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Original paper

Morphological variability of *Nebela collaris* s.s. (Arcellinida: Hyalospheniidae) from Krečko Brdo Hill, East Herzegovina

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Summary. *Nebela collaris* s.l. is the best studied species complex within the family Hyalospheniidae. This complex includes 11 taxa established on the basis of small differences in shell shape and size. The type species for this complex (*N. collaris* s.s.) was defined based on only four specimens. Here we present morphological and morphometric data from moss-dwelling population of *N. collaris* s.s. from Krečko Brdo Hill (East Herzegovina), based on 765 specimens. Shell length ranged from 87 to 115 µm (previously reported 95-115 µm), shell width from 53 to 73 µm (previously reported 74-81 µm) and aperture width from 21 to 31 µm (previously reported 28-32 µm). In the literature, reported shell length/shell width ratios range from 1.38 to 1.47, however subsequent morphometric analysis yielded values from 1.33 to 1.91. These data show that *N. collaris* s.s. can be considered to be a very variable pseudocryptic species among testate amoebae. Coefficients of variation were low for all measured characters in the studied population. Minimal variability (4.28%) was observed for shell length, while maximal variability (6.45%) was recorded for aperture width/shell length ratio. Analysis of the size frequency distribution suggests that the studied population of *N. collaris* s.s. is size-monomorphic.

Keywords: biometry, morphometry, protists, pseudocryptic species, taxonomy, testate amoebae.

INTRODUCTION

Hyalosphenid testate amoebae include species characterized by rigid, colorless or yellowish-brown, flask-vase shaped, oval or pyriform, dorso-ventrally compressed shells, which are composed only of an organic matrix or with addition of self-secreted siliceous plates or recycled shell plates of small euglyphid testate amoebae, and rarely with other similar material such as diatom frustules. The aperture is terminal and bordered by a thin organic collar (Kosakyan et al. 2012). This group includes some well-studied genera of the testate amoebae because it includes comparatively large species, most of which live in mosses: a well-studied habitat important for the distribution, taxonomy and ecology of testate amoebae.

Species of hyalosphenid testate amoebae in most cases are clearly differentiated and well-distinguished from each other. Recently, some species were described based on clear morphological differences (Todorov 2010; Bobrov and Kosakyan 2015). However, most species were last described at the end of 19th or beginning of the 20th century, based on only a small number of specimens. For this reason, many species remain poorly described and without any information about their morphological variability. In light of this, several studies have been devoted to the morphological variability of earlier described hyalosphenid testate amoebae (Török 2001; Todorov 2002; Heger et al. 2011; Luketa 2015a, 2016; Nicholls 2015). The increasing use of molecular methods has led to the description of new pseudocryptic species (Kosakyan et al. 2012, 2013; Singer et al. 2015; Qin et al. 2016), but these studies often underestimates intraspecific variability on intrapopulation and interpopulation levels. Findings of molecular and morphometric studies represent a considerable contribution to the clarification of the taxonomy and phylogeny of hyalosphenid testate amoebae, and for the solution of taxonomic problems with closely related and incorrectly described species.

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Based on comprehensive phylogenetic analysis, Kosakyan et al. (2016) confirmed that the genus *Nebela* is paraphyletic and divided into six genera. In addition, these authors included all members of hyalosphenid testate amoebae in the family Hyalospheniidae with 13 genera, while Luketa (2015b) included 3 genera with elongated necks in the family Padaungiellidae. These taxonomic changes show that beta taxonomy of hyalosphenid testate amoebae is not stable. According a very recent report (Kosakyan et al. 2016), the genus *Nebela* s.s. comprises species with rounded, ovoid-pyriform or wide-pyriform shells covered by circular to elongated plates. These plates are apparently recycled mostly from euglyphid testate amoeba prey. The shell sometimes also includes fragments of diatom frustules or other small mineral elements. This genus includes eleven taxa of *N. collaris* complex (*N. acolla*, *N. aliciae*, *N. collaris*, *N. flabellulum*, *N. gimlii*, *N. guttata*, *N. pechorensis*, *N. rotunda*, *N. subsphaerica*, *N. tincta* and *N. tincta* f. *galeata*) and some well-defined morphospecies (*N. carinatella*, *N. d'ydevallei*, *N. pulchra*). In the *N. collaris* complex, small differences in shell shape can be used to discriminate species (Kosakyan et al. 2013; Singer et al. 2015). However, these pseudocryptic species are often described based on less than ten specimens. For this reason, we know very little about its intraspecific morphological variability. In the present paper, we report the morphology and morphometry of *N. collaris* (Ehrenberg, 1848) sensu Kosakyan et Gomaa, 2013 from epigenous mosses collected on Krečko Brdo Hill, East Herzegovina.

