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A revision of chiggers of the *minuta* species-group (Acari: Trombiculidae: *Neotrombicula* Hirst, 1925) using multivariate morphometrics

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Abstract We revise chiggers belonging to the *minuta*-species group (genus *Neotrombicula* Hirst, 1925) from the Palaearctic using size-free multivariate morphometrics. This approach allowed us to resolve several diagnostic problems. We show that the widely distributed *Neotrombicula scrupulosa* Kudryashova, 1993 forms three spatially and ecologically isolated groups different from each other in size or shape (morphometric property) only: specimens from the Caucasus are distinct from those from Asia in shape, whereas the Asian specimens from plains and mountains are different from each other in size. We developed a multivariate classification model to separate three closely related species: *N. scrupulosa*, *N. lubrica* Kudryashova, 1993 and *N. minuta* Schluger, 1966. This model is based on five shape variables selected from an initial 17 variables by a best subset analysis using a custom size-correction subroutine. The variable selection procedure slightly improved the predictive power of the model, suggesting that it not only removed redundancy but also reduced 'noise' in the dataset. The overall classification accuracy of this model is 96.2, 96.2 and 95.5%, as estimated by internal validation, external validation and jackknife statistics, respectively. Our analyses resulted in one new synonymy: *N. dimidiata* Stekolnikov, 1995 is considered to be a synonym of *N. lubrica*. Both *N. scrupulosa* and *N. lubrica* are recorded from new localities. A key to species of the *minuta*-group incorporating results from our multivariate analyses is presented.

Introduction

Chiggers of the *minuta* species-group of the genus *Neotrombicula* Hirst, 1925 are very common ectoparasites of rodents in Asia and certain European regions (Kudryashova, 1979, 1998). Species identification in the *minuta*-group is extremely difficult because of large intraspecific and low interspecific variation (Stekolnikov, 1995). Previous original diagnostic characters or small type-series may not capture extensive variation commonly found in the *minuta*-group and may not be sufficient for accurate identification of unknown specimens. Because almost all differences between closely related species of chiggers are metric, appropriate statistical treatment

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is necessary for them. There is one widely distributed and extremely morphologically variable species (*N. scrupulosa* Kudryashova, 1993) and 13 morphologically similar species with restricted ranges (our data). A revision of the group can only be accomplished if the variability of *N. scrupulosa* across its entire geographical range is carefully examined.

Here we conduct morphometric analyses of inter- and intraspecific variation of a representative taxon set of the *minuta*-group using Principal Component Analysis (PCA). Our analyses contrasts size-and-shape (raw variables) and shape variance (Darroch & Mosimann, 1985), because size-related variation usually represents non-genetic intraspecific variation related to differences in environmental conditions, whereas shape-related variation may represent genetic interspecific variation (Jungers et al., 1995). *N. scrupulosa* Kudryashova, 1993 and the closely related *N. minuta* Schluger, 1966 and *N. lubrica* Kudryashova, 1993, as delimited by the exploratory PCA, are further analysed using Discriminant Function Analysis (DFA) conducted on size-corrected (shape) variables. To distinguish between these three species, we develop a classification rule based on a best-subset analysis which uses classification accuracy as the explicit criterion for variable selection. Finally, we incorporate this rule into our key to species of the *minuta*-group and give a synonymy list along with extensive collection data for each species of the group.

Materials and methods

Definition of the group

The *minuta*-group includes medium-sized mites having two genualae I, mostly nude galeala, nude dorsal and mostly nude lateral palpal tibial setae, fPp = B/B/NN(B)B, and the basic variant of the dorsal idiosomal setae arrangement fD = 2H-6(8)-6-6-4(2)-6(4)-2. The synthetic identification formula is 7BS-N-3-2111.1000; fCx = 1.1.1; fSt = 2.2. This group was first revised by Kudryashova (1993), who described several new species closely related to *N. minuta*. In the next revision, *N. scrupulosa* was shown to be the most widespread species, as indicated by extensive new collection data (Stekolnikov, 1995). Kudryashova (1998) redescribed *N. minuta* and seven other closely related species, including those she described in 1993. Recently two more species from the *minuta*-group were found in the Caucasus (Stekolnikov, 2008). In addition, based on literature records, the following three species can also be attributed to the *minuta*-group: *N. anax* Audy & Womersley, 1957 from India and China, *N. balcanica* Kolebinova, 1973 and *N. corvi* Kolebinova, 1971 from Bulgaria.

Collections

Our investigation was based on the collections deposited in the Zoological Institute of the Russian Academy of Sciences, St Petersburg, Russia (ZIN) and the Zoological Museum of the Moscow State University, Moscow, Russia (ZMUM). Other depositories cited in this paper are as follows: Institute of Zoology of the Bulgarian Academy of Sciences, Sofia, Bulgaria (ZISB); South Australian Museum, Adelaide, Australia (SAM); Natural History Museum [formerly British Museum (Natural History)], London, UK (BMNH); National Museum of Natural History (formerly, United States National Museum), Washington DC, USA (USNM); and National Institute of Virology, Pune, India (NIV). Abbreviations are given following Evenhuis (2009).

Geographical localities were verified in the US National Geospatial-Intelligence Agency (NGA) database (http://geonames.nga.mil/ggmagaz/geonames4.asp). Altitudes of the collection localities were estimated using topographic maps (scale 1:150,000 and 1:100,000), and verified in Google Earth, ver. 4.3 (http://earth.google.com). Names of mammalian hosts are given after Wilson & Reader (2005). The name of the senior author is abbreviated as AAS when presenting collection information.

The numbers of measured specimens in the two most common species of the *minuta*-group are as follows:

N. scrupulosa (a total of 244 larvae): *Plain Asian form* (48): Bayanjargalan (3); Ishim (4); Imantau (7); Algabas (4); Ufa (15); Uksunay (4); Novotaraba (3); Eronda (2); Novaya Barda (4); Babiy Log (2); *Mountain Asian form* (82): Almaty (9); Saur (8); Orto-Syrt (11); Semis-Bel’ (6); Alamedin (3); Kosh-Agach (15); Aldyy-Shynaa (15); Barlyk (15); *Caucasian form* (114): Marsis (15); Haghartsin (6); Guzeripl (17); Teberda (6); Baduk (15); Bezengi (20); Buron (5); Mazada (5); Ashil’ta (6); Kurush (17); Tlokh (1); Khnov (1).

N. lubrica ($n = 54$): Koytezek (9); Rovand (5); Sarikhosor (15); Tirich Mir (6); Galaligez (10); Aydere (8); Parkhay (1).

Morphometrics

Mite specimens were mounted in Faure-Berlese medium under uniform conditions and examined under a compound microscope with phase contrast optics. Measurements were made with an ocular micrometer and converted to micrometres. Drawings were made using a camera lucida for each specimen in order to count idiosomal setae and document their arrangement.

The terminology follows Goff et al. (1982), with some modifications. The following 11 morphometric variables commonly employed in chigger systematics (e.g. Kolebinova, 1992; Kudryashova, 1998; Fernandes & Kulkarni, 2003) were measured: AM, length of anteromedian seta of scutum; AL, length of anterolateral setae of scutum; PL, length of posterolateral setae of scutum; H, length of humeral setae; AW, distance between ALs; PW, distance between PLs; SB, sensillary bases and distance between sensilla; ASB, distance from SB to extreme anterior margin of scutum; PSB, distance from SB to extreme posterior margin of scutum; P-PL, distance from PL to posterior-most scutal margin; AP, distance from AL to PL on one side. We used also six original indices, namely: D_{min}, length of the shortest dorsal idiosomal seta; D_{max}, length of the longest dorsal idiosomal seta; D_{1-4}, number of humeral setae and dorsal idiosomal setae in rows 1–4; VC, number of all other idiosomal setae excluding sternal; TaIII, length of tarsus III; and dmt, distance from mastitarsala to the base of tarsus III.

