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A MORPHOMETRIC AND MOLECULAR STUDY OF THE GENUS *PSEUDOPODISMA* (ORTHOPTERA: ACRIDIDAE)

Máté Kisfali[1], Péter Sólymos[2], Antal Nagy[3], István A. Rácz[1]

Debrecen, P.O. Box 3, Hungary; E-mails: mkisfali@gmail.com; racz.istvan@science.unideb.hu
[2]Alberta Biodiversity Monitoring Institute and Department of Biological Sciences
CW 405, Biological Sciences Bldg., University of Alberta, Edmonton, Alberta, T6G 2E9, Canada
E-mail: solymos@ualberta.com
[3]Institute of Plant Protection, University of Debrecen
H-4032 Debrecen, Böszörményi út 138, Hungary; E-mail: nagyanti@agr.unideb.hu
[4]Department of Botany, University of Debrecen, H-4010 Debrecen, Egyetem tér 1, Hungary
E-mails: horsolya@gmail.com; sramko.gabor@science.unideb.hu
[5]MTA-DE "Lendület" Evolutionary Phylogenomics Research Group
H-4010 Debrecen, Egyetem tér 1, Hungary

The specific status of three described taxa belonging to the genus *Pseudopodisma* (*P. fieberi, P. transilvanica, P. nagyi)* is based on a single character, because the original descriptions separate the species solely on the basis of genital structure of the males, and the three species are seemingly indistinguishable based on external morphology. To test this taxonomic separation we conducted morphometric and molecular investigations in the genus. Males were *a priori* identified by the original descriptions of the taxa, and from 18 external morphometric characters collected, the six most eligible characters of males and females were selected for linear discriminant analysis. The linear discriminant based classification agreed for 35 (85.3%) male and 30 (88.2%) female specimens out of the 41 *a priori* identified males and 34 females. Besides morphometrics, we sequenced the cytochrome *b* gene with newly devised primers in all species plus two outgroups. The phylogenetic analysis of resulting sequences indicated a strong species-delimitation within the genus *Pseudopodisma* (i.e., multiple individuals of the *a priori* species formed monophyletic clades). Thus, taking the morphometric and molecular results into consideration, we conclude the validation of specific status of *Pseudopodisma fieberi*, *P. nagyi* and *P. transilvanica* within the genus.

Key words: *Cyt*B, mitochondrial primers, grasshopper systematics, linear discriminant analysis, multivariate morphology.

INTRODUCTION

The genus *Pseudopodisma* (Orthoptera: Acrididae) includes three species. *Pseudopodisma fieberi* (Scudder, 1897) is the nominal species of the genus (Mistshenko 1947). Scudder (1897) originally described this species from "Carniola" (today Slovenia), but also reported from Serbia and Transylvania. Galvagni and Fontana (1991) described the same species again as *Pseudopodisma disconzii* (junior synonym) from Italy. The original holotype of *P. fieberi*

was lost, therefore Harz designated a neotype from Cluj-Napoca (Romania) in 1957 (Harz 1957). This incorrect neotypification was corrected by Galvagni and Fontana (1993), who appointed a new neotype from Slovenia, which corresponds to the original type locality (Fig. 1).

At the same time, Galvagni and Fontana (1993) also described *P. transilvanica* based on the individuals collected near Cluj-Napoca (West Romania) (Fig. 1). *P. transilvanica* was separated from *P. fieberi* based on morphology of male genitalia. Later, *P. transilvanica* was also found in Slovakia (Kočárek *et al*. 1999), and other parts of Romania.

As part of their taxonomic revision, Galvagni and Fontana (1996) described *P. nagyi* from Hungary (type locality: peak "Galya-tető" in the Mátra

