Biogeography of the cosmopolitan terrestrial diatom *Hantzschia amphioxys* sensu lato based on molecular and morphological data

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Until now, the reported diversity of representatives from the genus *Hantzschia* inhabiting soils from different parts of Eurasia was limited to the few species *H. amphioxys*, *H. elongata* and *H. vivax* and some of their infraspecific taxa. We have studied the morphology, ultrastructure and phylogeny of 25 soil diatom strains, which according to published description would be assigned to “*H. amphioxys* sensu lato” using 18S rDNA, 28S rDNA and *rbcL*. We show that strains are made up of seven different species of *Hantzschia*, including five new for science. Five strains were identified as *H. abundans*. This species has a slight curvature of the raphe near its external proximal ends. Four of the examined strains were represented by different populations of *H. amphioxys* and their morphological characteristics fully correspond with its isolecotype and epitype. The main specific features of this species include 21–25 striae in 10 μm, 6–11 fibulae in 10 μm, 40–50 areolae in 10 μm and internal proximal raphe endings bent in opposite directions. *H. attractiva* sp. nov., *H. belgica* sp. nov., *H. parva* sp. nov., *H. pseudomongolica* sp. nov. and *H. stepposa* sp. nov. were described based on differences in the shape of the valves, significant differences in dimensions, a lower number of striae and areolae in 10 μm and the degree and direction of deflection of the internal central raphe endings.

Based on the study of the morphological variability and phylogeny of soil *Hantzschia*-species from different geographical locations we conclude that while some species such as *H. amphioxys* are truly cosmopolitan in their distributions, some sympatric populations of pseudocryptic taxa exist in the Holarctic.

Diatoms are one of the most widespread group of unicellular algae, being reported from all types of water bodies and from all continents1. Currently, two hypotheses about freshwater diatom distribution are being discussed; one suggesting that all or most diatom species have a cosmopolitan distribution2,3 and the second that restricted geographic distributions and even endemicity are more common4,5. On the one hand, capacity for dispersal, wide environmental tolerances and interbreeding between morphologically distinct units should result in wide, perhaps ubiquitous distributions6-8. On the other hand, current careful investigation of taxonomy and distribution of diatoms9-12 and other microorganisms13,14 have shown that unicellular organisms can be specific to restricted areas. Due to the long history of references that *Hantzschia amphioxys* (Ehrenberg) Grunow has a cosmopolitan distribution15-17, an investigation of the global distribution of this species is an important tool for our understanding of global diatom biogeography.

Until recently, the genus *Hantzschia* Grunow included less than 50 species known from different parts of the world and from different water ecosystems like freshwater and saline waterbodies, terrestrial and soil ecosystems1. Many new species from this genus were described during the last decade. Over 250 described taxa are currently known from the genus *Hantzschia*18. Main morphological features that are used for species delimitation are

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H. amphioxys in the same clade there is strain D27_008, which has been designated as epitype20. One of the larg-
ert clades is represented by different strains of 

H. abundans is the clade with 

H. parva and 

H. pseudomongolica and 

H. attractiva 



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Results and discussion 

In most of the forest soil samples used in this survey, specimens belonging to the genus Hantzschia are quite 

common. Based molecular as well as on light microscopy (LM) and scanning electron microscopy (SEM) obser-

vations of 25 strains, seven different taxa were recognized. Figure 1 contains the locations of the strain’s habitats. 

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Molecular data. The obtained phylogenetic tree for representatives of the different strains Hantzschia con-

tains several large clades, some of which are monophyletic, while others contain several different species names (Fig. 2). In the analyzed tree, the largest clade is represented by different strains of H. amphioxys, the structure of which is described in the corresponding molecular analysis section. At the same time, the most significant is that in the same clade there is strain H. amphioxys D27_008, which has been designated as epitype20. One of the larg-
est is the clade with H. abundans, which, in addition to our strains, and some that have already been published, 

includes the group of strains referred to as “Hantzschia sp. 3” (Sterre6)e, (Sterre6)f from Souffreau et al.16. We 

propose to refer to all of these strains as H. abundans. The next clade consists of the new species of H. attractiva 

and three strains of Hantzschia sp. 2 (Mo1)α, (Mo1)e, (Mo1)m from Souffreau et al.16, the latter we propose to 

merge into the new species named H. pseudomongolica, which is sister to H. attractiva. Given the topology of the 
tree and the morphological features of the representatives, we can conclude that there is a close relation between 

H. abundans and H. attractiva plus H. pseudomongolica. A separate group consists of two clades with sufficient 
statistical support (likelihood bootstrap, LB 76; posterior probability, PP 100), one of which is represented by 

two strains of H. parva, and the other with strains of H. cf. amphioxys (Sterre1)l, (Sterre1)k. Another large clade 

represents a set of strains of Hantzschia sp. 1 and Hantzschia sp. 2 (Mo1)b, (Mo1)l from Souffreau et al.16, among 

which there are both large cells (86–95 µm length) and smaller ones (37–39 µm length); strains also differ by 

striation – from 18–20 striae in 10 µm (strain (Mo1)b) to 21–22 in 10 µm (strain (Ban1)b). It is possible that 

Hantzschia sp. 1 and Hantzschia sp. 2 (Mo1)b, (Mo1)l may be several closely related species. Besides the large
clades, there are a number of separate branches in the tree, representing separate strains: Hantzchia sp. 1 (Ban1) d, and the new species H. belgica (H. cf. amphioxys (Sterre3)a from Souffreau et al. [16]) and H. stepposa. Interesting is the position of the H. abundans (Tor3)c strain, which is very distant from other representatives of H. abundans and probably is a cryptic taxon, whose taxonomic status needs to be revised.

The absence of nucleotide sequences of the 18S rDNA gene for the Hantzchia strains discussed in Souffreau et al. [16] did not allow us to carry out phylogenetic analysis based on three genes (18S rDNA, 28S rDNA and rbcL). However, in GenBank, 18S rDNA sequences are available for other Hantzchia strains, including strain D27_008, for which the nucleotide sequence of the 18S V4 rDNA region is known (NCBI Accession FR873247).

Comparison of this sequence with similar ones in our strains shows a similarity of 98.4% (368 bp) with H. amphioxys MZ–BH9, MZ–BH14 and MZ–BH15. At the same time, the 18S V4 rDNA regions of these three strains are identical and they differ from the epitype by four transitions and one transversion. The similarity with the MZ-BH8 strain is somewhat less with 98.1%, due to one transition (transitions), in which it differs both from our strains and from the epitype. Also, for the 18S V4 rDNA region of the H. amphioxys D27_008 strain, compared with all of our H. amphioxys strains, a specific feature is characteristic—the position of one nucleotide (insertion of thymine T). The level of similarity of 98.1–98.4% significantly exceeds the previously calculated rate of 97% for 399/387 bp [20] between the strain H. amphioxys D27_008 and the strain H. amphioxys var. major A4 (NCBI Accession HQ912404) from Ruck and Theriot [42].

