**H Rabebiella Periglandulata** (Annelida: “Polychaeta”) – Do Apparent Differences in Chaetal Ultrasound Structure Indicate the Existence of Several Species in Europe?

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**H Rabebiella Periglandulata** (Annelida: “Polychaeta”), one of only two known exclusively terrestrial non-clitellate annelids (“polychaetes”), has been found at an increasing number of sites in Europe and has also recently been reported from Korea. Until recently it was known only from a zone from southern Sweden across central Europe to central Italy. Looking at SEM micrographs, the ultrastructure of the unique chaetae of *H Rabebiella Periglandulata* appeared to differ rather markedly among different populations, which gave rise to the question of whether they all belonged to a single species. To resolve this question, specimens from a total of 17 sites were collected, ranging from Spain in the West to Romania in the East. Specimens from these populations were processed by two different methods and examined using SEM. The results obtained indicate that apparent differences, as published for some Italian and German populations, can be caused by even small differences in the fixation procedure preceding the SEM examination. Such causes might lead to false conclusions when judging morphological differences visible by SEM within taxonomical studies. We conclude that chaetal morphology and ultrastructure point at the existence of only one species of *H Rabebiella* that is widely distributed. We present 19 previously unpublished localities for the species (not fully overlapping with the 17 collection sites used in our SEM examination), including the first records for four countries: Spain, Denmark, Hungary, and Romania.

Keywords: *H Rabebiella Periglandulata*, Polychaeta, structure of chaetae, SEM investigation

**Introduction**

*H Rabebiella Periglandulata* Pižl et Chalupský, 1984, is one of only two truly and exclusively terrestrial non-clitellate annelids, the other being *Parergodrilus heideri* Reisinger, 1925. Both species are found in soils, the former preferring the upper mineral layer of slightly acidic forest and meadow soils, the latter the litter layer of moderately acidic forest soils (see Graefe & Schmelz 1999, Graefe & Beylich 2003, and authors cited below with regard to distribution data). The systematic position of *H. Periglandulata* has been an enigma and has
not yet been fully resolved, despite numerous investigations touching this
matter (e.g. Struck & Purschke 2005, Struck et al. 2008, Rousset et al. 2007,
Zrzavý et al. 2009). The interest in both species has been also driven by the
fact that many questions regarding annelid and clitellate phylogeny remain
open. They resemble terrestrial clitellates to some extent, but lack several of
their characters, foremost a clitellum, making them interesting models for
considerations of convergent adaptations to life in soil (Purschke 1999), but
also potential basal members of a hypothetical lineage within the paraphyletic
“Polychaeta” that may lead towards Clitellata (Purschke et al. 2000, Purschke
2003, Jördens et al. 2004). Within these studies it was convincingly shown that
H. periglandulata and P. heideri were not closely related (Rota 1998, Rota et al.
2001, Jördens et al. 2004). Based on an ultrastructural examination, Purschke
(2003) suggested that H. periglandulata might be the sister group of the Cli-
tellata, but the molecular study of Jördens et al. (2004) did not provide any
definitive support for this hypothesis. Nevertheless, Christoffersen (2012)
included H. periglandulata as the sister group of Euclitellata within Clitellata
sensu lato. Rousset et al. (2007) questioned the notion of a close relationship
of H. periglandulata to Clitellata or Aphanoneura, whereas Zrzavý et al. (2009)
confirmed the notion of a taxon (Clitellatomorpha) including H. periglandulata
and Aphanoneura as one sister group and the true Clitellata as the other.