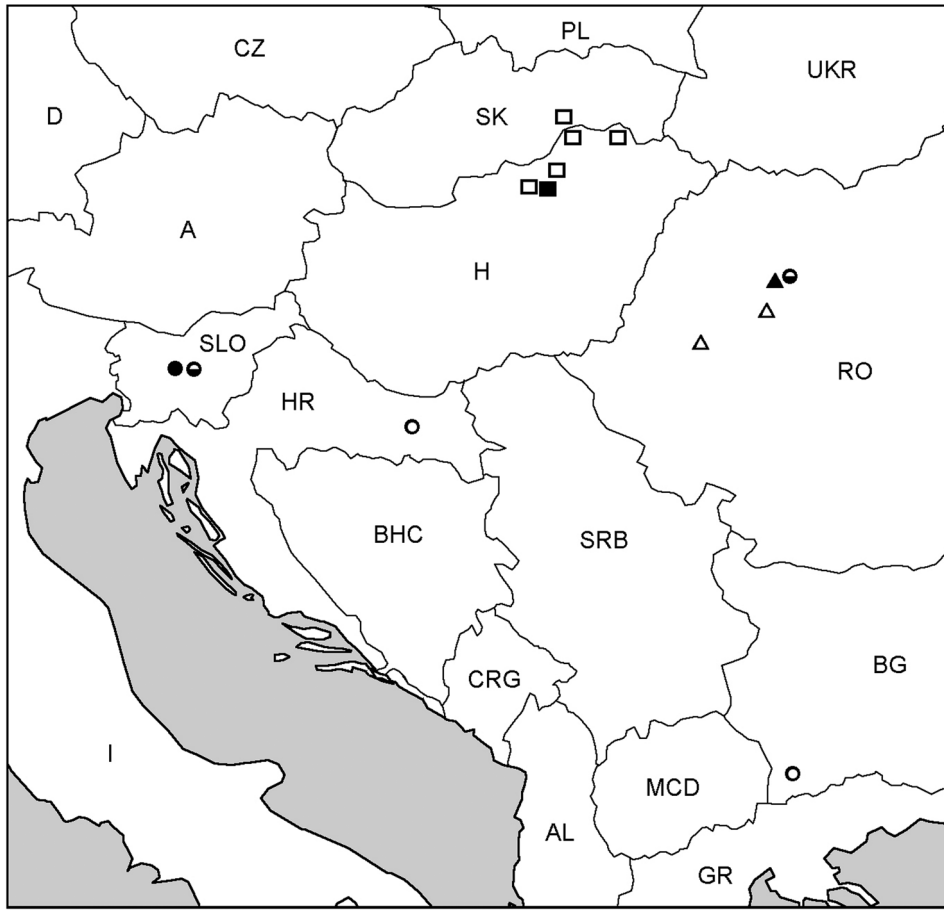

Fig. 1. Sampling sites, holotype and neotype localities of the studied *Pseudopodisma* species. *Pseudopodisma fieberi*: circle, *P. nagyi*: square, *P. transilvanica*: triangle; filled symbols: holotype, half–filled symbols: neotype, empty symbols: sampling sites for this study

Mts) (Fig. 1), and it was also reported from Slovakia (Kočárek *et al*. 2005). *P. nagyi* does not occur in Czech Republic (Holuša *et al*. 2013), so the northern border of the distribution area of the genus is in Slovakia. The differential diagnosis of *P. nagyi* was also based on the morphology of male genitalia. The females of all three species are reported to be undistinguishable (Galvagni & Fontana 1996).

Beyond these species descriptions only two papers provided some phylogenetic data on the genus *Pseudopodisma*. Litzenberger and Chapco (2001) mentioned *P. nagyi* in their study on the phylogenetic relationships and origin of the North American subfamily Melanoplinae (Orthoptera: Acrididae). They used *P. nagyi* as a representative species of the *Pseudopodisma* genus in their analysis. Chintauan-Marquier *et al*. (2013) analyzed the evolutionary history of the Eurasian and European Podismini including only *Pseudopodisma transilvanica* out of the three *Pseudopodisma* species. The geographic distribution of the three *Pseudopodisma* species is little known and only sporadic records are available in the literature (e.g., Kiss 1961, Nagy *et al*. 2005). In Hungary, both habitat preference and geographic distribution of *P. nagyi* are well studied (Nagy & Rácz 2007, Nagy *et al*. 2007, Nagy *et al*. 2010). In Romania, the checklist of orthopterans (Iorgu *et al.* 2008) mentions *P. fieberi* and *P. transilvanica* from the same region, whereas Hoffmann (2009) reports the third species, *P. nagyi*, from Zarand Mts (SW Romania).

Male genitalia are thought to be extremely successful in species-delimitation in certain insect groups (e.g., Mutanen & Pretorius 2007, Ilango 2011, Corrêa *et al*. 2012, Tóth & Varga 2012) including grasshoppers (López *et al*. 2007). However, the geographic distribution – including the supposed sympatric occurrences of different species in Romania – together with the weak morphological differentiation among the species question their differentiation at the species level argues for a synonymization under *P. fieberi*. It is noteworthy that, unlike other orthopteran groups whose taxonomy is intensively studied based on song patterns (e.g., Orci *et al*. 2010), this genus cannot be studied using these means as they do not stridulate. Therefore, in the current paper we examine the morphometric and molecular differentiation in the species of the *Pseudopodisma* genus based on external morphology and cytochrome *b* (*Cyt*B) gene sequences of specimens collected from various localities within the distribution range of the genus.