MATERIAL AND METHODS

Material for the present study was extracted from epigenous mosses collected on the edge of the forest. Samples were taken from Krečko Brdo Hill (43°18'55.2"N, 18°12'00.8"E, ca. 1150 m a.s.l.), municipality Nevesinje, East Herzegovina on 10 May 2016. Krečko Brdo is a limestone hill whose peak is ca. 1250 m above sea level. Morphological characters and morphometric variables were studied using a light microscope (Zeiss Axio Imager A1). Images were captured using an AxioCam MRc5 (Zeiss) digital color camera. Measurements were conducted in the program AxioVision 4.9.1. The following measurements were taken for 765 shells: shell length, shell width and aperture width. The following descriptive statistics were calculated: extreme values (minimum and maximum), median, arithmetic mean, standard error of the arithmetic mean, standard deviation, coefficient of variation (in percentage), skewness and kurtosis. For description of bag plot (a bivariate generalization of the univariate boxplot) see Rousseeuw et al. (1999). Statistical analysis was conducted in the programs PAST 2.17c and STATISTICA 13.0.

RESULTS

The shell is large, narrow to wide pear-shaped, slightly yellowish or brownish, laterally compressed, with small lateral pores (the number of which can vary) that can be difficult to observe. The shell is composed mainly of oval or circular plates or only of organic cement. The shell neck is very short or almost absent. The aperture is terminal, oval, linear, slightly curved or strongly curved, surrounded by a thin organic lip. Figure 1 shows light micrographs of different specimens in broad lateral view showing their general shell shape and outline.

Morphometric characters of 765 specimens from Krečko Brdo Hill were measured and the results are presented in Table 1. The most frequent shell length (103 μm) was registered in 75 specimens (Fig. 2A); the most frequent shell width (62 μm) was registered in 100 specimens (Fig. 2B), and the most frequent aperture width (26 μm) was registered in 197 specimens (Fig. 2C). Coefficients of variation were low for all measured characters, ranging from 4.28% to 6.45%. For basic characters, minimal variability was observed for shell length (4.28%), while the maximal variation coefficient was observed for aperture width (5.83%). For ratio characters, minimal variability was observed for shell length/shell width ratio (5.37%), while the maximal variation coefficient was observed for aperture width/shell length ratio (6.45%).

Analysis of the size frequency distribution of the measured specimens indicates that studied population of *Nebela collaris* s.s. is a size-monomorphic. For example, shell lengths ranged from 87 to 115 μm. However, 64.84% of all the specimens had a shell length of 101-108 μm, whereas only 23.40% were smaller than 101 μm and only 11.76% were larger than 108 μm. Histogram analysis revealed nearly the same regularity with respect to shell width distribution. Namely, all measured specimens had a shell width between 53 and 73 μm. In this case, 65.36% of all specimens had a shell width of 59-64 μm, whereas only 34.64% were narrower than 59 μm and wider than 64 μm. Figures 2D-F show bag plots analyses of the correlation between shell length, shell width and aperture width.

The negative values of skewness for shell length, shell length/shell width ratio and aperture width/shell width ratio suggest an asymmetrical distribution with a long tail toward lower values. The asymmetry of aperture width/shell width ratio was low, with a skewness value of -0.182. The asymmetry of shell length was moderate low (-0.375), while a high negative value was observed for shell length/shell width (-0.595). Both aperture width and aperture width/shell length ratio were found to have low positive skewness values, while high positive value were observed only for shell width (0.560). Negative values of kurtosis were not observed for any characters. In fact, all characters had positive values of kurtosis, indicating a distribution sharper than a standard Gaussian distribution. Low positive values (between 0.154

Fig. 1. Light micrographs of *Nebela collaris* s.s. from Krečko Brdo Hill, East Herzegovina - broad lateral view of different specimens showing general shell shape and outline. Scale bars: 20 µm.

Table 1. Morphometric characterization of *Nebela collaris* s.s. from Krečko Brdo Hill (East Herzegovina) based on 765 specimens (measurements in μm).