Pižl and Chalupský (1984) described H. periglandulata from South Bo-
heemia, i.e. the south-west of the Czech Republic (= Czechia). Until recently
it was only known from Europe, ranging from southern Sweden (Erseüs &
Rota 1998) in the North to central Italy in the South (Rota & Lupetti 1996,
1997, Ascher et al. 2012) and from Germany in the West (Jans & Römbe 1989,
Austria (Bauer 2003, 2004) to south-eastern Poland (Dumnicka & Rožen 2002)
Recently, the species was also found in Korea (Dózsa-Farkas & Hong 2010),
indicating a much wider distribution than had been assumed before. Investi-
gations under the light microscope did not show any conspicuous morpho-
logical differences between populations, the chaetae resembling a trident
with the three prongs interconnected by a thinner lamina into a small shovel-
or ice-spoon-shaped blade. However, investigations of external morphology
by scanning electronic microscope (SEM) revealed striking differences in the
ultrastructure of the chaetae. Jans and Römbe (1989) presented some SEM
photographs with a brief description of chaetal morphology. They found the
ectal blades of the chaetae to be curved, sail-shaped, and scaly, with three
apical tips (according to Jans & Römbe 1989, Rota & Lupetti 1996, call this
shape “flame-like”) and a joint-like base. Rota and Lupetti (1996), in contrast,
found chaetae resembling a handled brush: a “shaft, which is devoid of a nod-
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Ulus, supports distally a flattened, quadrangular cushion, which gives off on one side numerous, densely packed ‘bristles’. Thus it seemed questionable whether all of the investigated populations did indeed belong to a single species or if there were at least two distinct species within Europe. Already Rota and Lupetti (1996) suggested that a reinvestigation of topotypes of H. periglandulata was “needed to clarify the extent of variation of chaetal morphology and to establish whether the Italian or the German populations might be recognized as a separate species rather than a variant of the Bohemian ones”. Purschke (1999) investigated specimens from northern Germany and found chaetae similar to the Italian specimens under SEM. However, he suggested that the apparent differences between chaetae of different populations could be the effect of different handling of the specimens prior to their examination by SEM (Purschke 1999).

In the present study, we try to answer the question of the species-identity of European Hrabeiella populations, based on the investigation of the chaetal ultrastructure (as shown by SEM) of specimens from several European populations (including the type locality), using various fixation procedures. We also present previously unpublished localities for the species from seven European countries, including first records for the fauna of four countries, thus substantially expanding the known range of Hrabeiella within Europe. Our study was triggered by the first finds of Hrabeiella periglandulata in Hungary, which we investigated under the light microscope as well as by SEM, based on our initial examinations the latter showed a chaetal ultrastructure resembling that described by Jans and Römbke (1989), but as shown below this case was not that straightforward.

MATERIALS AND METHODS

Hrabeiella specimens collected in Hungary (in the Kőszeg and in the Bakony Mts), and Romania (Gutin Mts) were extracted from soil samples by the wet funnel method (O’Connor 1962). They were kept in water, examined and measured alive under the light microscope. The important morphological structures were noted, drawn and photographed with a Zeiss Axio Imager.A2 microscope, using DIC (differential interference contrast) illumination and an AxioCam MRc 5 (Zeiss) digital camera with Axiovision software. Subsequently some specimens were preserved in 70% ethanol and some were permanently mounted in Euparal on a microscope slide. Twenty-one specimens were then prepared for scanning electron microscopy by dehydration through an ethanol series (96%, absolute), critical-point dried (CO2), mounted on stubs, and sputter-coated with gold-palladium. Scanning electron micrographs were taken in the Hungarian Natural History Museum, Budapest, with a Hitachi SN 2600 scanning electron microscope.

To obtain a more general overview of the variability of chaetal morphology in Hrabeiella, additional specimens from populations across Europe (ranging from north-western Spain to north-western Romania) were collected by the authors or obtained from fellow...
Table 1. Collecting sites of specimens used for the SEM investigation and other, hitherto unpublished, localities where Hrabeiella periglandulata was recorded. Set in bold are countries for which the listed records are the first published ones, geographic coordinates of hitherto unpublished localities, and collectors of material for those sites from which specimens were used for our SEM investigation.