MATERIAL AND METHODS

Samples – 41 males and 34 females of the three species were measured (Table 1). The *a priori* species identification was based on the morphology of male genital structure as described by Galvagni and Fontana (1996). We assumed that the males and females from same locality belong to the same species. A subset of the above specimens (stored in

80 V/V% ethanol) was used in molecular characterization (Table 1). Because only relatively recent samples were suitable for PCR-amplification of the selected mitochondrial region, some samples were omitted from the molecular work. Altogether, we used six samples of the three species of *Pseudopodisma*, and one sample of *Odontopodisma rubripes* (Ramme, 1931) as closely related outgroup in the molecular work (Table 1).

Measurements – Eighteen morphometric characters were used in this study (Fig. 2): body length (BL), eyes distance (ED), fore tibia length (FTL), head length (HL), head width (HW), mesosternum lobe length (MLL), mesosternum lobe width (MLW), pronotum length (PL), pronotum width (PW), pronotum lateral side length (PLL), pronotum lateral side width (PLW), sternum length (SL), sternum width (SW), third femur length (TFL), third femur width (TFW), third tibia length (TTL), tegmen length (WL), tegmen width (WW). A Motic SMZ-168 stereomicroscope was used for all morphometric measurements. The specimens used in this study were preserved separately in 80 V/V% ethanol, and are deposited in the Entomological Collection at Department of Evolutionary Zoology and Human Biology, University of Debrecen, Hungary.

Morphometric data analysis – We performed a stepwise forward variable selection (Hocking 1976) using the Wilk's λ criterion separately for both genders. Wilk's λ is a generalization of the *F*-distribution and is a statistical test used in multivariate analysis of variance to show whether there are differences between the means of identified groups of subjects on a combination of dependent variables (Everitt & Dunn 2001). The initial model

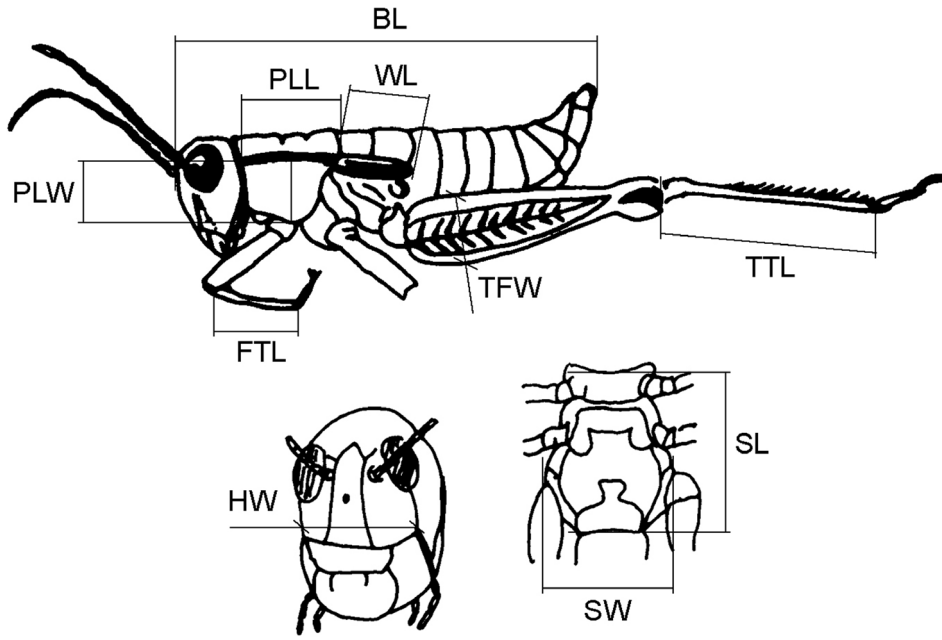

Fig. 2. Location of ten measured variables out of the 18 examined on the *Pseudopodisma* specimens. BL = body length, FTL = fore tibia length, HW = head width, PLL = pronotum lateral side length, PLW = pronotum lateral side width, SL = sternum length, SW = sternum width, TFW = third femur width, TTL = third tibia length, WL = tegmen length (Original drawing by Harz 1957)

Table 1. Sampling areas and locations, sampling date, the collector(s) name, male / female ratio of the measured *a priori* identified *Pseudopodisma* species, *a priori* identification and *a posteriori* group membership (classification) on the basis of morphometric analysis.