Characters	Min	Max	M	x̄	SE	SD	CV	Sk	Ku
Shell length	87	115	104	103.48	0.16	4.43	4.28	-0.375	0.183
Shell width	53	73	62	62.40	0.12	3.40	5.46	0.560	0.154
Aperture width	21	31	26	26.45	0.06	1.54	5.83	0.073	0.369
Shell length/shell width	1.33	1.91	1.67	1.66	0.00	0.09	5.37	-0.595	0.537
Aperture width/shell length	0.19	0.31	0.25	0.26	0.00	0.02	6.45	0.148	0.690
Aperture width/shell width	0.33	0.50	0.43	0.42	0.00	0.02	5.60	-0.182	0.201

Abbreviations: Min and Max – minimum and maximum values, M – median, **x̄**– arithmetic mean, SE – standard error of the arithmetic mean, SD – standard deviation, CV – coefficient of variation in %, Sk – skewness, Ku – kurtosis.

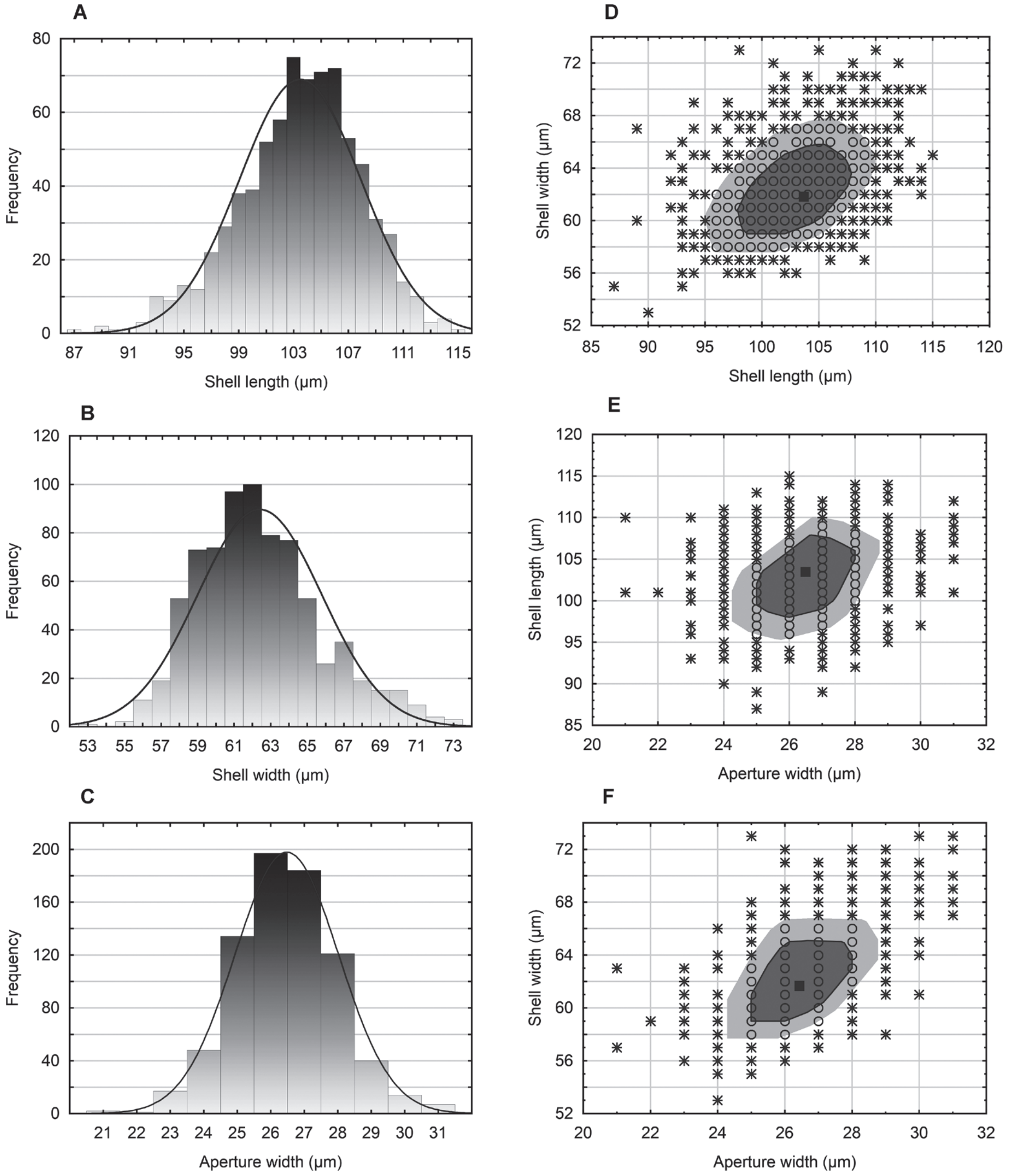

Fig. 2. Graphs created based on 765 measured specimens of *Nebela collaris* s.s. from Krečko Brdo Hill, East Herzegovina. Histograms show the size frequency distribution of the shell length (**A**), shell width (**B**), and aperture width (**C**); bag plots shows the correlation between shell length and shell width (**D**), aperture width and shell length (**E**), and aperture width and shell width (**F**). Legend for bag plots: depth median ■, characters on Y axes ○, outliers *.