<table>
<thead>
<tr>
<th>Country</th>
<th>Locality (situation and habitat)</th>
<th>Collector and collecting date</th>
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<tbody>
<tr>
<td>Spain</td>
<td>Galicia, Prov. Coruña, Sobrado dos Monxes, 43°02'12&quot;N, 08°00'33&quot;W, ca 530 m a.s.l.; pasture near a lake</td>
<td>R. M. Schmelz; April 1994</td>
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<tr>
<td>Spain</td>
<td>Galicia, Prov. Coruña, west of Cesuras, near River Mendo: 43°10'36&quot;N, 08°10'27&quot;W, ca 214 m a.s.l.</td>
<td>R. M. Schmelz; Oct. 2007</td>
</tr>
<tr>
<td>Denmark</td>
<td>Eastern Jutland, Hestehaven Forest near Aarhus (former IBP study area), ca 56°17'14&quot;N, 10°28'24&quot;E, ca 15 m a.s.l.; beech forest</td>
<td>R. M. Schmelz; Nov. 1998</td>
</tr>
<tr>
<td>Germany</td>
<td>Waldpark Falkenstein near Hamburg; 53°33'54&quot;N, 9°45'56&quot;E, ca 20 m a.s.l.; landscape park with mixed forest (deciduous trees and pine)</td>
<td>U. Graefe; 31 July 1972</td>
</tr>
<tr>
<td>Germany</td>
<td>Eifel Mts. near Aachen, Soil Monitoring Site “BDF NRW Lammersdorf Buche”; ca 50°39'57&quot;N, 6°14'57&quot;E, ca 450 m a.s.l., deciduous forest (beech, oak) with interspersed spruce</td>
<td>U. Graefe; 2009 (first record from 22 Oct. 2004)</td>
</tr>
<tr>
<td>Germany</td>
<td>Lehndorf-Wattenbüttel near Braunschweig (land of the Thünen-Institute), 52°17'02&quot;N, 10°26'36&quot;E, 85 m a.s.l.; oak-beech wood</td>
<td>J. Schlaghamerský; 16 July 2010</td>
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<td>Austria</td>
<td>Salzburg state, permanent soil monitoring plot (Bodendauerbeobachtungsfläche 2) near Sankt Koloman, ca 47°39'N, 13°11' E, 1005 m a.s.l.; grassland</td>
<td>R. Bauer; 12 Sept. 2002</td>
</tr>
<tr>
<td>Austria</td>
<td>Eastern Austria, Rax Mountains, 47°43'01&quot;N, 15°46'32&quot;E, 1563 m a.s.l.; spruce forest</td>
<td>J. Farkas; 15 May 2012</td>
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<tr>
<td>Czechia</td>
<td>NW Bohemia, Bohemian Switzerland Nat. Park, alluvium of the stream Brtnický potok; 50°56'01&quot;N, 14°24'19&quot;E, 313 m a.s.l.; open maple-ash stand on sandy alluvial deposits</td>
<td>J. Schlaghamerský; 3 Oct. 2009 &amp; 22 June 2010</td>
</tr>
<tr>
<td>Czechia</td>
<td>NW Bohemia, Bohemian Switzerland Nat. Park, Häsenschgrund ravine; 50°52'36&quot;N, 14°22'21&quot;E, 324 m a.s.l.; sandy alluvial deposits along a small, intermittent brook in a spruce forest</td>
<td>J. Schlaghamerský; 3 Oct. 2009</td>
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<tr>
<td>Country</td>
<td>Locality (situation and habitat)</td>
<td>Collector and collecting date</td>
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<tr>
<td>Czechia</td>
<td>S-Moravia, Brno municipal forest: Holendá Game Park at Jundrov; 49°12'17&quot;N, 16°32'38&quot;E, 300 m a.s.l.; mixed forest (pine, oak, beech)</td>
<td>J. Schlaghamerský; April 2010</td>
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<tr>
<td>Czechia</td>