The studied Hantzchia strains presented low similarities between the clades indicated in the Fig. 2 and the evolutionary distance matrix based on the partial 28S rRNA gene including the D1-D3 region showed that nine H. abundans strains shared 99.20–99.87% similarities inside the clade, but they shared 95.61–96.67% similarities with H. amphioxys strains, 95.74–96.25% with H. stepposa MZ–BH12 and 96.14–96.54% with H. belgica (Sterre3) a (Supplementary Table S1). Thirteen H. amphioxys strains shared more than 99.07% similarity inside the clade with the greatest differences between SCII and SCIII subclades. Simultaneously, the evolutionary distance matrix showed that H. pseudomongolica strains shared low similarities with Hantzchia species which have central raphe endings internally bent to opposite directions with similarity 96.01–96.54% for H. amphioxys, 96.11% for H. stepposa and 96.54% for H. belgica.

Pairwise comparisons with putatively related taxa showed that strains of H. amphioxys were more than 99.13% similar, however they shared 98.01–98.26% similarities with H. attractiva and less than 98.55% amino acids similarity of ribulose-1,5-bisphosphate carboxylase with H. abundans strains in the analysis (Supplementary Table S2). Amino acids similarity between H. belgica, H. parva and H. stepposa had value less 99%. The percent similarity among aligned amino acids between Hantzchia species had a range of 97.95–100%.

Calculated evolutionary distances for the region D1–D3 of the 28S rRNA gene and amino acid sequence of ribulose-1,5-bisphosphate carboxylase provided strong evidence for H. attractiva, H. belgica, H. parva, H. pseudomongolica and H. stepposa as species separate from H. amphioxys and H. abundans, since the intraspecies percent dissimilarity for Hantzchia species was 0.13–0.93% (D1–D3) or 0.59–0.89% (rbcL).

**Morphological comparisons.** In order to obtain an overview of the diversity and distribution of the diatoms with Hantzchia amphioxys-like morphology, we took soil samples for diatom analysis from steppe, meadow and forest biogeocenoses of Eurasia. In total, we studied 20 soil samples and from these thirteen strains of Hantzchia were isolated. H. attractiva sp. nov., H. belgica sp. nov., H. parva sp. nov., H. pseudomongolica sp. nov. and H. stepposa sp. nov. were described based on differences in the shape of the valves, significant differ-

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*Figure 1.* Map with the habitat locations of the studied strains.
ences in dimensions, a lower number of striae and areolae in 10 µm and the degree and direction of deflection of the internal central raphe ends. Molecular investigations based on two-gene phylogeny and comparison of 18S V4 rDNA sequences supported our results that new species were clearly separated from other *Hantzschia* species. The remaining species belonged to *H. abundans* and *H. amphioxys*. *H. abundans* was split off from *H. amphioxys* by Lange-Bertalot 28. The main morphological features of the species (compared to *H. amphioxys*) were the large sizes of the valves (length up to 80 µm, width up to 10 µm), fewer striae in 10 µm (15–20) and one-sided deviation of the internal proximal raphe ends (Table 1). A soil isolate from a wet meadow dominated by *Phalaridetum*, which was located 30 m from the river Nidder, Windecken, Hessen, Germany, was chosen as holotype. In general, our strains correspond to the diagnosis of the *H. abundans* species according to all major morphological characteristics. An exception, however, is the slightly smaller valve length in strain MZ–BH6 (37.5–39 µm, in diagnosis—not less than 40 µm), a smaller valve width in strains MZ–BH1 and MZ–BH6 (6–7 µm, in diagnosis—7–10 µm). We also noted an increase in areolae density up to 50 in 10 µm (40 in 10 µm in diagnosis), although close values (45 in 10 µm) were noted by Zidarova et al. 19 for the population of *H. abundans* from Byers Peninsula, South Shetland Islands. We have not noted the formation of the keel near the raphe from the outer side of the valve in our strains, which, according to Lange-Bertalot 28, could indicate an excess of silica in the substrate. From the outer side of the valve in all the studied strains, we noted a slight curvature of the raphe near its central endings, which was not previously noted (Fig. 3m). This

Figure 2. Bayesian tree for representatives of the different strains *Hantzschia*, from an alignment with 40 sequences and 1785 characters (partial *rbcL* gene and 28S rDNA fragments). Type strains indicated in bold. The epitype of *Hantzschia amphioxys* is underlined. Values above the horizontal lines (on the left of slash) are bootstrap support from RAXML analyses (< 50 are not shown); values below the horizontal lines (to the right of slash) are Bayesian posterior probabilities (<80 are not shown). *100% statistical support.
feature is more characteristic for different strains of *H. amphioxys*. According to morphological characteristics such as the width and length of the valve, the number of striae and fibulae 10 μm (supplementary Table S2 in Souffreau et al.16), as well as the position in the phylogenetic tree rbcL–28S rDNA (Fig. 2), we suggest to include the *Hantzschia* sp. 3 strains (Sterre6)e and (Sterre6)f from Souffreau et al.16 to *H. abundans*.

*H. attractiva* sp. nov. is visually and parametrically similar with some *Hantzschia* species but differs in a number of ways. In terms of the ratio of the length and width of the valve, the shape of its ends and concavity in the center of the ventral side of the valve, *H. attractiva* is most similar to *H. subrupestris* Lange-Bertalot28. However, these species can be distinguished from one another by a smaller number of striae (14–16 in 10 μm) and areolae (24 in 10 μm) in *H. subrupestris* (Table 1). In the case of *H. subrupestris*, the central raphe ends are straight or barely noticeably deflected in one direction, whereas in *H. attractiva* they deviate in one direction very clearly. Compared with *H. abundans*, the diagnostic features of *H. attractiva* include: less pronounced concavity of the middle part of the valve from the ventral side, vertical position of the valve ends, which are also less pronounced;