These 17 variables were included in analyses of the general dissimilarities between samples. Moreover, some other standard measurements and indices proved to be useful for comparison between several species: $SD = ASB + PSB$; pa, length of leg I; pm, length of leg II; pp, length of leg III; $Ip = pa + pm + pp$; NDV, number of idiosomal setae excluding sternal (can be computed as $D_{1-4} + VC$); m-t, ratio between distance from mastitarsala to the base of tarsus III and length of tarsus III ($m\text{-}t = dmt/TaIII$).

The advantage of D_{1-4} and VC over the traditional indices DS and VS was discussed previously (Stekolnikov, 2008). We used variable TaIII instead of other variables characterising the length of legs (pa, pm, pp and Ip) to equalise the magnitude of variation with other variables. The character dmt is proposed in the present paper for the first time. In our previously accumulated morphometric data the index m-t was present instead of the measurement of this distance. Therefore, dmt was restored from these data as a product of m-t and TaIII.

Morphometric data for *N. anax* were taken from Audy & Womersley (1957) and Fernandes & Kulkarni (2003). As the authors did not give values of P-PL, we calculated a rough estimate of this character, approximating the scutum as a pentagon, using the formula:

$$P - PL = SD - \sqrt{AP^2 - \left(\frac{PW - AW}{2}\right)^2}$$

The character dmt was measured using figures given in the above works. All morphometric data are available in the supplementary MS Excel file (Online Resource 1).

In morphometric analyses, we used both raw variables and the size-corrected variables. Size adjustment was performed according to the procedure successfully used previously in a study of cheyletid mites (Klimov et al., 2006), where size was defined as the geometric mean of all variables and size-free or ‘shape’ variables were computed by dividing raw variables by the geometric mean of all variables (Darroch & Mosimann, 1985). This method follows Mosimann’s strategy of shape investigation, interpreting shape as an intrinsic property of an organism rather than a characteristic of differences between samples. This algorithm was proven to be more statistically consistent as compared with other methods of size adjustment (Jungers et al., 1995).

Multivariate analyses

The pattern of multivariate distances between the three putative groups of *N. scrupulosa* (Caucasian, plain Asian and mountain Asian) was estimated by covariance-based principal component analysis (PCA), where each group was presented by the group centroid. The analysis was performed using 17 raw and size-corrected variables. As the intraspecific nature of the three geographical forms of *N. scrupulosa* was clearly demonstrated (see below), distances determined for these geographical forms were used as a scale for assessing morphometric differences for other species.

In the subsequent analysis, samples of *N. scrupulosa* were merged into three OTUs according to their geographical origin. The distances between samples of all examined species and the three groups of *N. scrupulosa* were then summarised using the UPGMA clustering algorithm applied to the matrix of Euclidean distances. This was done solely for the purpose of simplifying data presentation as opposed to PCA. Because PCA extracted several principal components (not shown), data are needed to be presented in multidimensional graphs which may not be very easy to read. At this point UPGMA trees should not be considered as phylogenies but a way to show hierarchically similarities and dissimilarities between putative species. The single linkage clustering method was also used for the three geographical forms of *N. scrupulosa*.

Despite the fact that counts, as opposing to measurements, are usually entered to the analysis as a separate datatype, a comparison of scores of the first principal components from four different PCAs, including *N. sculpurosa*, *N. minuta* and *N. lubrica* of the *minuta*-group, suggested that counts behave almost the same as metric variables and that their treatment as such is fully justified. The four PCAs considered the following situations: (i) measurements and counts were treated as metric and categorical variables, respectively; (ii) ditto, but measurements were size-corrected; (iii) measurements and counts were treated as metric variables; and (iv) ditto, but all variables were size corrected. Because plots of PCA scores were not substantially different for each analysis, we concluded that counts (D_{1-4} and VC) indeed behave as metric variables.

The Mann-Whitney U test was used to evaluate whether individual morphometric variables are significantly differ in putative species. This test checks for equality of the group mean ranks in two groups (Bergmann et al., 2000), which can be interpreted as checking whether the distribution in one group has values that are systematically larger than those in the other group (Moore & McCabe, 2005). Working with ranks makes this method independent of specific assumptions about the shape of the distribution, allowing comparing heteroscedastic samples of very unequal size. We evaluated three algorithms of this test in Statistica ver. 8.0 (StatSoft Inc., Tulsa, OK, USA): (1) with no corrections, (2) with a correction for ties, and (3) a simplified 'exact' algorithm that is valid only when there are no ties (Bergmann et al., 2000). The outcomes of these algorithms were not significantly different in our work.

For discriminating between *N. scrupulosa*, *N. lubrica* and *N. minuta*, a best subset discriminant analysis was run using a custom Visual Basic script (Klimov et al., 2006) in SPSS ver. 18 (http://insects.ummz.lsa.umich.edu/acari/Tools/Best_Subset/Best_Subset_SPSS.htm). The script performs Darroch and Mosimann size-correction at each iteration of the best subset analysis. Prior to the analysis, our dataset was randomly split into training sample (to calculate discriminant functions) and holdout sample (to validate these functions independently). We also accessed potential upward bias of discriminant functions using jackknife (leave-one-out) statistics. Subsets generated by a best subset analysis can be sorted by these three measures of classification accuracy in order of their importance (external validation, leave-one-out statistics and analysis only). A decision about using a particular subset can be made based on these data and expert knowledge of the variables (e.g. taxonomically informative, can be easily and accurately measured).

Multivariate normality was tested using a generalised Shapiro-Wilk's test (Villasenor-Alva & Gonzalez-Estrada, 2009) and Mardia's test (Mardia, 1985). Computations were performed in mvShapiroTest (Gonzalez-Estrada & Villasenor-Alva, 2009) and dprep (Acuna, 2009) modules implemented in the 'R' software environment ver. 2.10.1 (http://www.r-project.org).

Results and discussion

Intraspecific variation as exemplified by *Neotrombicula scrupulosa*

PCA analysis of 17 raw variables (see 'Materials and methods') yielded two first components accounting for 51.52 and 15.04% of the total variance, respectively. There is a clear geographical structure in the data (Fig. 1). Samples from Caucasus and Turkey (putative 'Caucasian form'), mountains of Central Asia (putative 'Mountain Asian form') and plains of Central and Northern Asia (putative 'Plain Asian form') comprise three distinct clusters formed by the first two principal components. One sample from Armenia (Haghartsin) and one from Russia (Altaiskiy