country	sampling area	sampling site	year	collector	♂/♀	APC	males			females		
							F	N	T	F	N	T
H	Mátra Mts	Mátrakeresztes	2004	Nagy	1 / 0	N		1				
	Aggtelek Karst	Jósvafő	2003	Nagy & Kisfali	10 / 7	N	1	4		1	4	
		Nagy-oldal				N		1	1		1	
		Kerek-Gárdony-tető				N		2	1	1		
	Bükk Mts	Leány-töbrök	2003	Nagy & Rácz	6 / 5	N		5			4	
		Török-rét				N		1			1	
	Zemplén Mts	Gyertyánkúti-rétek	2003	Nagy	5 / 5	N		5			5	
	no locality info		missing	Varga	2 / 0	N		2				
BG	Pirin Mts		1970	Varga	4 / 3	F	4			1	2	
HR	Papuk Mts	Gornj Vrhovci	2008	Szövényi & Puskás	2 / 7	F			2	7		
RO	Trascău Mts	Piatra Secuiului	2008	Varga	2 / 3	T			2			3
	Zarand Mts	Debela Gora	2007	Szövényi & Puskás	4 / 1	T		1	3			1
SK	Slovakian Karst	Hačava	2003	Nagy	5 / 3	N		5			3	
						Σ	5	27	9	10	20	4

Abbreviations: APC = a priori categorisation, H = Hungary, BG = Bulgaria, HR = Croatia, RO = Romania, SK = Slovakia, F = *Pseudopodisma fieberi*, N = *P. nagyi*, T = *P. transilvanica*

was defined starting with the variables that provided the best separation between groups. The model was then extended considering further variables depending on the Wilk's λ criterion: select the one which minimizes the Wilk's λ of the model including the variable if its *p*-value still shows statistical significance (Mardia *et al*. 1979). This variable selection was performed by the 'klaR' package (Weihs *et al*. 2005) of the R software (R Core Development Team 2009).

This selection procedure resulted in a set of variables that were further analyzed by linear discriminant analysis (LDA; Fisher 1936). We used pre–defined group membership (species based on identification and location, as given in Table 1) to find linear combinations of the original variables that has a maximal ratio of separation of the class means to the within–class variance (Venables & Ripley 2002). For three groups (and more than two variables), the number of linear discriminants that can be calculated is two. Resulting eigenvalues are the proportions of the variance between classes explained by the linear discriminants. LDA was performed by the MASS package (Venables & Ripley 2002) of the R software (R Core Development Team 2009).

Molecular characterization – DNA was extracted by homogenizing the third femur of ethanol-preserved specimens in 800 µl extraction buffer described by Gilbert *et al*. (2007). The samples were incubated for 24 h at 56°C with gentle agitation and then centrifuged at 14 000 rpm for 1 min. The supernatant was washed twice with an equal volume of chloroform–isoamyl alcohol (24:1) to remove proteins. The DNA was precipitated by adding the mixture of 80 µl ammonium acetate (7.5 M) and an equal volume of ice–cold isopropanol and storing the samples at –20°C for 4 h. The DNA was pelleted by centrifugation at 14 000 rpm for 10 minutes at 4°C. After centrifugation, the supernatant was discarded and the DNA pellet was washed twice with 70 V/V% ice–cold ethanol. The pellet was air dried for 1 h at room temperature, and was re–dissolved in 50 µl elution buffer (10 mM Tris HCl, pH 8.0 and 0.5 mM EDTA, pH 9.0).

The cytochrome *b* gene (*Cyt*B) of the mitochondrion, which can provide an alternative to the widely used cytochrome *c* oxidase I (COI) for recently diverged taxa (Hebert *et al*. 2003) because it has comparable resolution at terminal branches of insect phylogenetic trees (Simmons & Weller 2001), offers an adequate tool to test the putative separation of *Pseudopodisma* species. Although Litzenberger and Chapco (2001) provided primers for the amplification of the *Cyt*B region in grasshoppers, they performed poorly in our amplification experiments. To overcome this, we devised new primers to specifically amplify the *Cyt*B region by determining conservative regions in two closely related species (*Ognevia*, *Prumna*) with complete mitochondrial genome available (GenBank identifiers: EU914848, NC_013835, respectively). The following primers were devised: "CytB–SaskaIFw" (5′ – CCG TTC ATG CTA ATG GAG C – 3′) as forward, and "CytB–SaskaRv" (5′ – GGA CTT TAC CTC GTT TTC G – 3′) as reverse primer. With these primers, we were able to specifically amplify a 1047 bp long region located between bp 10686–11718 (as compared to *Ognevia*–EU914848) that covers 852 bp of the 3′ end of the *Cyt*B region and small portion of the 5′ end of the ND1 gene in the following PCR-conditions. The reaction mixture contained: 0.2 mM dNTP, 2 mM Mg, 1 mg/ml BSA, 0.2 µM of each primer, 0.02 U Phusion Hot Start II High–Fidelity DNA Polymerase (Thermo Fisher Scientific Ltd.) and 2× of the corresponding reaction buffer (Phusion HF). The amplification was performed in a Veriti 9600 thermal cycler (Applied Biosystem) programmed for: initial denaturation at 98°C for 45 sec, followed by 35 cycles of denaturation at 98°C for 15 sec, annealing at 58°C for 30 sec, extension at 72°C for 1 min, and a final extension at 72°C for 7 min. The amplification success was checked on 1% agarose gel stained with ethidium-bromide. Successfully