| Species, strains | Length (μm) | Width (μm) | Number of striae in 10 μm | Number of fibulae in 10 μm | Number of areolae in 10 μm | Central raphe endings structure references |
|------------------|-------------|------------|---------------------------|---------------------------|----------------------------|------------------------------------------|
| *H. abundans* MZ–BH1 | 45.5–48     | 6–7        | 16–20                     | 7–9                       | 40–45                      | Internally unilaterally bent this study |
| *H. abundans* MZ–BH2 | 48.5–53.5   | 6.5–7.5    | 18–20                     | 6–9                       | 40–45                      | Internally unilaterally bent this study |
| *H. abundans* MZ–BH5 | 48.5–50     | 6.5–7.5    | 18–20                     | 7–9                       | 40–50                      | Internally unilaterally bent this study |
| *H. abundans* MZ–BH6 | 37.5–39     | 6–6.5      | 18–20                     | 7–9                       | 45–50                      | Internally unilaterally bent this study |
| *H. abundans* MZ–BH17 | 50–53.5     | 7–7.5      | 16–18                     | 7–8                       | 40–45                      | Internally unilaterally bent this study |
| *H. abundans* | 40–80       | 7–10       | 15–20                     | 5–8                       | 40                         | Internally unilaterally bent            |
| *H. achroma* | 12–58       | 4–4.5      | 34–35                     | 11–14                     | n.a                        | Li and Volcani49                        |
| *H. acuticapitata* | 57–67       | 7.8–9.1    | 22–23                     | 6–9                       | 50                         | Zidarova et al.17                       |
| *H. amphioxys* MZ–BH8 | 36–38       | 4.8–5      | 23–24                     | 6–8                       | 45–48                      | Internally bent to opposite directions |
| *H. amphioxys* MZ–BH9 | 40–41.5     | 5–6        | 23–24                     | 6–8                       | 40–50                      | Internally bent to opposite directions |
| *H. amphioxys* MZ–BH14 | 44–46       | 5.5–6      | 21–24                     | 7–8                       | 50–60                      | Internally bent to opposite directions |
| *H. amphioxys* MZ–BH15 | 40–41.5     | 5–6        | 22–23                     | 7–10                      | 45–50                      | Internally bent to opposite directions |
| *H. amphioxys* isoelectotype | 38–42.5     | 4.4–6.4    | 23–25                     | 7–11                      | n.a                        | n.a                                      |
| *H. amphioxys* epitype | 36.8–44.6   | 5.2–6.6    | 20–26                     | 5–7.5                     | 48–53                      | Internally bent to opposite directions |
| *H. attractiva* MZ–BH11 | 66–70       | 7.7–8.9    | 17–18                     | 5–7                       | 40–50                      | Internally unilaterally bent this study |
| *H. bardii* | 40–80       | 6–10       | 20–24                     | 6–9                       | 36                         | Lange-Bertalot et al.46                 |
| *H. belgica* (Sterre3)a | 36–38       | 6–7        | 22–23                     | 8–9                       | 50–55                      | Internally bent to opposite directions |
| *H. compactoides* | 60–80       | 10–12      | 14–16                     | 5–6                       | 30                         | Lange-Bertalot et al.46                 |
| *H. delicatula* | 43–50       | 5.5–6      | 18                        | 7–9                       | 30                         | Metzeltin et al.44                      |
| *H. giessiana* | 50–100      | 6.5–11     | 16–20                     | 4–7                       | 26–30                      | Lange-Bertalot28                       |
| *H. mongolica* | 80–127      | 15–18      | 11–14                     | n.a                       | n.a                        | Metzeltin et al.44                      |
| *H. nematoda* | 60–90       | 8          | 18                        | 7–9                       | n.a                        | Metzeltin et al.43                      |
| *H. parva* MZ–BH3 | 41–42.5     | 6–7        | 17–20                     | 7–8                       | 40–45                      | Internally unilaterally bent this study |
| *H. parva* MZ–BH4 | 37–38.5     | 5–6        | 19–20                     | 8–9                       | 40–45                      | Internally unilaterally bent           |
| *H. psammicola* | 50–68       | 8          | 10                        | n.a                       | 28                         | Metzeltin et al.41                      |
| *H. pseudomongolica* | 83.2–91.4   | 7.8–9.4    | 19–21                     | 6–8                       | 40–45                      | Internally straight This study; souffreau et al.16 |
| *H. stepposa* MZ–BH12 | 40–42.5     | 6–6.5      | 22–25                     | 5–9                       | 44–52                      | Internally bent to opposite directions |
| *H. subrupestris* | 50–80       | 7–9        | 14–16                     | 5–7                       | 24                         | Internally straight Lange-Bertalot28    |
| *H. valdeventricosa* | (41) 55–70  | 9–10       | 18–19 (20)                | 5,5–7                     | n.a                        | n.a                                      |

Table 1. Comparison of species and strains of *Hantzschia* from studied material with similar taxa.
a larger number of areolae in 10 μm and one-sided deviation of the central raphe ends on both sides of the valve (they are located straight in H. abundans). Our species differs from H. bardi Lange-Bertalot, Cavacini, Tagliaventi et Alfinito in the shape of the valve ends (in H. bardi, they are obliquely cuneate and finally protracted narrow–rostrate to subcapitate), a smaller number of striae in 10 μm and a larger number of areolae. Of nearly similar size to H. attractiva is H. compactoides Lange-Bertalot, Cavacini, Tagliaventi et Alfinito, which can be distinguished by a more pronounced bend of the valve from the ventral side, the direction of the valve ends in the same direction and their rostrate shape. Also, H. attractiva is characterized by smaller values of the width of the ends of the raphe and up to 100 μm in length is H. giessiana Lange-Bertalot et Rumrich, which is characterized by capitate valve ends, a smaller number of areolae in 10 μm (26–30) and the central raphe ends from inner part deviate in opposite directions in comparison with H. attractiva. The lengths 66–70 μm and breadth 7.7–8.9 μm also correspond to the type of H. nematoda Metzeltin, Lange-Bertalot et García-Rodríguez and H. psammicola García-Baptista, which are distinguished from H. attractiva in addition to the shape of the valves by the presence of costae on the outer side of the valve.

We obtained the light and scanning electron micrographs based on the study of the original materials of the strains (Mo1)a, (Mo1)e and (Mo1)m and described H. pseudomongolica sp. nov. Originally the (Mo1)a, (Mo1)e and (Mo1)m strains were presented in Souffreau et al. as “H. amphioxys sp. 2”, however, the authors presented only the length and breadth characteristics of the valves without clarifying the taxonomic status or providing an explanation of the isolated position of the strains in the phylogenetic tree in relation to the clade with H. amphioxys (Fig. 4 in Souffreau et al.). H. mongolica Metzeltin, Lange-Bertalot et Nergui was described from freshwater habitats from Mongolia. This species differs from H. pseudomongolica sp. nov. by its larger valve size (length 80–127 μm and width—15–18 μm) and significantly smaller striae density (11–14 in 10 μm). H. pseudomongolica sp. nov. differs from H. attractiva in both its dimensional characteristics (longer valves and more dense arrangement of striae) and the shape of the valve ends. In H. attractiva the ends are narrowed, broadly-rounded and directed vertically, in H. pseudomongolica the ends are more attenuate, almost capitate, and deflected towards to the dorsal side.

The paratype of H. parva differs from the holotype by somewhat larger valve sizes, namely, 41.5–42.5 μm long and 6–7 μm wide. The large width of the valves, a smaller number of striae in 10 μm and one-sided deviation of the central end of the raphe underlines its differences with H. amphioxys (in comparison with the isolectotype). Compared with H. abundans, H. parva is characterized by having narrower valves (for H. abundans in the range of 7–10 μm), a more dense areola arrangement, lack of a keel near the raphe externally and by the position of the central raphe ends (for H. abundans they are straight internally).