and 0.201) were observed for shell width, shell length and aperture width/shell width ratio. A moderate positive value (0.369) was observed for aperture width, while high positive values were observed for shell length/shell width ratio (0.537) and aperture width/shell length ratio (0.690).

DISCUSSION

It is widely accepted that natural selection acts directly on phenotype and not genotype. Also, species are usually defined as a phylogenetic unit. For these reasons, it is necessary to find the phenotypic differences between genetically discrete units on cladograms if these genetically separate clades are to be defined as different species. The term "pseudocryptic diversity" refers to the existence of species that are genetically distinct, but morphologically very close. Taxonomic studies based on molecular and morphological data have led to the recognition that pseudocryptic diversity is widespread among hyalosphenid testate amoebae (Kosakyan et al. 2013, 2016; Oliverio et al. 2014). These pseudocryptic species are described based on only a small number of specimens and we do not know anything about their intraspecific morphological variability at the intrapopulation and interpopulation level. However, many studies based on a high number of specimens reported high intraspecific morphological variability in many testate amoebae species (Wanner 1999; Blanco 2001; Bobrov and Mazei 2004). Moreover, based on morphometric analysis of 24 testate amoebae taxa, Bobrov and Mazei (2004) concluded that the character of variability (its amplitude and correlativity) differs not only in different species, but also in different populations of the same species. For these reasons, current descriptions of pseudocryptic species in the *Nebela collaris* complex are highly questionable, since they are based on studies using only small morphological and morphometrical differences and a small number of specimens.

Kosakyan et al. (2013) concluded that morphological characters of proven taxonomic validity within *N. collaris* complex are: size and shape of the shell, the presence or absence of a lateral ridge, the length of the neck, and the shape and size of the aperture. In addition, they concluded that characters of no proven taxonomic validity are: the composition of the shell, the size and shape of the shell plates, the presence or absence of an organic layer on the shell, and the presence or absence of lateral pores. These authors noted that shells of *N. collaris* s.s. possess very short or almost absent necks. Results from the present study confirm this observation. Kosakyan et al. (2013) observed diversity of aperture shape within *N. collaris* s.s.: linear, slightly curved or strongly curved. In the present study, shells were also found with differently shaped apertures. Results from the present study confirm previously reported conclusions that the number of lateral pores vary, and that pores can be difficult to observe. In addition, the composition of shells in previously studied populations and in the population from Krečko Brdo Hill is similar: shells are composed mainly of oval or circular plates or can be composed only of organic cement.

Kosakyan et al. (2013) for *N. collaris* s.s. noted the following measurements: shell length 95-115 µm (mainly 109-112 µm), shell width 74-81 µm and aperture width 28-32 µm. The authors sequenced only four specimens with a shell length of 109-112 µm, while extreme values were taken from specimens without molecular data. Results from the present study show that the population from Krečko Brdo Hill have similar variability of shell length (87-115 µm), but values of shell width are extremely different (74-81 µm versus 53-73 µm). This difference may be caused by interpopulation variability or may be an indicator that these populations are not conspecific. A second, unlikely possibility may be that Kosakyan et al. (2013) measured a small number of specimens and higher variability can be expected in the population from Le Cachot bog (Switzerland). Based on shell length/shell width ratios it is possible to distinguish two groups within the *N. collaris* complex: shells are wider than long (*N. acolla* Cash, 1909 and *N. flabellulum* Leidy, 1874) and shells are longer than wide (nine other taxa, e.g. *N. collaris* s.s.). According to Kosakyan et al. (2013) and Singer et al. (2015), the values of shell length/shell width ratio range from 1.2 to 1.5 within a second group of *N. collaris* complex. Kosakyan et al. (2013) for *N. collaris* s.s. noted the value of this ratio 1.4 (recalculated to two decimals: 1.38-1.47). The values of shell length/shell width ratio in the population from Krečko Brdo Hill range from 1.33 to 1.91. These data show that *N. collaris* s.s. can be considered an example of a very variable pseudocryptic species among testate amoebae.

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