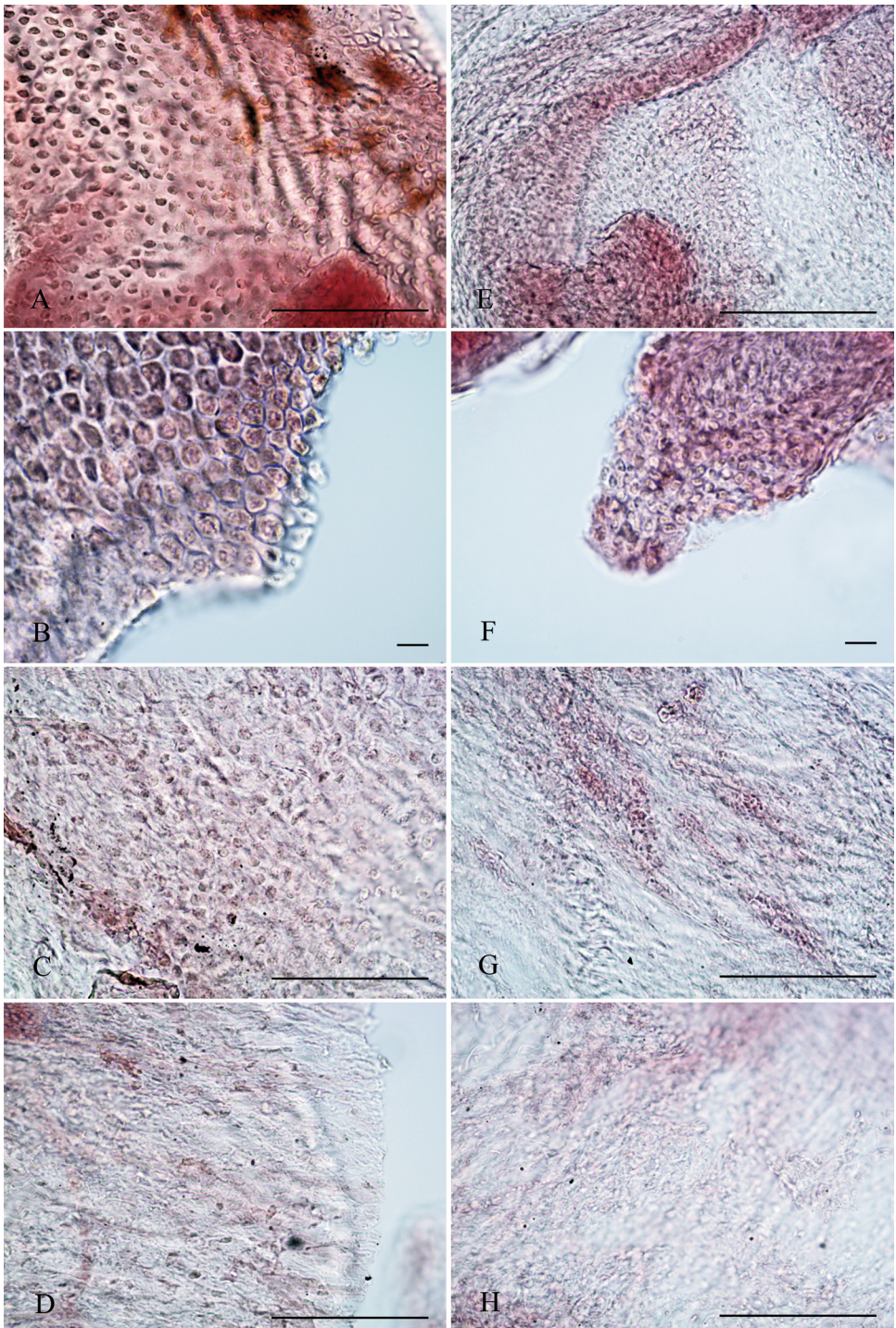
Fig. 1: Inflated and stained (Alizarin Red S) everted hemipenes of different specimens of
 A-D – *Ablepharus kitaibelii* (BIBRON & BORY SAINT-VINCENT, 1833) and
 E-H – *Ablepharus budaki* GÖCMEN, KUMLUTAŞ & TOSUNOĞLU, 1996.
 A, C, D, E – sulcal side; B, G, H – asulcal side; F – lateral view.
 A, B, C – overinflated, H – incompletely everted. Scale bar = 1 mm.