The smallest species of the genus Hantzschia is H. achnoma B.E. Volcani et C.-W. Li, which differs from H. parva, in addition to the narrower shape of the valve and tapering valve ends, by a smaller width (up to 4.5 μm), more dense striaion (34–35 striae in 10 μm) and a higher fibular density (11–14 in 10 μm). In spite of the close dimensions of the valve, described from Uruguay H. delicatula Metzeltin, Lange-Bertalot et García-Rodríguez has an almost flat ventral side of the valve and its ends are in almost the same plane as the cuneate, as well as lower density of areolae (30 to 10 μm), which distinguishes it from H. parva (Table 1). The greater width of the valve (9–10 μm), as well as its flattened form on the ventral side, distinguish H. parva from another Uruguayan species H. valdeventricosa Metzeltin, Lange-Bertalot et García-Rodríguez. According to its morphometric features, H. stepposa sp. nov. is similar to H. amphioxys. Also, these two species are similar in the deflection of the central raphe ends on the inner side of valve—they curve in opposite directions. Despite this, the two species are easily distinguished by the shape of the valves: H. amphioxys is characterized by a pronounced concavity of the ventral side of the valve in the middle, whereas in H. stepposa, the ventral side is almost straight; in H. amphioxys, the valve ends are clearly directed to the dorsal side of the valve, while in H. stepposa they are arranged vertically. The presence of central raphe ends at the inner side that are deflected in opposite directions distinguishes H. stepposa from most of the known Hantzschia species, whose valve length do not exceed 100 μm (including H. attractiva and H. parva). The only species with a similar position of the central ends of the raphe and up to 100 μm in length is H. giessiana. However, along with the large size of the valve (50–100 μm), it is also characterized by a completely different shape of the valves—a strongly concave ventral side and ends curved towards it (Table 1).

Originally strain (Sterre3)a was presented in Souffreau et al. as “H. cf. amphioxys”, however, the authors presented only the morphometric characteristics and the light micrograph of one valve without clarifying its taxonomic status and explaining its isolated position in the phylogenetic tree in relation to the Hantzschia amphioxys clade (Fig. 4 in Souffreau et al.). We present light and scanning electron micrographs based on a study of the original material with description of a new species H. belgica sp. nov. In comparison with other Hantzschia species that have internal proximal raphe ends that are deflected in opposite direction (H. amphioxys and H. stepposa), H. belgica differs in shape and direction of the valve ends. If H. amphioxys and H. stepposa are characterized by extended and capitated ends, which are directed to the dorsal side of the valve in H. amphioxys and arranged vertically in H. stepposa, in H. belgica the ends of the valves are not distinctly protracted and not directed to either the dorsal or ventral margins.

A comparative analysis of the main morphological characteristics (length and width of the valves, the number of striae, the number of fibulae and areolae in 10 μm) in the four H. amphioxys strains isolated by us with the proposed diagnoses of Jahn et al. of the lectotype and epitype show a coincidence in most data. Small differences can be attributed to the slightly larger maximum length of the valves in strain MZ–BH14 (46.0 μm with a maximum of 44.6 μm in the epitype). The density of the areolae for epitype is in the range of 48–53 areolae in 10 μm (submitted in this study). Some studies indicate the number of areolae in 10 μm for different populations of H. amphioxys in the range of 40–50 areolae in 10 μm. In this regard, the excess of this range is observed only in the strain MZ–BH14, whose density of areolae in the valve can reach 60 in 10 μm.
Based on a detailed study of morphology and ultrastructure, as well as the use of phylogenetic analysis using the 28S rDNA and rbcL genes, our results found 25 soil strains that represent geographically distant populations and formally could be classified as H. amphioxys sensu lato, containing seven different Hantzschia species, including five new for science. Five strains were identified as H. abundans and are fully consistent with the diagnosis of all major morphological characteristics for that taxon. For these strains, we noted areolar densities up to 50 in 10 μm (the original diagnosis reported 40 areolae in 10 μm). Externally, in all the studied strains, we noted a slight curvature of the raphe near its proximal endings, which was not previously reported. In addition, another four of the studied strains represent different populations of H. amphioxys and their morphological characteristics fully correspond to the adopted isolectotype and epitype20. The main specific features of these types include: the presence of 21–25 striae in 10 μm, 6–11 fibula in 10 μm, 40–50 areolae in 10 μm and internal proximal raphe ends curved in opposite directions (Table 1). Five new species were identified on the basis of differences in the shape of valves, the size characteristics of the valves, the number of striae and areolae in 10 μm and the position of the central raphe ends compared to the already known Hantzschia species.

Phylogenetic analysis using the 28S rDNA and rbcL genes showed that adding our strains does not change the tree topology obtained by C. Souffreau et al.16 for different lines of H. amphioxys. At the same time, the presence of a detailed morphological description of our strains using SEM results made it possible to distinguish morphological differences between the available clades. The connection of the topology of the obtained tree with the morphological features of the isolated strains shows that morphological features reflect phylogenetic relatedness. These morphological features that also reflect relationships based on genomic data are shape of the valve, the raphe structure (presence or absence of the central node and direction of the central branches relative to each other in it), the number of striae and areolae in 10 μm. These morphological features were correlated with the clades in the ML (Maximum Likelihood) and BI (Bayesian inference) trees constructed from fragments of the 28S rDNA and rbcL genes.

Description of new species and emendations. Hantzschia abundans Lange–Bertalot emended by Maltsev et Kulikovskiy (Fig. 3). Morphology. Valves are dorsiventral with evidently concave central part of ventral side of the valve. Dorsal side is slightly convex, sometimes straight (Fig. 3a–l). Valve ends are slightly tapered and capitate (strains MZ–BH2, MZ–BH5) or more cuneate and capitate (MZ–BH1, MZ–BH6). Length 37.5–53.5 μm, breadth 6.0–7.5 μm. Fibulae are of different sizes, 6–9 in 10 μm. Two central fibulae are rather widely spaced. Striae are radiate, parallel at the valve ends, 16–20 in 10 μm. Areolae are not visible in LM. Fibulae are connected with 1–4 striae or interstriae.

Ultrastructure. In outside view central raphe ends are straight or slightly curved to the same side (Fig. 3m). In inside view central raphe ends are curved to dorsal side (Fig. 3n). Striae are uniseriate, 40–50 in 10 μm. Helicoglossae are small.

Molecular analysis. In the phylogenetic tree based on the 28S rDNA and rbcL genes, H. abundans strains form a separate clade (Fig. 2). In addition to our 5 strains, we also included two other strains of H. abundans, (Wes1)b and (Wes3)b, that were isolated from De Panne, Belgium (Souffreau et al.16). This clade also includes soil strains (Sterre6)e and (Sterre6)f, named by Souffreau et al. as “Hantzschia sp. 3”, isolated from Gent, Belgium.

Ecology and distribution. The strains H. abundans MZ–BH1, MZ–BH2, MZ–BH5, MZ–BH6 and MZ–BH7 isolated by us belong to soil populations (Fig. 1, Table 2).

Hantzschia attractiva Maltsev et Kulikovskiy sp. nov. (Fig. 4). Holotype. Slide no. 02508/MZ–BH11 (Holotype represented by Fig. 4) in the collection of Maxim Kulikovskiy, K.A. Timiryazev Institute of Plant Physiology RAS, 10.05.2015, collected by Y. Maltsev.

Type locality. Sod-medium podzolic soil, horizon 0–5 cm, plantation Betula pendula Roth, Yaroslavl region, Russia, N57°50 ’38″, E38°4′23.83″, 10.05.2015.

Registration. https://phycobank.org/102516.