<td>Locus typicus: South Bohemia, meadow on Vencová hora between the villages Kmin, Dolní Třeboň and Zlatá Koruna; 48°51'26&quot;N, 14°23'37&quot;E, 550 m a.s.l.; meadow at forest edge</td>
<td>J. Schlaghamerský; 27 April 2010</td>
</tr>
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<td>Hungary</td>
<td>Western Hungary, Kőszeg Mts. (foothills of the Alps), near Velem; 47°22'00&quot;N, 16°29'00&quot;E, ca 600 m a.s.l.; pine forest</td>
<td>K. Dózsa-Farkas; 24 April 1985</td>
</tr>
<tr>
<td>Hungary</td>
<td>Western Hungary, Kőszeg Mts. (foothills of the Alps), near Velem; 47°21'07&quot;N, 16°29'09&quot;E, 430 m a.s.l.; mixed forest (hornbeam-oak-beech-pine)</td>
<td>K. Dózsa-Farkas; 6 March 2009</td>
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<tr>
<td>Hungary</td>
<td>Western Hungary, Kőszeg Mts., Steier Houses, 47°21'10&quot;N, 16°28'30&quot;E, 563 m a.s.l.; mixed forest (pine and deciduous trees)</td>
<td>G. Szövényi; 22 Jan. 2011</td>
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<td>Hungary</td>
<td>Bakony Mts., near Nyirád; 47°01'21&quot;N, 17°23'34&quot;E, 209 m a.s.l.; oak forest (Turkey oak with some hornbeam, with <em>Pteridium aquilinum</em> in undergrowth)</td>
<td>K. Dózsa-Farkas &amp; J. Farkas; 8 May 2008</td>
</tr>
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<td>Hungary</td>
<td>Bakony Mts., Hárskút, Esztergáli-Valley, 47°10'58&quot;N, 17°48'49&quot;E, 490 m a.s.l.; meadow</td>
<td>K. Dózsa-Farkas &amp; J. Farkas; 8 May 2008</td>
</tr>
<tr>
<td>Hungary</td>
<td>Bakony Mts., Öreg Szarvard Graben; 47°17'13&quot;N, 17°42'54&quot;E, 450 m a.s.l.; deciduous forests</td>
<td>G. Boros &amp; A. Kunszeri; 14 Nov. 2009</td>
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<tr>
<td>Romania</td>
<td>Gutin Mts., (Munții Gutău; Eastern Carpathians), near Mogosa (ski resort) and lake (lacul) Bodí, 47°40'52&quot;N, 23°47'14&quot;E, 698 m a.s.l.; beech forest and a clearing</td>
<td>K. Dózsa-Farkas; 17 Sept. 2005</td>
</tr>
<tr>
<td>Romania</td>
<td>Apuseni Mts., Márișel; 46°40'06&quot;N, 23°08'43&quot;E, 900 m a.s.l.; hornbeam-beech forest</td>
<td>Cs. Csuzdi, J. Kontschán, V.V. Pop &amp; Zs. Ujvári; 24 Oct. 2009</td>
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</table>
researchers from other countries, who either submitted fixed specimens (some already collected many years ago) or soil samples (Table 1). The investigated populations included the locus typicus of *Hrabeiella periglandulata* at Vencova hora (South Bohemia, Czechia). Unfortunately, we did not succeed in obtaining live specimens from Tuscany, Italy, despite many attempts. In those cases in which live specimens were obtained by one of the authors (those listed as such in Table 1 and from the Sax Mountains, Austria), these were also examined under the light microscope. Twenty-two specimens preserved in 96% ethanol or, in a few cases (specimens from the Spanish sites), in Bouin solution or formaldehyde were prepared for the SEM as described above.