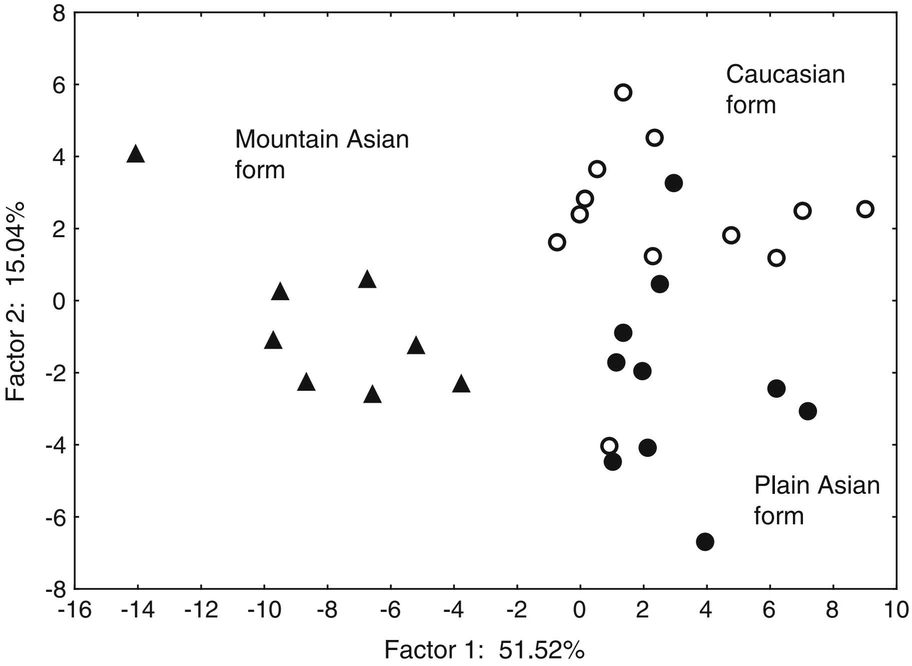

Fig. 1 Results of PCA for samples of *Neotrombicula scrupulosa* (17 raw variables), factor coordinates of cases

Kray, Eronda) are exceptions. The first sample was grouped with the plain Asian cluster, and the second one with the Caucasian cluster, whereas, according to their geographical positions, the contrary grouping would be expected. The geographical distribution of the Mountain Asian and Plain Asian forms is presented on Fig. 2.

Results of the PCA confirm that size (Darroch & Mosimann, 1985) greatly contributes to the morphometric variation within *N. scrupulosa*. The first principal component accounts for more than 50% of the total variance and the loadings of 10 variables are high (>0.6) for this component (Table 1). Raw variables show that the Plain Asian form and the Caucasian form are close to each other and segregated from the Mountain Asian form. In relation to the size-free variables, the two Asian forms cluster together (as expected) and this group is different from the Caucasian form (Fig. 3). Thus, the differences between the two Asian forms are mainly in relation to size: the Mountain form includes larger specimens than the Plain form, whereas the Caucasian form is characterised by unique shape (as a morphometric property). The Caucasian form differs from the other groups by lower absolute and relative values of TaIII and PSB (Fig. 4). Relatively low values of these differences and their overlaps suggest the intraspecific nature of these groups. Complete discrimination for any of these forms is not possible.

Size difference between the Asian forms is a clear example of the eco-geographical rules previously recorded for chigger mites: mites from mountainous regions are larger than individuals of the same species collected in plains, and size increases with altitude directly or is concomitant with the rise of altitude of surrounding mountains (e.g. Stekolnikov, 1998, 2002, 2006).

Interspecific variation

UPGMA dendrograms were obtained using both size- and shape and size-free variables. In the size-and-shape analysis (Fig. 5A), most of the *N. lubrica* samples (from Koytezek, Galaligez, Aydere, Sarikhosor and Rovand) form a separate cluster that includes the type-material (from Koytezek), specimens from Turkmenistan, and the holotype of *N. dimidiata* Stekolnikov, 1995 (from Galaligez and Aydere). However, the forms of *N. scrupulosa* are separated from each other.

In contrast, the size-free analysis (Fig. 5B) renders the OTUs of *N. scrupulosa* together, which seems to

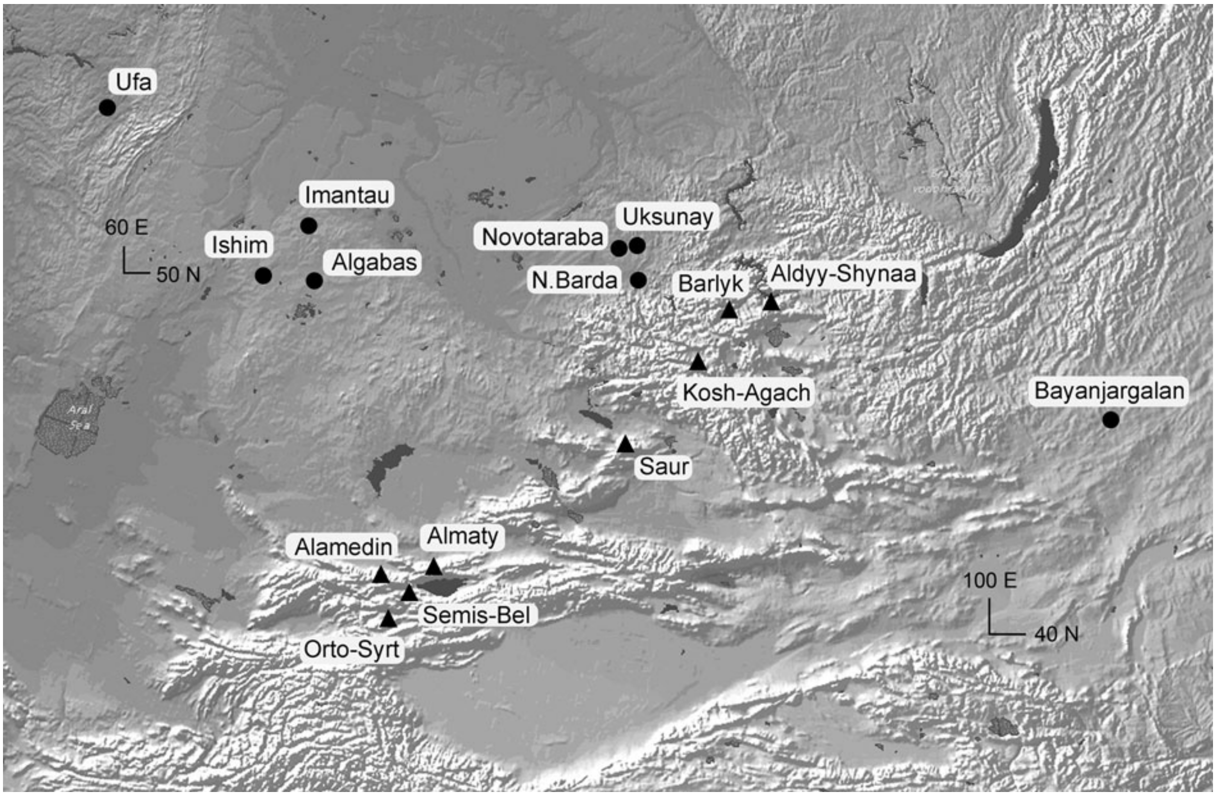

Fig. 2 Geographical distribution of the *Neotrombicula scrupulosa* samples of the Plain Asian (circles) and Mountain Asian (triangles) forms

Table 1 Results of PCA for samples of *Neotrombicula scrupulosa*, factor-variable correlations (factor loadings), based on covariances

	Factor 1	Factor 2		Factor 1	Factor 2
AW	−0.738*	0.298	PL	−0.834*	0.197
PW	−0.823*	0.395	H	−0.833*	0.028
SB	−0.713*	0.315	D_{min}	−0.832*	0.072
ASB	−0.437	0.357	D_{max}	−0.749*	0.295
PSB	−0.725*	−0.351	D_{1-4}	−0.297	0.443
P−PL	−0.440	−0.082	VC	−0.535	−0.286
AP	−0.870*	−0.068	TaIII	−0.798*	−0.555
AM	−0.546	0.537	dmt	−0.134	−0.219
AL	−0.551	0.485			

Note. Loadings with absolute values greater than 0.6 are indicated by an asterisk.

be consistent with the taxonomy of this species. The distance between type-specimens of *N. lubrica* (from Koytezek) and *N. dimidiata* (from Galaligez) is about the same, as the distance between geographical forms of *N. scrupulosa*, and is much smaller than distances between such well-defined and easily recognisable species as *N. kubanensis* Stekolnikov, 2008, *N. uraliensis* Kudryashova, 1993 and *N. urartensis* Stekolnikov, 2008. Thus, *N. dimidiata* should be considered as a synonym of *N. lubrica*. The sample from Pakistan (from Tirich Mir) is situated well within the *N. lubrica* cluster. Species *N. agriotricha* Stekolnikov, 1994 and *N. minuta* are very close morphometrically to *N. scrupulosa* and *N. lubrica* respectively, but they also have distinct non-metric characters which make their validity beyond doubt.