Diagnosis. Length 66–70 μm, breadth 7.7–8.9 μm. Valves slightly asymmetrical to the apical axis, with dorsal margin concave or straight, ventral margin slightly convex. Apices protracted, rounded. Fibulae are different in size and placed irregularly, 5–7 in 10 μm. Central fibulae are more widely spaced than the others. Striae 17–18 in 10 μm. Areolae are not evident in LM.

Ultrastructure. Raphe with two branches, not continuous, internal proximal raphe ends curved in the dorsal direction. Fibulae of varying sizes, connected with 1–5 striae. Striae are uniseriate with 40–50 areolae in 10 μm. Internally, the areolae are rectangular in shape.

Etymology. The epithet refers to the attractive nature of the shape of the valve.

Molecular analysis. In the phylogenetic tree based on the 28S rDNA and rbcL genes, the strain H. attractiva MZ–BH11 occupies an isolated position in the clade with three strains of Hantzschia sp. 2 (Mo1)a, (Mo1)e and
(Mo1)m (Fig. 2), described by Souffreau et al.\textsuperscript{16} from Kangai-Nuruu (Mongolia). Clade \textit{H. attractiva} plus \textit{H. pseudomongolica} (\textit{Hantzschia} sp. 2(Mo1)a, (Mo1)e and (Mo1)m) has high BI support and is sister to the clade of \textit{H. abundans} (Fig. 2).

Ecology and distribution. The strain \textit{H. attractiva} MZ–BH11 was isolated from the soil (sod-medium podzol) (Table 2).

\textit{Hantzschia pseudomongolica} Maltsev et Kulikovskiy sp. nov. (Fig. 5). Holotype. Slide no. CSO77/(Mo1)a (holotype represented by Fig. 5a, d–g) in the collection of Universiteit Gent, collected by Pieter Vanormelingen. Type locality. Soil, Kangai-Nuruu, Mongolia. N47°50′20.0″ E99°59′59.9″.

Paratype. Slide no. CSO78/(Mo1)e (paratype represented by Fig. 5b–c) in the collection of Universiteit Gent, collected by Pieter Vanormelingen. Slide no. CSO81/(Mo1)m in the collection of Universiteit Gent, collected by Pieter Vanormelingen.

Registration. https://phycobank.org/102517.

Diagnosis. Valves nearly straight, slightly asymmetrical to the apical axis and some specimens are also slightly asymmetrical to the transapical axis (Fig. 5d,g). Apices protracted, rounded to slightly capitate. Length 83.2–91.4 µm, breadth 7.8–9.4 µm. Fibulae are of different sizes, 6–8 in 10 µm. Two central fibulae are more widely spaced than the others. Striae 19–21 in 10 µm. Areolae are not discernible in LM.

Ultrastructure. Raphe not continuous. In inside view central raphe ends are straight (Fig. 5h). Areolae 40–45 in 10 µm.
Molecular analysis. In the phylogenetic tree based on the 28S rDNA and rbcL genes, H. pseudomongolica strains form a common clade with H. attractiva with high statistical support (LB 90; PP 100) (Fig. 2). In this case, the strains (Mo1)a and (Mo1)e form a separate subclade, slightly isolated from the clade with strain (Mo1)m.

Etymology. The species is named according to the region where it was found and indicating superficial similarities with H. mongolica.

Hantzschia parva Maltsev et Kulikovskiy sp. nov. (Fig. 6). Holotype. Slide no. 02509/MZ–BH4 (Holotype here represented by Fig. 6a–g) in the collection of Maxim Kulikovskiy, K.A. Timiryazev Institute of Plant Physiology RAS, 19.04.2015, collected by Y. Maltsev.

Type locality. Chernozem, horizon 0–5 cm, mixed deciduous stands, Poltava region, Ukraine, N49°8’31.69”, E34°13’1.51”, 19.04.2015.

Figure 4. Valve morphology of the strain Hantzschia attractiva MZ–BH11. (a–d) Light micrographs of the natural population. (e–i) Light micrographs of monoculture. (j) Scanning electron micrographs, interior of valve. (k) Scanning electron micrograph, showing the raphe keel. (l) Scanning electron micrograph, interior of valve at valve center, showing the bent of the raphe near the central raphe endings. Scale bars (a–j) = 10 µm, (k–l) = 1 µm.
| Strains | Sample locality | Collection date | Latitude | Longitude | Short description | Slide No | GenBank Accession Numbers (BOLD ID) |
|---------|-----------------|-----------------|----------|-----------|------------------|----------|-------------------------------------|
| Hantzschia abundans MZ–BH1 | Henichek, Kherson region, Ukraine | 01 Mar 2013 | N46°10'31.0" | E34°46'12.1" | Steppe, chestnut soil, horizon 0–5 cm | 02502 | MW396843 MW396827 MW387960 |
| Hantzschia abundans MZ–BH2 | Henichek, Kherson region, Ukraine | 01 Mar 2013 | N46°9'57.72" | E34°47'34.78" | Cemetery, chestnut soil, horizon 0–5 cm | 02503 | MW396844 MW396828 MW387961 |
| Hantzschia abundans MZ–BH5 | Kalivokva village, Zhytomyr region, Ukraine | 18 Apr 2015 | N50°16'4.03" | E28°47'54.57" | Betula pendula forest, chernozem, horizon 0–5 cm | 02510 | MW396847 MW396831 MW387964 |
| Hantzschia abundant MZ–BH6 | Dmitrovka village, Kyiv region, Ukraine | 18 Apr 2015 | N50°26'49.26" | E30°11'8.13" | Pinus sylvestris forest, chernozem, horizon 0–5 cm | 02511 | MW396848 MW396832 MW387965 |
| Hantzschia abundant MZ–BH7 | Boryspil, Kyiv region, Ukraine | 19 Apr 2015 | N51°0'19.99" | E31°0'0.50" | Concrete pavement with biofilm, moist | CSO92 | n.a | (HANT021-11) |
| Hantzschia abundant (Sterre6)e | Gent, Oost-Vlaanderen (East Flanders), Belgium | 21 Nov 2007 | N51°0'30.8" | E3°42'55.2" | Concrete pavement with biofilm, moist | CSO93 | n.a | (HANT022-11) |
| Hantzschia attractiva (Sterre6)f | Yaroslavl region, Russia | 10 May 2015 | N57°50'23.83" | E3°4'13.02" | Betula pendula and Pinus sylvestris forest, sod-medium podzolic soil, horizon 0–5 cm | 02508 | MW396852 MW396836 MW387969 |
| Hantzschia pseudomonogelica (Mo1)a | Kangai-Nuruu, Mongolia | n.a | N47°50'20.0" | E99°59'59.9" | Sediment (lit-toral zone) | CSO77 | n.a | (HANT006-11) |
| Hantzschia pseudomonogelica (Mo1)e | Kangai-Nuruu, Mongolia | n.a | N47°50'20.0" | E99°59'59.9" | Sediment (lit-toral zone) | CSO78 | n.a | (HANT007-11) |
| Hantzschia pseudomonogelica (Mo1)m | Kangai-Nuruu, Mongolia | n.a | N47°50'20.0" | E99°59'59.9" | Sediment (lit-toral zone) | CSO81 | n.a | (HANT010-11) |
| Hantzschia parva MZ–BH3 | Henichek, Kherson region, Ukraine | 01 Mar 2013 | N46°9'57.72" | E34°47'34.78" | Cemetery, chestnut soil, horizon 5–10 cm | 02504 | MW396845 MW396829 MW387962 |
| Hantzschia parva MZ–BH4 | Kobyllyak village, Poltava region, Ukraine | 19 Apr 2015 | N49°8'31.69" | E34°13'1.51" | Quercus robur L, forest, chernozem, horizon 0–5 cm | 02509 | MW396846 MW396830 MW387963 |
| Hantzschia stepposa MZ–BH12 | Kamenka-Dniprovska, Zaporizha region, Ukraine | 20 Oct 2014 | N47°30'23.46" | E3°42'15.99" | Planting of Quercus robur, fertilization sub-horizon of forest floor | 02516 | MW396853 MW396837 MW387970 |
| Hantzschia belgica (Sterre3)a | Gent, Oost-Vlaanderen (East Flanders), Belgium | 21 Nov 2007 | N51°0'18.6" | E3°42'58.4" | The upper half cm of bare soil under Quercus in meadow, moist | CSO86 | n.a | (HANT015-11) |
| Hantzschia amphibians MZ–BH8 | Mishutilino village, Moscow region, Russia | 05 Oct 2015 | N56°22'55.12" | E38°6'36.13" | Populus nigra L. and Betula pendula forest, sod-medium podzolic soil, horizon 0–5 cm | 02505 | MW396850 MW396834 MW387967 |