To clarify the question if different fixation procedures were responsible for apparent differences in chaetal ultrastructure, further live specimens from the Kőszeg Mountains, Hungary, were collected and eight of those were treated following the procedure as described by Rota and Lupetti (1996), namely: the specimens were fixed in Karnovsky’s fluid for 1 h, washed twice in cacodylate buffer (pH 7.4) and postfixed in a cacodylate-buffered 0.5% osmium tetroxide solution (at 4 °C, for 90 min). For SEM, after rinsing twice in cacodylate buffer, they were dehydrated in ethanol, critical-point dried, mounted on aluminium stubs and sputter-coated with gold. They were examined and photographed with a Hitachi S-2360N scanning electron microscope at the Department of Plant Anatomy of Eötvös University, Budapest.

**RESULTS**

**New recordings**

Below we list (from West to East) 19 so far unpublished records (localities) of *Hrabeiella periglandulata* from Europe (Table 1). These include the first country records for Spain, Denmark, Hungary and Romania. The site from western Austria presented below is based on an unpublished report of Bauer (2004), the records from Hamburg (Amsinckpark and Waldpark Falkenstein) on the unpublished report of Graefe et al. (2003) and on additional information received from U. Graefe (in lit. 2012). European localities with known occurrence of *H. periglandulata* (previously published or presented here) are shown in Fig. 1. One locality from Bohemian Switzerland (Czechia) and the Amsinckpark in Hamburg (Germany) had been previously shown in a distribution map (Schlaghamerský & Šidová 2009), but without locality names or any further details, these are therefore shown with the symbol for “previously published sites” in Fig. 1 but listed as new localities in Table 1.

**Chaetal morphology**

The morphology of specimens under the light microscope was identical with the earlier descriptions, therefore only a few characteristic photos are presented here (Figs 2A–D).
The SEM analysis of Hungarian and the other European specimens dehydrated in an ethanol series and sputter-coated with gold-palladium showed that chaetae of *H. periglandulata* can be found in segments II–XV, distributed mostly in two ventro-lateral and two ventral bundles per segment, each containing 2–3 chaetae (Figs 3A, C, D, 4A). A closer examination reveals that the chaetae were not completely uniform: The posterior chaetae and those of the (medio-) ventral pairs were shovel-shaped (Fig. 4C), the ventro-lateral ones more elongated and pointed (Fig. 3B, 4B). Our photos were compared with the results of JANS and RÖMBKE (1989), ROTA and LUPEITI (1996) and PURSCHKE (1999), and it was observable, that they resembled mostly JANS’ and RÖMBKE’S (1989, p. 161. Fig. 4) results, and did not show the very different “handled brush” (consisting of parallel fibres) described by ROTA and LUPEITI (1996, p.

![Fig. 1. Distribution of *Hrabeiella periglandulata* in Europe based on records previously published (circles) or reported in this study (squares).](image)
232, Fig. 8) and also found by Purschke (1999, p 3, Fig 1F). The examination of the chaetal ultrastructure of specimens from the other European populations included in the study (Table 1) showed the same chaetal morphology in all investigated specimens with only small differences among them (Figs 3B–D, 4A, B). The observed chaetal morphology was very similar to that shown by the SEM photographs of Jans and Römke (1989). This was also true for the specimens from Spain, the most remote and also the furthest south of the populations investigated.

However, the SEM analysis of Hungarian specimens fixed by the method of Rota and Lupetti (1996) showed a chaetal ultrastructure that to a fair extent resembled the one found in Italian specimens by Rota and Lupetti (1996) and in specimens from northern Germany by Purschke (1999). Although not identical, it was apparent that the two flattened laminae bending towards each other were not built up by sheets but in all cases by thin fibres, clinging to each other, that were emerging from flattened quadrangular cushions (Fig. 5A, B). In several cases, their real structure was more clearly visible at the beginning or at the end of the chaetae (Fig. 4D, 5C, D). Also here, we observed that the ventro-lateral chaetae were somewhat more elongate and pointed than the ventral ones.