The original description of *N. dimidiata* states that this species differs from *N. lubrica* in the shape of the scutum and its punctation (Stekolnikov, 1995). The discovery of new specimens showed, however, that the original diagnostic characters are variable. At the same time, our morphometric analysis did not reveal any additional differences.

The taxonomic status of *N. irata* Kudryashova, 1993 remains uncertain. This species differs from

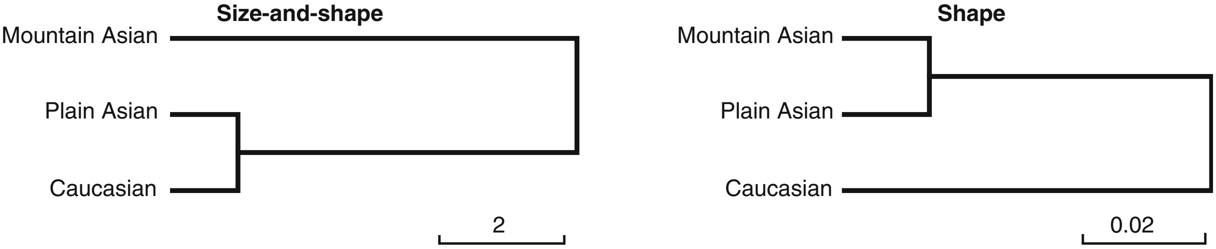

Fig. 3 Groupings of the *Neotrombicula scrupulosa* geographical forms by size-and-shape and shape variables (Euclidean distances, single linkage). Scale-bars are given for linkage distances

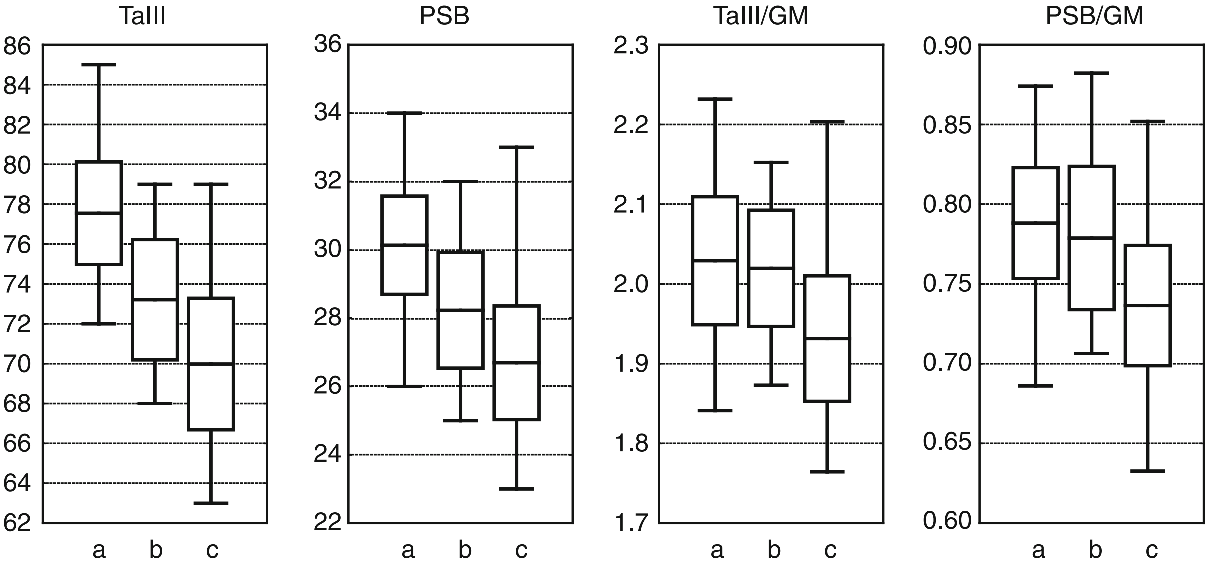

Fig. 4 Box plot of main diagnostic variables of the *Neotrombicula scrupulosa* geographical forms (range, mean ± SD; a, Mountain Asian; b, Plain Asian; c, Caucasian)

N. lubrica in its slightly shorter scutum (lower PSB, SD and AP), longer setae (AM, AL, H, D_{min} and D_{max}) and shorter legs (Ip). These variables are significantly different across the two species ($p < 0.05$, the Mann-Whitney test). Descriptive statistics for these variables are presented in Table 2. The small sample sizes for this species pair do not permit the construction of a reliable diagnostic function.

Neotrombicula anax differs from *N. scrupulosa* in having greater values for most measurements, including variables characterising the size of the scutum, lengths of setae and lengths of legs. Moreover, *N. anax* has six setae on the first row of D, whereas *N. scrupulosa* usually has more. Values of three measurements (lengths of setae) for *N. anax* lie outside the ranges for *N. scrupulosa*, i.e. AL = 45–46 *vs* 32–44 µm, PL = 55–56 *vs* 36–54 µm and D_{max} = 54 *vs* 36–50 µm. However, it should be noted that these values for *N. anax* are means, and in reality the ranges for these two species may overlap. Thus, potential differences between these two species outlined above require further investigation.

In most cases, *N. lubrica* can be discriminated from *N. scrupulosa* by the presence of two setae in the fourth row of D versus four in *N. scrupulosa*. However, this character may vary. For example, in 54 specimens of *N. lubrica*, five specimens had four setae and seven had three setae. In 244 measured specimens of *N. scrupulosa*, two had two setae and 12 had three setae. Because this single character cannot readily distinguish between these two species, and the fact that *N. scrupulosa* also cannot be distinguished from *N. minuta* based on univariate characters,