amplified products were submitted to be sequenced from both directions by commercially available service provider (Macrogen Inc., South Korea) using the original amplification primers as sequencing primers.

Molecular phylogenetic analysis – The forward and reverse sequences of the same sample were manually checked for errors, then continuous reads ("contigs") were made by hand using the software Chromas Lite v.2.1 (Technelysium Pty Ltd.). Sequences were aligned manually in BioEdit v.7.1.3 (Hall 1999). Phylogenetic analysis of the sequences employed an exhaustive search under the maximum parsimony criterion as implemented in PAUP v.4.0b*10 (Swofford 2003). The number of sequences allowed an exhaustive search to be conducted, thus we performed this analysis, which examine all possible trees in the virtual tree–space. The overlapping part of sequence of *Ognevia* (EU914848) was used as distantly, while that of *Odontopodisma rubripes* (Ramme, 1931) as closely related outgroup. With the inclusion of two, differently related outgroup taxa we also tested the cohesiveness of the genus *Pseudopodisma*. The search treated every character as unordered and unweighted, gaps as missing. The statistical robustness of the resulted topology was checked in a bootstrap test with 1000 pseudo-replications.

RESULTS

Morphometrics

For males, six variables were selected (BL, FTL, PLW, SL, SW, WL) (Fig. 2) out of the 18 measured variables based on Wilk's λ values. The first discriminant (LD1) separated *P. nagyi* from *P. transilvanica* and *P. fieberi* (Fig. 3). Standardized coefficients of LD1 were the highest for predictors SL, SW and PLW (Table 2). This indicates that sternum length, sternum width and the pronotum lateral side width are suitable for discriminate between *P.nagyi* and the other two species, *P. transilvanica* and *P. fieberi*. The second discriminant (LD2) led to a separation between *P. transilvanica* and *P. fieberi* (Fig. 3). Standardized

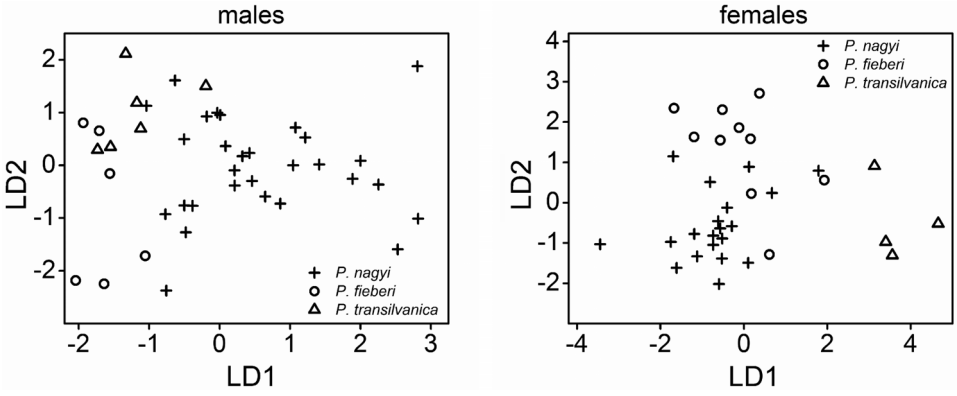

Fig. 3. Linear discriminant analysis results for males (left) and females (right). Each point represents an individual

Table 2. Six most important discriminating characters between groups, mean and standard deviation (±SD) (mm). LD1 and LD2 shows standardized coefficient values for each character, larger absolute values mean higher discriminating ability.