Abb. 1: Aufgeblähte, mit Alizarinrot S gefärbte ausgestülpte Hemipenes unterschiedlicher Individuen von
 A-D – *Ablepharus kitaibelii* (BIBRON & BORY SAINT-VINCENT, 1833) und
 E-H – *Ablepharus budaki* GÖCMEN, KUMLUTAŞ & TOSUNOĞLU, 1996.
 A, C, D, E – sulkale Seitenansicht; B, G, H – asulkale Seitenansicht; F – laterale Ansicht.
 A, B, C – übermäßig aufgebläht, H – unvollständig ausgestülpt. Maßstab = 1 mm.

Fig. 2 (opposite page) / Abb. 2 (gegenüberliegende Seite)

Keratinous microstructures on the surface of everted hemipenes of
 A-D – *Ablepharus kitaibelii* (BIBRON & BORY SAINT-VINCENT, 1833) and
 E-H – *Ablepharus budaki* GÖCMEN, KUMLUTAŞ & TOSUNOĞLU, 1996.
 A, E – apical surface; B, F – apical surface enlarged; C, G – truncus, asulcal surface;
 D, H – pedicel, asulcal surface. Scale bars: A, C, D, E, G, H = 100 µm; B, F = 10 µm.

Verhornte Mikrostrukturen auf der Oberfläche ausgestülpter Hemipenes von
 A-D – *Ablepharus kitaibelii* (BIBRON & BORY SAINT-VINCENT, 1833) und
 E-H – *Ablepharus budaki* GÖCMEN, KUMLUTAŞ & TOSUNOĞLU, 1996.
 A, E – Oberfläche des Apex; B, F – Oberfläche des Apex, vergrößert;
 C, G – asulkale Oberfläche des Truncus; D, H – asulkale Oberfläche des Pedicels.
 Maßstab: A, C, D, E, G, H = 100 µm; B, F = 10 µm.



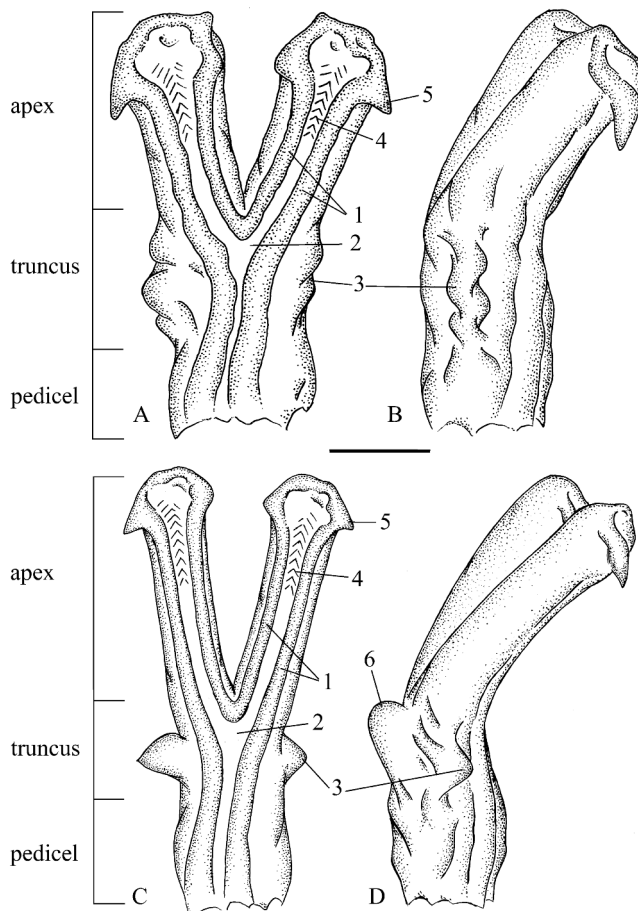


Fig. 3: Inflated hemipenes of
 A-B - *Ablepharus kitaibelii* (BIBRON & BORY SAINT-VINCENT, 1833) and
 C-D - *Ablepharus budaki* GÖÇMEN, KUMLUTAŞ & TOSUNOĞLU, 1996.
 1 - labia, 2 - sulcus spermaticus, 3 - bulbous lobes, 4 - v-shaped folds,
 5 - terminal awls, 6 - asulcal protrusion. Scale bar = 1 mm.