Continued
Paratype. Slide no. 02504/MZ–BH3 (Paratype here represented by Fig. 6h–n) in the collection of Maxim Kulikovskiy, К.А. Timiryazev Institute of Plant Physiology RAS, 1.03.2013, collected by Y. Maltsev.

Registration. https://phyco bank.org/102518.

Diagnosis. Valves linear-lanceolate, asymmetrical about the apical axis, with dorsal margin more or less straight to slightly convex, ventral margin concave, with apices small, protracted, rounded to subrostrate. Length 37.0–42.5 µm, breadth 5–7 µm. Fibulae irregularly-placed along the ventral margin, 7–9 in 10 µm, with a distinct central nodule. Striae 17–20 in 10 µm; areolae not visible with LM.

Ultrastructure. Raphe not continuous, with internal proximal raphe ends deflected slightly in the same direction. Areolae 40–45 in 10 µm, rectangular in shape internally.

Etymology. The epithet "parva" chosen according to size of the valve and compared with most species in the genus.

Molecular analysis. In the phylogenetic tree based on the 28S rDNA and \( rbcL \) genes, two strains of \( H. \) parva, MZ–BH3 and MZ–BH4, form a single clade (Fig. 2). With high bootstrap support, a sister clade joins this lineage, which includes two soil strains of \( H. \) cf. amphioxys (Sterre1)f and (Sterre1)h, isolated from the region of Gent, Belgium16. Compared to \( H. \) parva, these strains are characterized by somewhat larger valve sizes (35–49 µm long, 8–9 µm wide) and the number of striae is about 20–22 in 10 µm. It is possible that strains (Sterre1)f and (Sterre1)h represent another new species of \( Hantzschia \), closely related to \( H. \) parva.

Ecology and distribution. In the course of this research, two \( H. \) parva soil strains were isolated from geographically distant biogeocenoses (Fig. 1, Table 2). The strain \( H. \) parva MZ–BH4 (holotype) was isolated from the soil in the broadleaf forest biogeocenosis. The strain \( H. \) parva MZ–BH3 (paratype) BH2 was isolated from the soil of the steppe biogeocenosis at the city cemetery. The dominant grass cover consisted of \( Elytrigia repens \) (L.) Desv. ex Nevski, \( Hordeum leporinum \) Link and other grasses.

Table 2. List of all strains examined in this study with their GenBank accession numbers (BOLD ID). Geographic position and description of sample sites indicated.

| Strains                      | Sample locality | Collection date | Latitude   | Longitude   | Short description | Slide No | GenBank Accession Numbers (BOLD ID) |
|------------------------------|-----------------|-----------------|------------|-------------|-------------------|----------|-------------------------------------|
| \( Hantzschia \) amphioxys   | Kursk region, Russia | 02 May 2015     | N51°41‘8.11“ | E36°35’57.79“ | Quercus robur forest, gray forest soil, horizon 0–5 cm | 02506    | MW396851 MW396835 MW387968          |
| \( Hantzschia \) amphioxys   | Bogatyr village, Zaporizhia region, Ukraine | 04 May 2015     | N46°37’8.87“ | E35°16’27.13“ | planting of \( Pinus pallasiana \), fermentation sub-horizon of forest floor | 02513    | MW396854 MW396838 MW387971          |
| \( Hantzschia \) amphioxys   | Kamenka-Dniprovka, Zaporizhia region, Ukraine | 07 Oct 2014     | N47°30’32.50“ | E34°23’17.87“ | Planting of \( Pinus sylvestris \), chernozem with low humus, horizon 0–5 cm | 02514    | MW396855 MW396839 MW387972          |

Hantzschia stepposa Maltsev et Kulikovskiy sp. nov. (Fig. 7). Holotype. Slide no. 02516/MZ–BH12 (Holotype represented by Fig. 7) in the collection of Maxim Kulikovskiy, К.А. Timiryazev Institute of Plant Physiology RAS, 20.10.2014, collected by Y. Maltsev.

Registration. https://phyco bank.org/102519.

Diagnosis. Valves linear-lanceolate, straight to extremely weakly asymmetrical to the apical axis, apices protracted, knob-like to nearly capitate. Length 40.0–42.5 µm, breadth 6.0–6.5 µm. Fibulae irregular in size, 5–9 in 10 µm. Striae 22–25 in 10 µm.
Ultrastructure. Raphe not continuous externally and internally; central raphe endings are straight in outside view and curved in opposite directions in inside view. Areolae fine, 44–52 in 10 µm, rectangular in shape internally.

Etymology. The species named according to the ecological region where it was found.

Molecular analysis. In the phylogenetic tree based on the 28S rDNA and rbcL genes, *H. stepposa* MZ–BH12 occupies a separate position (Fig. 2). *H. stepposa* is the sister taxon to the *H. amphioxys* clade, which emphasizes the close relation of the two species. The *H. stepposa* plus *H. amphioxys* clade is sister to the soil-dwelling *Hantzschia belgica* (Sterre3)a, characterized as a small cell species (length 36–37 µm and width 6.0–6.5 µm) with a number of striae close to *H. stepposa* (22–23 in 10 µm).

Ecology and distribution. The strain *H. stepposa* MZ–BH12 was isolated from the fermentation sub-horizon of the forest floor in the *Quercus robur* plantation with *Celtis occidentalis* L. in the second tier. The grass ground cover was represented by *Stellaria holostea* L., *Anthriscus sylvestris* (L.) Hoffm., *Glechoma hederacea* L. and *Urtica dioica* L.