Sex	Characters	*P. fieberi*	*P. nagyi*	*P. transilvanica*	LD1	LD2
Male	WL	3.62 (±0.37)	3.87 (±0.37)	3.39 (±0.39)	0.81	-1.80
	FTL	3.41 (±0.25)	3.58 (±0.20)	3.59 (±0.06)	0.65	5.22
	SL	4.97 (±0.34)	4.90 (±0.25)	4.91 (±0.26)	-3.70	-1.85
	SW	3.53 (±0.47)	3.96 (±0.38)	3.74 (±0.22)	2.29	0.67
	PLW	2.18 (±0.19)	2.52 (±0.46)	2.30 (±0.22)	1.41	0.12
	BL	18.97 (±1.59)	19.74 (±2.17)	17.84 (±0.67)	0.28	-0.18
Female	PLW	2.69 (±0.20)	2.99 (±0.15)	2.85 (±0.24)	-3.92	-2.90
	WL	4.49 (±0.46)	4.93 (±0.50)	4.06 (±0.46)	-2.46	-0.12
	TTL	10.02 (±0.46)	10.36 (±0.56)	10.90 (±0.48)	1.42	-0.29
	TFW	2.89 (±0.29)	3.06 (±0.17)	2.90 (±0.34)	-4.05	0.27
	PLL	4.35 (±0.36)	4.78 (±0.25)	4.83 (±0.28)	3.04	-1.06
	HW	2.60 (±0.35)	2.93 (±0.27)	3.03 (±0.05)	1.73	-1.51

Abbreviations: BL: body length, FTL: fore tibia length, HW: head width, PLL: pronotum lateral side length, PLW: pronotum lateral side width, SL: sternum length, SW: sternum width, TFW: third femur width, TTL: third tibia length, WL: tegmen length; LD: linear discriminant function axes

coefficients of LD2 were highest for predictors FTL, SL and WL (Table 2). This indicates that fore tibia length, sternum length and the tegmen length are suitable variables to discriminate between *P. transilvanica* and *P. fieberi*. For males, LD1 accounted for 77.27%, while LD2 accounted for 22.73% of the variance between classes, singular values (giving the ratio of the between– and within–group standard deviations on the linear discriminant variables) were 4.17 and 2.26 for the two axes, respectively.

For females, the same six variables were selected (HW, PLL, PLW, TFW, TTL, WL) (Fig. 2) out of the 18 measured variables based on Wilk's λ values. The first discriminant (LD1) led to a separation between *P. transilvanica* and the other two species, *P. nagyi* and *P. fieberi* (Fig. 3). Standardized coefficients of LD1 were highest for predictors TFW, PLW, PLL and WL (Table 2). Consequently the third femur width, pronotum lateral side width, pronotum lateral side length and the tegmen length were appropriate variables to discriminate between *P. transilvanica* and the other two species. The second discriminant (LD2) separated *P. nagyi* and *P. fieberi* (Fig. 3). Standardized coefficients of LD2 were the highest for predictors PLW and HW (Table 2). This point out that pronotum lateral side width and head width had highest discriminatory power to distinguish *P. nagyi* and *P. fieberi*. For females, LD1 accounted for

71.31%, while LD2 accounted for 28.68% of the variance between classes, singular values were 5.66 and 3.59 for the two axes, respectively.

Out of the six males, *a priori* identified as *P. fieberi*, four specimens were correctly classified; the remaining two Croatian specimens were classified together with the *P. transilvanica* group. The proportion of correctly identified specimens was 66.6%. Out of the 29 males, *a priori* identified as *P. nagyi*, 26 specimens were correctly classified; two specimens from the Aggtelek Karst (Hungary) were classified with the *P. transilvanica* and one with the *P. fieberi* group. The accuracy of classification was 89.6%. Out of the six males, *a priori* identified as *P. transilvanica*, five specimens were correctly classified; the remaining sample from Zarandi Mts (Romania) was classified together with the *P. nagyi* group. The accuracy of classification was 83.3% (Table 1).

Out of the ten females, *a priori* identified as *P. fieberi*, eight specimens were correctly classified; the remaining two samples from Bulgaria were assigned to *P. nagyi* group. The proportion of correctly classified specimens was 80%. Out of the 20 *P. nagyi* females, only two specimens from the Aggtelek Karst were classified together with the *P. fieberi* group. The proportion of correctly classified specimens was 90%. Out of the four females, *a priori* identified as *P. transilvanica*, all four specimens were correctly classified, the proportion of correctly classified specimens was 100% (Table 1).

Out of the 41 *a priori* identified males, 35 specimens were correctly classified (85.3%), out of the 34 *a priori* identified females, 30 specimens were correctly assigned to its group (88.2%) (Table 1).