Abb. 3: Aufgeblähte Hemipenes von
 A-B - *Ablepharus kitaibelii* (BIBRON & BORY SAINT-VINCENT, 1833) und
 C-D - *Ablepharus budaki* GÖÇMEN, KUMLUTAŞ & TOSUNOĞLU, 1996.
 1 - Lippen, 2 - sulcus spermaticus, 3 - bulböse Lappen,
 4 - v-förmige Falten, 5 - endständige Dornen, 6 - asulkale Ausstülpung. Maßstab = 1 mm.

ovoid structure in the completely inflated hemipenis, for which we propose the term 'asulcal protrusion'. The branches are of the length of the hemipenial body. They are more slender and more elongate than in *A. kitaibelii*. Length to width branches ratio is ca. 4:1. The total length of the hemipenes is roughly the same as in the previous species,

i.e., 4-4.5 mm. The microscopic observation of the slides reveals that similar keratin structures cover most of the surface of the hemipenis (Fig. 2). They appear smaller and more densely distributed than in *A. kitaibelii*, but apparently, they are of the same keratinous composition and shape. Similarly, no calcified structures and orna-

ments were detected. *Ablepharus budaki* hemipenes are also deeply bilobed and the two branches of each hemipenis are equally long and symmetrical, with thick labia surrounding the sulci. Several v-shaped folds

are observed inside the sulci in the distal area of the branches. The sulci open on the tip of the branches of each hemipenis. On each side on the labium, at the end of the sulcus, there is a terminal awl.

DISCUSSION

COPE (1896) stated that the hemipenial characters 'have a constant systematic value', which 'differs with the character and varies from generic to superfamily in rank'. In the comprehensive revision of the subfamily Lygosominae (Scincidae), GREER (1979) considered the deep bifurcation of hemipenes with a long narrow base and two equally long branches as deterministic characters for some genera. The author regarded the bifurcated hemipenial type with elongate branches as a synapomorphy for the *Sphenomorphus* group. *Scincella reevesii* (GRAY, 1838) (fig. 321 in ZIEGLER 2002) also has bifurcated hemipenes with thin branches as long as the body (truncus plus pedicel). The hemipenes of the studied species of *Ablepharus* are similar in general shape.

LINKEM et al. (2011) described lateral lobes on the hemipenial truncus ('main shaft' according to the authors' terminology) claiming that they are unique for a Philippine skink group, for which they created a new genus, *Pinoyscincus* LINKEM, DIEMOS & BROWN, 2011. The authors referred to these protuberances as 'bulbous lobe structures'. The structures are very similar to those observed in *A. kitaibelii*. Such processes are observed also in taxa from other genera: *S. reevesii* (fig. 321 in ZIEGLER 2002) and *Hemiergis gracilipes* (STEINDACHNER, 1870) (given as *Sphenomorphus gracilipes*, fig. 14 in GREER 1979). GREER (1989) mentioned that the genera *Carlia*, *Lampropholis*, *Lygisaurus*, and *Saproscincus* share the unique hemipenial morphology of an elongate projection from the base of the everted hemipenis, probably referring to the same structures, but did not provide additional information. In fact, EREMČENKO & PANFILOV (1990) were the first to describe such structures in the genus *Ablepharus* for *A. pannonicus* and *A. dervazi*, and called them 'suspending folds' (translated from Russian). The more neutral

term 'bulbous lobes' is preferred here as to their shape and still unknown function and origin. It should be emphasised that the bulbous lobes are not universal in *Ablepharus*, as it is clearly demonstrated by EREMČENKO & PANFILOV (1990: fig. 2a, hemipenis of *A. deserti*). Apparently, the presence or absence of bulbous lobes is a rather homoplastic character for skink genera with bifurcated hemipenes.

Although similar in location, the bulbous lobes of *A. kitaibelii* and *A. budaki* demonstrate substantial differences in shape and size. The lobes of *A. kitaibelii* hemipenes are similar in size to those in *A. pannonicus*; the lobes of *A. dervazi* are apparently larger but with similar shape. These structures in *A. budaki* do not resemble those of any species known to the authors.