Figure 5. Valve morphology of the strain *Hantzschia pseudomongolica* (Mo1)a. (a–c) Light micrographs of the natural population. (d–g) Light micrographs of monoculture. (h) Scanning electron micrograph, interior of valve at valve center, showing the position of the central raphe endings. Scale bars (a–g) = 10 µm, (h) = 1 µm.

Hantzschia belgica Maltsev et Kulikovskiy sp. nov. (Fig. 8; Fig. 1 L in Souffreau et al[16]). Holotype. Slide no. CSO86/(Sterre3)a (Holotype represented by Fig. 8) in the collection of Universiteit Gent.

Type locality. Soil, Gent, Oost-Vlaanderen (East Flanders), Belgium. N51°01′38.6″ E3°42′58.4″, 21.11.2007, collected by Caroline Souffreau.

Registration. https://phycobank.org/102520.

Diagnosis. Valves linear-lanceolate, weakly asymmetrical to the apical axis, with small, protracted apices. Length 36–38 µm, breadth 6–7 µm. Fibulae variable in size and spacing along the ventral margin. Central fibulae more widely spaced than the others, 8–9 in 10 µm. Striae 22–23 in 10 µm.
Ultrastructure. Raphe not continuous and eccentric, placed on the valve mantle for the entire length. In inside view central raphe endings curved in opposite directions. Externally, areolae are rounded; internally they are rectangular in shape. Areolae fine, 50–55 in 10 µm.

Etymology. The species named according to the region where it was found.

Molecular analysis. In the phylogenetic tree based on the 28S rDNA and rbcL genes, *H. belgica* (Sterre3) occupies a separate position, sister to *H. amphioxys* plus *H. stepposa* clade (Fig. 2). These three taxa are closely related, with high statistical support (LB 100; PP 100) and represent a group of *Hantzschia* species, in which the central raphe ends on the inner side of the valve and are deflected in opposite directions.

*Hantzschia amphioxys* (Ehrenberg) Grunow emend. Maltsev et Kulikovskiy (Fig. 9). Morphology. Valves linear-lanceolate, with apices narrowly protracted, deflected dorsally. Fibulae approximately of similar size and equally-spaced, with the two central fibulae more separate from one another as compared to others. Length 36–46 µm, breadth 4.8–6.5 µm. Fibulae 7–10 in 10 µm. Striae 21–24 in 10 µm.

Ultrastructure. Raphe not continuous. In outside view central raphe ends slightly curved to the same side (Fig. 9r). In inside view central raphe endings distinctly curved in opposite directions (Fig. 9s). Number of areolae are variable in different strains: from 40–50 in MZ–BH9 to 50–60 in MZ–BH14. Areolae range from 40 to 60 in 10 µm between the strains investigated.

Molecular analysis. In the phylogenetic tree, all the *H. amphioxys* strains isolated by us, as well as the sequences taken from BOLDSYSTEMS, form a separate clade (Fig. 2). This clade has several prominent subclades, formed with high statistical support. The first subclade (SCI) is represented by two strains of *H. amphioxys* (Sterre1) and
The second subclade (SCI), which is sister to SCI, is formed by three strains, also isolated from Gent (Belgium), which are 2–5 µm shorter than the valves compared to the first group, one of our strains, MZ–BH8 and the epitype *H. amphioxys* D27_008. A separate monophyletic group within *H. amphioxys* has the other two subclades. The third subclade (SCIII) consists of three strains of *H. amphioxys* isolated from Schirmacher Oasis (Antarctica)\(^\text{16}\). The fourth subclade (SCIV) is represented by our three strains: MZ–BH9, MZ–BH14 and MZ–BH15 from widely separated habitats in Russia and Ukraine.

**Ecology and distribution.** The four *H. amphioxys* MZ–BH8, MZ–BH9, MZ–BH14 and MZ–BH15 strains we have identified belong to different, geographically distant, soil populations (Fig. 1, Table 2). Strain MZ–BH8 and MZ–BH9 were isolated from soil in mixed deciduous forests. Strain MZ–BH14 was isolated from the fermentation sub-horizon of forest litter in the *Pinus pallasiana* (D. Don) Holmboe planting. In the algae flora of this litter, *H. amphioxys* is one of the dominants\(^\text{36}\). Strain MZ–BH15 was isolated from the forest floor in the *Pinus sylvestris* L. plantation.

**Biogeography.** The *H. abundans* clade contains strains isolated in different regions of Belgium: (Sterre6)e and (Sterre6)f from Gent, (Wes1)b and (Wes3)b from De Panne\(^\text{16}\), as well as our strains from Zhytomyr, Kyiv and Kherson regions of Ukraine (Fig. 2). Clade *H. attractiva* plus *H. pseudomongolica* ("Hantzschia sp. 2" of Souf-freau et al.\(^\text{16}\)) represents rather remote populations: *H. attractiva* MZ–BH11 was isolated in the Yaroslavl region of Russia, and strains *H. pseudomongolica* (Mo1)a, (Mo1)e, (Mo1)m—in the mountain region of Kangai-Nuruu Mongolia. However, the presence of a stable phylogenetic connection between these populations undoubtedly underlines their close relationship. Also quite interesting is the relationship between the Canadian strains of *Hantzschia* sp. 1 (Ban1)b, (Ban1)e, (Ban1)f, (Ban1)h and Mongolian *Hantzschia* sp. 2 (Mo1)h, (Mo1)l. As for the clade of *H. amphioxys*, it represents the most distant populations from the Antarctic, Belgium, Germany, Russia and Ukraine. Moreover, the selected subclades do not always correspond to the region: the SCI subclade...
includes two Belgian strains (Sterre1)e and (Sterre3)c; SCII subclade is formed by three Belgian strains (Sterre4)a (Sterre4)b, (Sterre6)b, one Berlin strain D27_008 and strain MZ–BH8 from the Moscow Region (Russia); SCIII subclade consists of three Antarctic strains SCHIR S11, SCHIR S15, SCHIR S16; SCIV subclade is represented by our three strains: MZ–BH9 (Kursk region, Russia), MZ–BH14 and MZ–BH15 (Zaporizhia region, Ukraine). Due to the absence of distinct differences in morphology and ultrastructure between representatives of the subclade SCI–SCIV (Fig. 2) we don’t distinguish new species which may correspond to the separate monophyletic subclades within *H. amphioxys*.