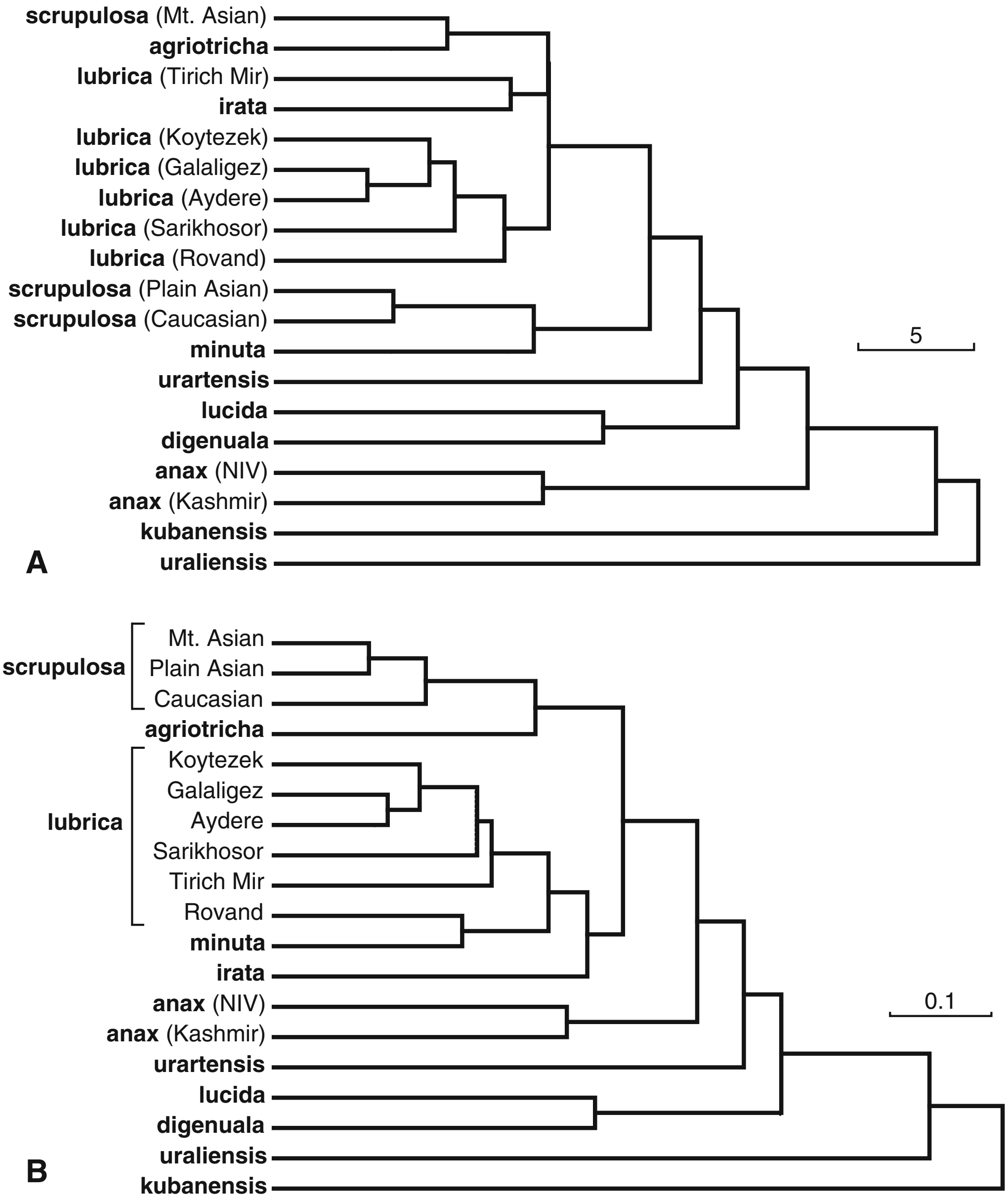

Fig. 5 Dendrograms of the species, forms and some individual samples from the *minuta* group (Euclidean distances, UPGMA); A, by 17 raw variables; B, by 17 size-corrected variables. Scale-bars are given for linkage distances

methods of multivariate discrimination should be employed for this problem (see below).

Discriminating between *N. scrupulosa*, *N. lubrica* and *N. minuta*

An analysis including all 17 variables resulted in 98.1, 94.2 and 94.3% classification accuracies as estimated by internal validation (training sample), external validation (holdout sample) and jackknife statistics, respectively. Misclassification may be due to incorrect original grouping or the presence of variables that do not contribute substantially in intergroup differences (Huberty, 1994). In the latter case, by forming a function of only a few predictors, and omitting redundant variables or variables that

Table 2 Univariate differences between *Neotrombicula lubrica* and *N. irata* (range, mean ± SE)

	N. irata (N = 6)	*N. lubrica* (N = 54)	p (Mann-Whitney test)
PSB	25–28, 26.3 ± 0.49	25–32, 28.7 ± 0.25	0.004
SD	52–56, 54.0 ± 0.68	52–63, 57.0 ± 0.38	0.014
AP	25–29, 26.8 ± 0.54	26–34, 28.9 ± 0.24	0.008
AM	34–44, 37.2 ± 1.42	29–40, 33.1 ± 0.27	0.0008
AL	41–44, 41.8 ± 0.48	33–43, 38.5 ± 0.32	0.0014
H	47–57, 52 ± 1.41	42–55, 48.3 ± 0.37	0.014
D_{min}	32–40, 36.8 ± 1.17	28–40, 33.6 ± 0.32	0.013
D_{max}	46–50, 47.5 ± 0.67	38–52, 44.2 ± 0.37	0.003
Ip	850–909, 885.2 ± 10.57	828–984, 919.2 ± 4.24	0.015
dmt	10–18, 13.6 ± 1.12	12–19, 15.8 ± 0.27	0.041

introduce ‘noise’ into the model, a discriminant function can be formed that maximises the separation of the groups.

Best subset analysis was used (see ‘Materials and methods’) to select a small set of highly informative variables. A total of 131,071 (2^17-1) subsets was generated by this analysis. Based on predictive power as evaluated by the three measures and the senior author’s expert knowledge of the variables, we selected a subset of five variables (AP, D_{max}, TaIII, dmt and D_{1-4}). This subset included taxonomically meaningful variables and combined high total classification accuracies in internal validation, external validation and jackknife statistics (96.2, 96.2 and 95.5%, respectively) and an acceptable rate of correct identifications for the smallest group (*N. minuta*).

The procedure of variable removal did not affect classification accuracy. The predictive power of the simpler 5-variable model even improved slightly, as compared to the 17-variable model (see above), suggesting that variable selection not only removed redundancy but also reduced ‘noise’ in the model. We present the 5-variable model, as the classification rule for the three species. It is described in more detail below.

Two highly significant ($p < 0.05$) discriminant functions (DF1–2), accounting for 90.6% and 9.4%, respectively, of the total variance, were produced by the 5-variable analysis. DF1 and DF2 display canonical correlations of 0.883 and 0.518, respectively, i.e. 78.0 and 26.8% of the variance in the dependent variable (species) can be accounted for by DF1 and DF2, respectively. Box’s M-test indicates that the assumption regarding the Canonical Variates Analysis in relation to the equality of the covariance matrices is not met ($p < 0.001$). However, many researchers (e.g. Hair et al., 1998) believe that discriminant analysis can be robust even when this assumption is violated. Discriminant loadings (Table 3) indicate that variable TaIII serves primarily to separate *N. scrupulosa* from *N. lubrica* + *N. minuta*, whereas the remaining four variables (D_{1-4}, AP, dmt and D_{max}) discriminate between *N. lubrica* and *N. minuta* (Fig. 6). Classification statistics (Table 4) exhibit rather low percentages of misclassified specimens by the analysis itself, leave-one-out and external validation. We estimate that the overall classification accuracy of this model may be as high as 96%; however, it can be as low as 80% when applied to *N. minuta* only (which has a small sample size) (Table 4).

Our data show a significant departure ($p < 0.001$) from multivariate normality as estimated by two tests. More specifically, generalised Shapiro-Wilk’s test gave a value for the multivariate W test statistic (MVW) of 0.937 ($p < 0.001$), and the Mardia’s test yielded insignificant ($p < 0.001$) third (257.283) and fourth (5.682) moments of the multivariate normal distribution. Because the criterion of multivariate normality is not met, unstandardised discriminant coefficients (Table 3) cannot be used to calculate group membership of an unknown specimen directly. Identification of unknown specimens should be based on the calculation of its posterior probabilities and a new discriminant analysis should be run every time for this purpose.