Fig. 2. Light microscope micrographs of Hrabeiella periglandulata specimens from Hungary (Bakony Mts), in vivo: A = entire specimen (a = anus, h = head), B = the characteristic epidermal glands, dorsal view, C–D: Light microscope micrographs of chaetae (specimens from Gutin Mts, Romania), in vivo: C = the first three rows of chaetae marked with arrows, D = two groups of chaetae.
DISCUSSION

Our study shows that *Hrabeiella periglandulata* has a much wider distribution in Europe than previously reported. The increasing number of known localities indicates that *H. periglandulata* is a rather common inhabitant of grassland and forest soils from lowland to montane-subalpine elevations. We found that *Hrabeiella* specimens from mutually distant populations from the western-most one in north-western Spain to the eastern-most one in north-western Romania have virtually the same ultrastructure of chaetae. We were not able to fully reproduce the brush-like state of the chaetae, with the fibres completely separated, as found by *Rota* and *Lupetti* (1996) and * Purschke* (1999). Even when we followed their fixation method, the fibres of which the chaetal blades were composed were more or less sticking together. Nevertheless, based on the results obtained, we are convinced that the degree to which the fibres are free, showing a brush-like structure, or stick together and be-

Fig. 3. *Hrabeiella periglandulata*, chaetae, SEM micrograph (the worms were fixed in ethanol, the specimen from Spain fixed in Bouin solution): A = anterior part of body with three rows of chaetae of a specimen from Bakony Mts, Hungary (ventro-lateral view), B = lateral chaetae in the second row (specimen from Brno, Czech Republic), C = chaetae in the second row (ventral view, specimen from Věncová hora (locus typicus), Czech Republic), D = chaetae in the fourth row (ventral view, specimen from Coruña province, Spain).

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come less visible, depends on differences in the fixation process. The apparent differences in chaetal ultrastructure of *Hrabeiella* specimens from different European populations, published by the above-mentioned authors or obtained by us, can be considered artefacts. Purschke (1999) had already hypothesized that the dissimilarities described by Jans and Römbke (1989) in comparison with the chaetal morphology found by Rota and Lupetti (1996) and himself were due to fixation artefacts rather than to genetic differences. We also believe that the brush-like structure is closer to the true structure than the scaly one with all fibres sticking together. However, one can not exclude the possibility that some fixation procedures eliminate adhesive properties of these fibres. Generally, fixation in alcohol may obscure many surface properties of the object. Unfortunately, most specimens available to us had been fixed in alcohol and a comparison across Europe therefore had to be based on these specimens. Only the Spanish specimens had been fixed either in Bouin or in formaldehyde solution, but we did not observe any difference between the

![SEM micrographs of *Hrabeiella periglandulata* chaetae showing a different shape (the worms were fixed only in ethanol): A = Lateral chaetae of a specimen from Hungary. B = Lateral chaetae in the third row of a specimen from north-western Czechia. C = Ventral chaetae of the last rows of a specimen from Hungary (Bakony Mts). D = Chaetae in the fifth row of a specimen from south-western Czechia (locus typicus, Věncová hora).](image-url)

*Fig. 4.* SEM micrographs of *Hrabeiella periglandulata* chaetae showing a different shape (the worms were fixed only in ethanol): A = Lateral chaetae of a specimen from Hungary. B = Lateral chaetae in the third row of a specimen from north-western Czechia. C = Ventral chaetae of the last rows of a specimen from Hungary (Bakony Mts). D = Chaetae in the fifth row of a specimen from south-western Czechia (locus typicus, Věncová hora).
appearance of their chaetae and the chaetae of specimens fixed in alcohol (cf. Figs 3C–D). We conclude that based on chaetal morphology alone, all investigated populations can be considered to belong to a single described species, *Hrabeiella periglandulata*, including the Italian population investigated by Rotà and Lupetti (1996). Apparent differences in ultrastructure thus might lead to false conclusions when not obtained by exactly the same fixation procedure.

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