KLAVER & BÖHME (1986) provided descriptions for various hemipenial structures in Chamaeleonidae but their homology with the structures described here is uncertain. The lobes observed in scincid lizards could not be associated with protuberances such as 'auriculae', 'papillae', 'pedunculi', 'projections' or 'rotulae', which denote different ornamentations (BOURGAT & BRYGOO 1968; KLAVER & BÖHME 1986).

Bifurcate hemipenes with long attenuate terminal elements (awls) are reported for the Palauan skink species *Sphenomorphus scutatus* (PETERS, 1867) and another undescribed taxon attributed to the same genus (CROMBIE & PREGILL 1999). These terminal structures are also present in both *A. kitaibelii* and *A. budaki*, but are not visible on the illustrated hemipenes of *A. pannonicus*, *A. deserti* and *A. dervazi* (EREMČENKO & PANFILOV 1990), which probably is due to incomplete eversion rather than real absence.

The phylogenetic relationships of the representatives of genus *Ablepharus* remain controversial, especially when relying on the data presented by PYRON et al. (2013).

In this work *A. budaki* and *A. kitaibelii* plus *A. chernovi* form a well-supported clade along with a set of other lygosomine species. In the cladogram, *A. pannonicus* is grouped with *Asymblespharus alaicus* ELPATJEVSKY, 1901, forming another well supported clade significantly distant from the previous one, but is a sister group to the large 'Sphenomorphus group'. This grouping obviously makes the genus *Ablepharus* appear paraphyletic. However, this is more likely a result of methodological approach and lack of matching of the mitochondrial gene sequences. According to the phylogenetic and phylogeographic data provided by POULAKAKIS et al. (2013) the *Ablepharus* specimens studied in the present paper fall into the geographic range inhabited by the 'Turkey' clade of *A. budaki*, which is consistent with the taxon *A. budaki anatolicus* described by SCHMIDTLER (1997).

No scincid species is reported to possess hemipenes with calcified structures, which is supported also by the present study. The absence could be used to some extent in the general diagnosis of family Scincidae. Similarly, they are absent in Anguillidae (THOMAS & HEDGES 1998) and Chamaeleonidae (KLAVER & BÖHME 1986). External calcified hemipenial structures (mainly spines) are detected in various but not all gymnophthalmid lizards (for review see NUNES et al. 2014), in several snakes (Colubroides sensu ZAHER et al. 2009), but surely are not present in the snake infraorders Scolecophidia and Alethinophidia (BRANCH 1981, 1986). The existing minute spines seen in Lacertidae are hypothesized to be seasonally developed (ARNOLD 1973, 1986a; IN DEN BOSCH 2001). Considering their sparse distribution among the squamate reptiles, the calcified hemipenial structures are tentatively understood as homologies, but this question is yet unsolved (NUNES et al. 2014).

Similar to observations in the current study, tissue containing microstructures is reported for other lizard families. The microstructures in these species are variable on both individual and population level (BÖHME 1971, 1988; IN DEN BOSCH 2001).

The hemipenial morphology provides a useful tool for interspecific discrimination of the studied members of genus *Ablepharus*. The differences between the hemipenes of *A. kitaibelii* and *A. budaki* are substantial and reveal that these species differ in more essential morphological characters than mentioned by GÖÇMEN et al. (1996) and SCHMIDTLER (1997), supporting their full specific status.

The application or the interpretation of the hemipenial morphology for higher taxonomic analyses however is less informative, an example being the results for the families Lacertidae (BÖHME 1971) and Chamaeleonidae (KLAVER & BÖHME 1986). This leads to the conclusion that the hemipenial differentiation should be used only after a stable phylogeny of the group of interest is available. NUNES et al. (2014) explicitly support this conclusion for Gymnophthalmidae. In this respect, evidently a phylogenetic study including more representatives of the genus *Ablepharus* is necessary in order to trace and interpret the evolutionary significance of the observed differences.

The hemipenial morphology of Scincidae provides an unexplored field which could help to elucidate the relationships in this group. The specific morphological peculiarities of hemipenes could be an important starting point for species recognition and description. This way the method presented here provides a necessary tool for future investigation of the hemipenial characteristics, especially in minute representatives of the squamate reptiles.

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