Table 3 Discriminant loadings and unstandardised canonical discriminant function coefficients

Variables	Discriminant loadings		Unstandardised coefficients	
	DF1	DF2	DF1	DF2
TaIII	0.894*	0.036	21.872	8.723
$D_{1–4}$	−0.393	0.682*	33.569	37.37
AP	0.186	0.639*	40.184	34.05
dmt	−0.226	−0.564*	65.265	36.198
D_{max}	0.015	−0.356*	22.728	9.347
(Constant)			−173.737	−108.842

Notes. Variables are ordered by absolute size of correlation within function. Discriminant loadings are pooled within-groups correlations between discriminating variables and standardised canonical discriminant functions. An asterisk indicates the greatest absolute correlation between each variable and any discriminant function

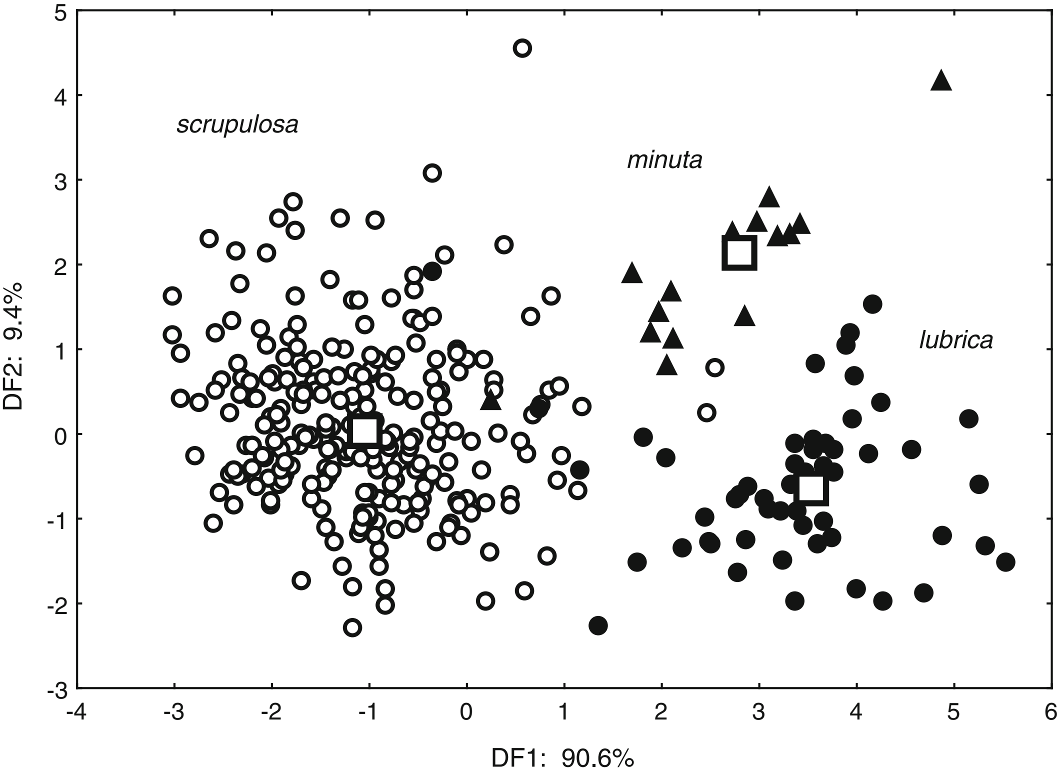

Fig. 6 Canonical discriminant analysis of *Neotrombicula scrupulosa* (open circles), *N. lubrica* (solid circles) and *N. minuta* (triangles). Group centroids are marked by the squares

The use of the 5-variable classification model

Below we provide a simple example how to use our 5-variable classification model:

1. Mount the unknown specimen as described in the ‘Materials and methods’.
2. Measure variables AP, D_{max}, $D_{1–4}$, TaIII and dmt. For metric variables, record the values in micrometres. For example, these values for one specimen, which we putatively identified as *N. scrupulosa*, are: 29, 43, 22, 85 and 15 µm, respectively.
3. Download our dataset (Online Resource 2) and input values from Step 2 in the appropriate columns (C:G) below the last record. The dependent variable may be any (according to our

Table 4 Classification accuracy of the 5-variable model as estimated by three methods

Validation	Observed	Predicted			Total (100%)
		scrupulosa	*lubrica*	*minuta*	
Internal	*scrupulosa*	117 (97.5%)	0	3 (2.5%)	120
(training sample)	*lubrica*	1 (3.7%)	24 (88.9%)	2 (7.4%)	27
	minuta	0	0	10 (100%)	10
Leave-one-out	*scrupulosa*	116 (96.7%)	1 (0.8%)	3 (2.5%)	120
(training sample)	*lubrica*	1 (3.7%)	24 (88.9%)	2 (7.4%)	27
	minuta	0	0	10 (100%)	10
External	*scrupulosa*	122 (98.4%)	1 (0.8%)	1 (0.8%)	124
(holdout sample)	*lubrica*	2 (7.4%)	24 (88.9%)	1 (3.7%)	27
	minuta	1 (20%)	0	4 (80%)	5

tentative identification we entered 1, for *N. scrupulosa*). The variable Validation (val3) should be set in such a way that it assigns the specimen to the holdout sample (0 in our dataset).

4. Once raw variables are entered, the Excel file will calculate Darroch and Mosimann shape variables automatically. For the data from step 1 these values are: 0.90, 1.33, 0.68, 2.63 and 0.46, respectively. (Calculations for the first variable: DM = 29/GM = 0.90, where GM (Geometric mean) = 32.27 (29*43*22*85*15)^(1/5)).
5. Repeat Steps 1–3 as many times as reasonable (we recommend measuring at least three specimens). Keep in mind that the overall classification accuracy of our model is about 96%, but it can be as low as 80% for *N. minuta*.
6. Run a Discriminant Analysis (Canonical Variates Analysis) in a statistical package of your choice. The dependent variable is Species_num (with the range 1–3), the independent variables are AP_DM, Dmax_DM, D1_4_DM, TaIII_DM and dmt_DM. The selection variable is val3, where value 0 = holdout sample and value 1 = analysis sample (should be selected). Request case-wise statistics.
7. Locate the unknown specimen in the case-wise statistics table. In our case it is specimen 320 at the end of the table. The analyses predicted it to be *lubrica* (2) not *scrupulosa* (1), with the probability of 0.990, thus invalidating our putative identification. If the probability of group membership is low, then more specimens are recommended to be measured and entered into the analysis. The same is recommended when multiple specimens from the same sample are classified as different species. A failure to achieve the estimated level of accuracy (80%) may be due to a species other than *scrupulosa*, *lubrica* or *minuta* (for example *N. anax*), a mixed sample, mistakes in measuring or failure of the model itself.