Molecular phylogenetic characterization

The newly devised primers produced unambiguous reads from both (forward and reverse) directions and allowed the concatenation of a 1047 bp long part of the *Cyt*B region in *Pseudopodisma*, 1043 bp in *Odontopodisma*. A final alignment of all sequences had a total number of aligned positions of 1054, out of which 183 were variable, and 59 parsimony-informative. For the *Pseudopodisma* samples, the above characteristics are 43 and 15, respectively. The exhaustive search under maximum parsimony criterion resulted in a single most-parsimonious phylogenetic tree (Fig. 4) with consistency index (CI) of 0.9563, homoplasy index (HI) of 0.0437, and retention index (RI) of 0.8929. The bootstrap procedure strongly supported (i.e., bootstrap above 85%) all but one branch, which – the one separating two Hungarian samples from the third – received weak bootstrap support (62%).

The 1047 bp long sequence of the cytochrome *b* region provided a basic insight into the phylogenetic relationships of the genus *Pseudopodisma*. The phylogenetic tree, which was rooted on *Ognevia*, placed the genus in a strong-

ly supported monophyletic clade with three main branches: (i) leading to the single sample of *P. fieberi* from Croatia, (ii) including two Romanian samples of *P. transilvanica*, and (iii) three samples of *P. nagyi* from Hungary. Within the latter clade it has identified a weakly supported sister relationship of the samples coming from the Aggtelek Karst and Zemplén Mts. The sample of the closely related species *Odontopodisma rubripes* was placed sister to the samples of *Pseudopodisma*.

DISCUSSION

We examined the morphological and molecular differentiation between three closely related putative species within the genus *Pseudopodisma*. While our sampling included specimens from the close vicinity of type localities in case of *P. nagyi* and *P. transilvanica* (Fig. 1), we could not include Slovenian specimen (i.e., from the type locality) of *P. fieberi*. This leaves us with some uncertainty regarding our taxonomic conclusions, although we believe we correctly extrapolated our *a priori* identification based on male genitalia.

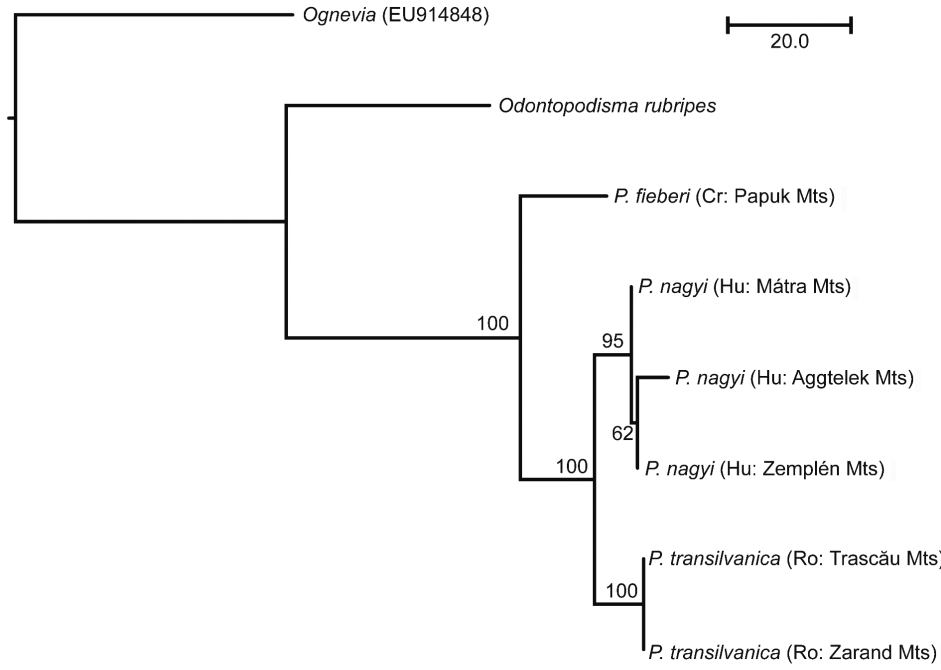

Fig. 4. The single most parsimonious phylogenetic tree for the genus *Pseudopodisma* obtained from an exhaustive search under maximum parsimony criterion and presented as a phylogram. Numbers above branches are bootstrap values resulting from 1000 pseudoreplicate. The scale bar represents 20 mutational changes

The morphometric analysis indicated (i) sternum length (SL), (ii) sternum width (SW), and (iii) the lateral side width of pronotum (PLW) as the best discriminating morphological characters to distinguish males of *P. nagyi* from *P. transilvanica* and *P. fieberi*. This discrimination was statistically significant but the characters could only be used jointly (i.e., not separately) to discriminate the species. Nevertheless, when using the values of the three most significant morphometric characters in a multivariate approach, the separation of *P. nagyi* from the other two species was highly significant on the first discriminant axis. There was a very little separation between the other two species, *P. transilvanica* and *P. fieberi*, both in terms of statistical significance and actual measurements.