Key to the species of the *minuta*-group (larvae)

1 fPp = B/B/NBB 2

– fPp = B/B/NNB 4

2 Anterior scutal margin convex; AM slightly anterior to or at level of ALs; NDV = 48–51 . . . . *digenuala*

– Anterior scutal margin sinuous; AM posterior to ALs; NDV > 55 3

3 More than six setae in first two rows of D; NDV = 90–93. . . . . *ovalis*

– Six setae in first two rows of D; NDV = 59–67 . . . . *agriotricha*

4 AM reaches posterior scutal margin; AM = 45–49 μm . . . .*kubanensis*

– AM does not reach posterior scutal margin; AM = 25–44 μm 5

5 Two setae in fourth row of D 6

– More than two setae in fourth row of D 7

6 NDV = 44–50; ASB = 32–35 µm; SB posterior to or at the level of PLs; galeala sometimes with one barb . . . . *lucida*

– NDV = 51–67; ASB = 25–31 µm; SB anterior to or at level of PLs; galeala nude . . . . *lubrica* (part) + *irata* (see Table 2)

7 Ip = 933–1053 µm; TaIII = 90–104 µm; NDV = 71–88 . . . . *uraliensis*

– Ip = 736–984 µm; TaIII = 63–95 µm; NDV = 51–80 8

8 VC ≥ 45 . . . . *urartensis*

– VC ≤ 44 9

9 DF1 and DF2 calculated from D_{max}, TaIII, dmt and $D_{1–4}$ fall within *N. scrupulosa* (see the use of the 5-variable classification model above) 10

– DF1 and DF2 calculated from D_{max}, TaIII, dmt and $D_{1–4}$ fall within *N. lubrica* or *N. minuta* (see the use of the 5-variable classification model) 11

10 AL = 32–44 µm; PL = 36–54 µm; D_{max} = 36–50 µm; usually 7–11 setae in first row of D (6 setae in 11.5% of all measured specimens) . . . . *scrupulosa*

– AL > 44 µm; PL > 54 µm; D_{max} > 50 µm; six setae in first row of D . . . . *anax*

11 Identified as *N. lubrica* by the 5-variable classification model; *c.*8 setae in anterior row of V . . . . *lubrica* (part)

– Identified as *N. minuta* by the 5-variable classification model; *c.*12 setae in anterior row of V . . . . *minuta*

Systematics

Neotrombicula agriotricha Stekolnikov, 1994

Neotrombicula agriotricha Stekolnikov, 1994: p. 198, figs. 3, 4 [Turkmenistan; holotype and paratypes in ZIN].

Material examined: 11 larvae (holotype and 10 paratypes), Turkmenistan, Balkan Welayat, Aydere, Oct. 1982, ex *Apodemus sylvaticus* (L.), E.V. Drugova.

Neotrombicula anax Audy & Womersley, 1957

Neotrombicula anax Audy & Womersley, 1957: p. 363, figs. 6, 7 [India, Kashmir; holotype in SAM, paratypes in SAM, BMNH, USNM, and other depositories]; Fernandes & Kulkarni, 2003: p. 269, fig. 87.

Distribution: India: Jammu and Kashmir, Himachal Pradesh, Uttaranchal (Fernandes & Kulkarni, 2003); China: Xizang (Tibet) (Wen, 1984).

Morphometric data: 1. Mean of holotype and 11 paratypes (Audy & Womersley, 1957). 2. Mean of 10 specimens deposited in NIV (Fernandes & Kulkarni, 2003).

Neotrombicula balcanica Kolebinova, 1973

Neotrombicula balcanica Kolebinova, 1973: p. 695, figs. 1–6 [Bulgaria; holotype and paratypes in ZISB]; 1992: p. 92. fig. 46.

Remarks: Based on the original description, this species is almost identical to *N. ovalis*. The differences in the scutal size (PW and SB) and lengths of scutal setae (AM, AL and PL) can be interpreted as geographical variation (Table 5). The differences in the leg lengths may result from the fact that Kolebinova probably measured legs without coxae (Stekolnikov, 2001). Thus, *N. balcanica* is a probable synonym of *N. ovalis*.

Neotrombicula corvi Kolebinova, 1971

Neotrombicula corvi Kolebinova, 1971: p. 788, fig. 2 [Bulgaria; holotype in ZISB]; 1992: p. 94, fig. 47; Stekolnikov, 2008: p. 24.

Remark: This species, known from a single specimen, is probably conspecific with *N. scrupulosa* (see Stekolnikov, 2008).

Table 5 Ranges of 9 potentially diagnostic variables for *Neotrombicula balcanica* and *N. ovalis*

	N. balcanica (n = 6)	*N. ovalis* (n = 2)		*N. balcanica* (n = 6)	*N. ovalis* (n = 2)
PW	88–91	89, 99	pa	263–290	335, –
SB	28–30	31, 34	pm	245–252	281, 288
AM	38–50	33, –	pp	278–283	322, 337
AL	38–47	34, 35	Ip	786–823	960, –
PL	50–58	47, –			

Note. For *N. balcanica* measurements are given after Kolebinova (1992). For *N. ovalis* two syntype specimens were measured

Neotrombicula digenuala Schluger, 1967

Neotrombicula digenuala Schluger, 1967: p. 45, fig. 4 [Ukraine, Odessa Oblast; syntypes in ZMUM]; Kudryashova, 1998: p. 193, fig. 151.

Distribution: Ukraine: Odessa Oblast, Kherson Oblast.

Material examined: Two syntypes (no. 2484), Ukraine, Odessa Oblast (label 'Izmail'skaya obl., Kantemir'), E.G. Schluger. No other data.

Neotrombicula irata Kudryashova, 1993

Neotrombicula irata Kudryashova, 1993: p. 6, fig. 2 [Kyrgyzstan, Osh Province; holotype and paratypes in ZMUM]; Stekolnikov, 1995: p. 264; Kudryashova, 1998: p. 194, fig. 152.

Material examined: Holotype and 5 paratypes, Kyrgyzstan, Osh Province, Uzgen District, Kara-Shoro, 20–21.ix.1974, ex *Apodemus sylvaticus* and *Cricetulus migratorius* (Pallas), S.N. Rybin.

Neotrombicula kubanensis Stekolnikov, 2008

Neotrombicula kubanensis Stekolnikov, 2008: p. 24, figs. 10–18 [Russia, Krasnodarskiy Kray; holotype and paratypes in ZIN].

Material examined: Holotype and 6 paratypes, Russia, Krasnodarskiy Kray.

Neotrombicula lubrica Kudryashova, 1993

Neotrombicula lubrica Kudryashova, 1993: p. 4, fig. 1 [Tajikistan; holotype and paratypes in ZMUM]; Stekolnikov, 1995: p. 263; Kudryashova, 1998: p. 195, fig. 153; Daniel & Stekolnikov, 2005: p. 284. *N. dimidiata* Stekolnikov, 1995: p. 258, figs. 6, 7 (syn. nov.) [Turkmenistan and Tajikistan; holotype and paratypes in ZIN].

Distribution: Tajikistan and Pakistan (Daniel & Stekolnikov, 2005), Turkmenistan (Stekolnikov, 1995).

Material examined: Type-material (depository and numbers of examined specimens are in parentheses): holotype and 8 paratypes of *N. lubrica* (ZMUM, 9), Tajikistan, Kuhistoni Badakhshon, 137 km E from Khorugh (probably near the Koytezek pass); holotype and 64 paratypes of *N. dimidiata* (ZIN, 65), Turkmenistan, Balkan Welayat, Western Kopetdagh, Syunt Mt., NE from Kara-Kala, Galaligez gorge; near Kara-Kala, Parkhay gorge; Aydere.

Additional material: Tajikistan, Kuhistoni Badakhshon, Vanj District, Vishkharvak (near Rovand) (ZIN, 5); Vahdat (Kofarnihon, Ordzhonikidzeabad) District, Sarikhosor (ZMUM, 15); Pakistan, East Hindukush, Tirich Mir Mt. (ZIN, 11).

Neotrombicula lucida Kudryashova, 1993

Neotrombicula lucida Kudryashova, 1993: p. 11, fig. 3 [Kyrgyzstan, Osh Province; holotype and paratypes in ZMUM]; Stekolnikov, 1995: p. 264; Kudryashova, 1998: p. 196, fig. 154.

Distribution: Kyrgyzstan, Tajikistan.

Material examined: Type-material*:* 1 paratype, no. K-196-1409, Kyrgyzstan: 7 km SW from Osh city, 11.x.1967, ex *Meriones lybicus* Lichtenstein, S.N. Rybin.

Additional material: Three larvae labelled as '*Neotrombicula minuta*' (ZMUM, nos 5220, 5222, 5226), Tajikistan, Khatlon Province, Danghara District, Oksu (Ak-Su), 29.xi.1965 and 1.xii.1965, ex *Blanfordimys afghanus* (Thomas), coll. G.S. Davidov, det. A.A. Stekolnikov.

Neotrombicula minuta Schluger, 1966

Neotrombicula minuta Schluger, 1966: p. 208 [Ukraine, Odessa Oblast; lectotype and paralectotypes in ZMUM]; Stekolnikov, 1995: p. 251, fig. 1; Kudryashova, 1998: p. 192, fig. 150 [lectotype designated].

Distribution: Ukraine: Odessa Oblast, Kherson Oblast.

Material examined: Lectotype and 14 paralectotypes, Ukraine, Odessa Oblast (labelled 'Izmail'skaya obl., Kantemir'), E.G. Schluger.

Neotrombicula ovalis Schluger & Davidov, 1967

Neotrombicula ovalis Schluger & Davidov, 1967: p. 24, fig. 8 [Tajikistan; syntypes in ZMUM]; Kudryashova, 1998: p. 197, fig. 155.

Material examined: Two syntypes (nos 2200 and 2201), Tajikistan (northern part), 28.ii.1960, ex *Apodemus sylvaticus*, coll. G.S. Davidov, det. E.G. Schluger. Both slides bear the inscription 'type'.

Neotrombicula scrupulosa Kudryashova, 1993

Neotrombicula scrupulosa Kudryashova, 1993: p. 21, fig. 8 [Mongolia; holotype and paratypes in ZMUM]; Stekolnikov, 1995: p. 252, fig. 2; Kudryashova, 1998: p. 197, fig. 156.

Distribution: Previously recorded in Mongolia, Kazakhstan (Aqmola Province, Almaty and East Kazakhstan), Russia (Adygeya, Dagestan, Bashkortostan, Altaiskiy Kray, Altai Republic and Tyva

Republic) (Stekolnikov, 1995). Recorded in Turkey, Armenia, Kyrgyzstan, Krasnodarskiy Kray, Karachai-Cherkessia, Kabardino-Balkaria and North Ossetia for the first time.

Material examined (Depository and numbers of examined specimens in parentheses; detailed data are given for new records only):

Plain Asian form (ZMUM, 48): holotype and 2 paratypes, Mongolia, Dundgovi Aimag, 20 km N from Bayanjargalan (3); Kazakhstan, Aqmola Province, Ishim River (4); Aqmola Province, Imantau Lake (7); ? Aqmola Province, Algabas (labelled 'Angabass') (4); Russia, Bashkortostan, Ufa (15); Altaiskiy Kray, Togul'skiy District, Uksunay (4); ? Kytmanovskiy District, Novotaraba (labelled 'Tarabarskiy') (3); Krasnogorskiy District, Eronda (Yeronda) (2); Krasnogorskiy District, Novaya Barda (4); ? 'Babiy Log' (2).

Mountain Asian form (114): Kazakhstan (ZMUM), Almaty (9); East Kazakhstan, Saur Mts. (8); Kyrgyzstan (ZIN), Naryn Province, Ak-Talaa District, Orto-Syrt (18), 21.vii.1990, ex *Apodemus sylvaticus* (L) and *Alticola roylei* (Gray), A.V. Bochkov; Kochkor District, Semis-Bel' (13), 25.vi.1990, ex *Lepus tolai* Pallas, A.V. Bochkov; Chuy Province, S from Bishkek, Kirgiz Range, Alamedin gorge (3), 2,000–2,400 m, 13.ix.1998, ex *A. sylvaticus* (including specimen deposited in ZIN, no. 83612), AAS; Russia, Altai Republic (ZMUM), Kosh-Agachskiy District (15); Tyva Republic, Ulug-Khemskiy District, Aldyy-Shynaa (ZMUM, 15); Mongun-Tayginskiy District, Barlyk (ZIN, 33).

Caucasian form (ZIN, 219): Turkey, Rize Province, Marsis Mt. (Gül Dagi Mts.) (24), 2,400 and 1,800 m, 24 and 26.vi.1998, ex *Microtus majori* Thomas and *Apodemus fulvipectus* (Ognev) (including two specimens deposited in ZIN, nos 83473 and 83478), AAS; Armenia, Tavush Marz, Haghartsin (Kuybyshev) (6), 3.viii.1956, ex *Coturnix coturnix* L., K.S. Akhumyan; 'Erivanskaya oblast, Sardyr-Bulak' (3), 1903, ex *Mesocricetus auratus* (Waterhouse), K.A. Satunin, mites collected 25.iv.1997, AAS; Russia, Adygeya, Guzeripl (22); Krasnodarskiy Kray, Chugush Mt., 2,400 m, upper reaches of Berezovaya River (2), 25.vii.1994, ex *Apodemus (Sylvaemus)* sp., AAS; Kamyshanov (at Laganaki Massif) (1), 1,273 m, 26.viii.1994, ex *A. (S.)* sp., AAS; Karachai-Cherkessia, Teberda (7), 1,370 and 2,000 m, 5 and 9.vii.1997, ex *A. uralensis* (Pallas), AAS; Baduk River (45), 2,110 m, 12.vii.1997, ex *A. uralensis*, *Chionomys gud* (Satunin) and *Microtus daghestanicus* Shidlovsky, AAS; Pkhiya (1), 1,770 m, 9.viii.1995, ex *A. uralensis*, AAS; Kabardino-Balkaria, El'brus (1), 1,900 m, 29.vi.1996, ex *A. uralensis*, AAS; Verkhniy Chegem (4), 1,700 m, 18.vi.1996, ex *A. uralensis*, AAS; Bezengi gorge (38), 1,500 and 1,750 m, 13 and 16.vi.1996, ex *A. uralensis*, *A. agrarius* (Pallas), and *M. majori*, AAS; North Ossetia, Buron (7), 25.vi.1976 and 13.vii.1976, ex *A. sylvaticus*, S.N. Rybin; Dagestan, Tlyaratinskiy District, Mazada (7); Untsukul'skiy District, Ashil'ta (7); Akhtynskiy District, Kurush (42), Khnov (1); Botlikhskiy District, Tlokh (1).

Neotrombicula uraliensis Kudryashova, 1993

Neotrombicula uraliensis Kudryashova, 1993: p. 11, fig. 4 [Kazakhstan, Atyrau Province; holotype and paratypes in ZMUM]; Stekolnikov, 1995: p. 264; Kudryashova, 1998: p. 198, fig. 157.

Material examined: Three paratypes (nos 51, 296 and 704), Kazakhstan, Atyrau Province (labelled 'Guryevskaya obl.'), 40 km NE from Makhambet, Oct. 1968, ex *Rhombomys opimus* (Lichtenstein), N.I. Kudryashova.

Neotrombicula urartensis Stekolnikov, 2008

Neotrombicula urartensis Stekolnikov, 2008: p. 22, figs. 1–9 [Armenia; holotype and paratypes in ZIN]. *N. corvi* (nec Kolebinova, 1971): Stekolnikov, 1995: p. 262 (misidentification).

Material examined: Armenia: holotype, 7 paratypes, and 9 additional specimens (6 from Amasia, 1 from Sarnakhpyur and 2 from Haghartsin).

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