The most important morphometric characters for the females were (i) the width of third femur (TFW), (ii) the lateral side width of pronotum (PLW), (iii) the lateral side length of pronotum (PLL), and (iv) the length of tegmen (WL). In this gender, *P. transilvanica* was distinguished from the other two species along the first axis, however, *P. nagyi* was not separable from *P. fieberi*. *P. transilvanica* was statistically distinguishable from *P. fieberi* and *P. nagyi*, and the statistical difference between *P. fieberi* and *P. nagyi* was again very small along the second axis.

It is worth noting the morphometric results concerning the Zarand Mts. population in our study, which was identified based on male genital organs as *P. transilvanica*. One specimen from this population was somehow close to *P. nagyi* in morphology (Table 1), while the rest was classified according to the *a priori* identification. The false classification of a single specimen from Zarand Mts makes the finding of Hoffmann (2008) – if that is made based solely on overall morphology and not genital structure – doubtful. Unfortunately, it is not clear from that paper if the author used genital characters to distinguish between the *Pseudopodisma* species, or the identification was based only on the descriptions of the cited literature.

In summary, individual morphometric characters were not suitable for the differentiation of the species contrary to the fact that we identified different degrees of morphological differences among the examined taxa. Nevertheless, combination of morphological characters in a morphometric analysis were able to distinguish male *P. nagyi* from the other congeners, whereas other characters made it possible to separate female *P. transilvanica* from the congeners. Male genital morphology was found to be supportive of the *a priori* identification within the genus *Pseudopodisma*, since the specimens examined were correctly classified into the *a priori* species with a success rate of 80–90% that we consider generally acceptable. These results gave us the confidence in concluding that the *a priori* taxa are morphologically subtly but significantly different, a finding what one would expect in case of slightly diverged species.

The molecular dataset was somewhat sparing but provided very robust results with negligible homoplasy. The phylogenetic analysis based on mitochondrial DNA sequences has indicated the cohesive nature of the genus *Pseudopodisma*. More samples are needed from Podismini, especially genera *Podisma* (Berthold, 1827), *Micropodisma* (Dovnar-Zapolskij, 1932), etc., to test monophyly of the genus. Within the genus, we found three, highly supported (all have bootstrap support of 100%) monophyletic clades, which corresponded to the *a priori* specific identification (i.e., *P. fieberi, P. nagyi, P. transilvanica*). The difference between the Mátra Mts sample and the reminders of *P. nagyi* is remarkable and, although only weakly supported, warrants further investigation. This result may indicate some phylogeographic structure within the dataset as the Mátra Mts is the highest mountain range in Hungary and geographically more isolated than Zemplén Mts or Bükk Mts. The preservation of this population therefore needs more attention. Altogether, we consider the phylogenetic results as supportive of the *a priori* species delimitation of Galvagni and Fontana (1993, 1996) within the genus *Pseudopodisma*.

It is notable however, that the molecular genetic data suggest a closer relationship of *P. nagyi* and *P. transilvanica*, whereas the morphometric results depict a somehow different pattern: in the morphometric space (Fig. 3) *P. fieberi* is impossible to distinguish from the congeners. This result might be explained by the different nature of the markers used; if any of the morphometric characters are under selection, the morphometric results can deviate from what we would expect if they were evolving neutrally—an advantage of the usage of (nearly) neutrally evolving molecular markers (Hebert *et al*. 2003). Secondly, if *P. fieberi* represents the earliest diverging lineage within the genus, we might expect less morphological divergence from more derived congeners.

Taking together the genital morphological, morphometric and genetic results, these data points towards a distinction at the species level among the three members within the genus *Pseudopodisma*. It must be, however, admitted that a denser sampling of the genus (i.e., phylogeographic survey) should be the next step in the work with this taxon, where only basic taxonomic work has been done before. Indeed, we cannot fully exclude the possibility that this genetic pattern is simply reflecting geographic variation (i.e., phylogeographic pattern), although we think the strong power of male genital organs and *Cyt*B sequences to characterize the species are suggestive of more profound isolation.

Our finding of some phylogeographic structure within our phylogenetic dataset can be the starting point of a more detailed molecular work in the genus, for which we provide here a useful molecular tool. Beyond the specific question, our results indicate that simple external morphometric techniques

alone are not enough to clearly separate these grasshopper species from each other. We should combine these methods with genital morphometry and/or molecular genetic investigation. A more straightforward approach is however the combination of genital morphometry together with molecular genetic analysis, which can be highly useful in orthopteran taxonomy, especially in non-stridulating groups like *Pseudopodisma*.

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