Analysis of the distribution of the studied strains showed the presence of two trends in the biogeography of *Hantzschia* species. The first one shows that within the genus there are a number of widely distributed species; the second one shows that there are also species with a probably limited distribution (see also for *Planothidium* by Jahn et al.12). Neither *H. abundans*, nor *H. amphioxys* had strains that are more closely related to local endemics, and given the habitats confirmed in our study, they are species with wide distribution. Also, *H. parva* can be considered as widely distributed, two strains of which were isolated from habitats distant more than 400 km apart. The second trend may indicate the presence of species with a limited distribution: *H. stepposa*, *H. abundans* (Tor3)c, and two strains of *H. cf. amphioxys* (Sterre1)f, (Sterre1)h. However, the question of endemism of these species has to be rethought after revising the characteristics of the morphology and ultrastructure of

Figure 8. Valve morphology of the strain *Hantzschia belgica* (Sterre3)a. (a–c) Light micrographs of the natural population. (d–g) Light micrographs of monoculture. (h) Scanning electron micrograph, exterior of valve. (i) Scanning electron micrograph, interior of valve at valve center, showing bent to opposite directions of the central raphe endings. Scale bars (a–g) = 10 µm, (h) = 5 µm, (i) = 0.5 µm.
phylogenetically close or new strains of *Hantzschia*. Our study has shown that samples of *H. amphioxys* strains can contain both *H. amphioxys* sensu stricto itself and a number of pseudocryptic taxa. When studying the biogeography of individual taxa, it is necessary to take into account the seasonal characteristics of their vegetation. We have shown, for example, that *H. amphioxys* as a dominant of all communities in the coniferous and white acacia litter in the spring, and in the summer—only in the white acacia. In the fall, *H. amphioxys* falls out of the dominant complex and may be absent in samples.

Today, there is no single and universal morphological or ultrastructural feature that would characterize the genus *Hantzschia* and the most effective way to verify membership to this genus is with a molecular phylogeny. Considering this, the data obtained here for 13 new soil strains of *H. amphioxys* and 6 related species not only significantly complement the previously obtained material, but also represent a significant step in understanding the diversity and biogeography of cryptic and pseudocryptic taxa in the genus *Hantzschia*.

**Materials and methods**

Diatom samples of *Hantzschia* were collected in 2014–2015 from different soil and forest floor horizons in the territory of steppe, meadow and forest biogeocenoses of Eurasia (Fig. 1, Table 2). The forest floor samples were taken at a distance of 1.0–1.5 m from tree trunks in the areas without large branches and accumulated bark from the A01 (fresh debris), A02 (0–5 cm, decomposed debris with partially preserved initial structure of some...
Monoclonal strains were established by micropipetting single cells under the inverted microscope Zeiss Axio Vert. A1. Nonaxenic monocultures of the algae were cultivated in the liquid WC medium in Petri dishes with alternating 12-h light and dark photoperiod with 70 μmol photons m⁻² s⁻¹ light intensity. Strains were analyzed after one month of culturing.

Parts of the original strains by Souffreau et al. such as Hantzchia sp. 1 (Ban1f)e and (Ban1f), Hantzchia sp. 2 (Mo1)a, (Mo1)e and (Mo1)m, Hantzchia sp. 3 (Sterre6)f, H. amphioxys SCHIR_S11, SCHIR_S16, (Sterre1)e, (Sterre3)c and (Sterre4)b, H. cf. amphioxys (Sterre3)a were provided by Ghent University (Belgium) and used for morphological studies with taxonomic revisions.

Samples and strains for light microscopy and scanning electron microscopy investigations were processed by means of a standard procedure involving treatment with 10% HCl and concentrated H₂O₂. The material was washed with distilled water. Permanent diatom preparations were mounted in Naphrax. LM observations of the natural population and monocultures were performed using a Zeiss Axio Scope A1 microscope (Germany) equipped with oil immersion objective (×100/n.a. 1.4, DIC). The ultrastructure of the valve was examined with the JSM-6510LV (Japan) scanning electron microscope. Cell measurements were based on at least 50 individuals and expressed as minimum and maximum values in the taxonomic description.

The sample selected and designated as lectotype was from the Labrador Peninsula (sample Nr. 1780) stored in the Ehrenberg collection, Museum für Naturkunde, Leibniz-Institut für Biodiversitäts- und Evolutionsforschung an der Humboldt Universität zu Berlin (BHUPM) with the mica-reference number 250502b blue. Additionally, in the Algae Collection, Botanischer Garten und Botanisches Museum Berlin-Dahlem (B) an islectotype (B 40 0040891) was selected, and the strain D27_008 was designated as epitype with its V4 domain of 18S rDNA already published[29]; rbcL and 28S rDNA sequences of Hantzchia amphioxys strain D27_008 have been submitted to NCBI in this study (for methods of extraction see Abarca et al. [8]).

Total DNA of monoclonal cultures was extracted using InstaGene Matrix according to the manufacturer’s protocol. Fragments of 18S rDNA (380 bp, including V4 domain), partial 28S rDNA gene including the D1–D3 region (753 bp) and partial rbcL plastid gene (1032 bp) were amplified using primers from Zimmerman et al. for 18S rDNA fragments, from Jo et al. for 28S rDNA fragments and from Ruck and Theriot for rbcL fragments. The rbcL and 28S rDNA sequences were used for phylogenetic reconstruction. V4 domains were used for the step–by–step comparison of the 18S rDNA sequences of our strains and other Hantzchia strains.

Amplifications of the 18S rDNA fragments, partial 28S rDNA and rbcL genes were carried out using the premade mix ScreenMix (Evrogen, Russia) for the polymerase chain reaction (PCR). The conditions of amplification for 18S rDNA fragments were: an initial denaturation of 5 min at 95 °C, followed by 35 cycles at 94 °C for denaturation (30 s), 52 °C for annealing (30 s) and 72 °C for extension (30 s), and a final extension of 10 min at 72 °C. The conditions of amplification for partial 28S rDNA were: an initial denaturation of 5 min at 95 °C, followed by 45 cycles at 94 °C for denaturation (30 s), 62 °C for annealing (30 s) and 72 °C for extension (80 s), and a final extension of 10 min at 72 °C. The conditions of amplification for partial rbcL were: an initial denaturation of 5 min at 95 °C, followed by 45 cycles at 94 °C for denaturation (30 s), 60 °C for annealing (30 s) and 72 °C for extension (80 s), and a final extension of 10 min at 72 °C.

The resulting amplicons were visualized by horizontal agarose gel electrophoresis (1.5%), colored with SYBR Safe (Life Technologies, United States). Purification of PCR products was carried out with a mixture of FastAP, 10 × FastAP Buffer, Exonuclease I (Thermo Fisher Scientific, USA) and water. 18S rDNA fragments and partial 28S rDNA and rbcL genes were decoded from two sides using forward and reverse PCR primers and the Big Dye system (Applied Biosystems, USA), followed by electrophoresis using a Genetic Analyzer 3500 sequencer (Applied Biosystems, USA).

Editing and assembling of the consensus sequences were carried out by comparing the direct and reverse chromatograms using the Ridom TraceEdit program (ver. 1.1.0) and Mega7. Newly determined sequences and DNA fragments from 27 other Hantzchia species, which were downloaded from GenBank and BOLD (taxa, strain numbers and Accession Numbers are given in Supplementary Table S3), were included in the alignments.

The nucleotide sequences of the 28S rDNA and rbcL genes were aligned separately using the Mafft v7 software and the E-INS-i model[34]. For the protein–coding sequences of the rbcL gene, we checked that the beginning of the aligned matrix corresponded to the first position of the codon (triplet). The resulting alignments had lengths of 752 (28S rDNA) and 1033 (rbcL) characters and can be found in the Supplementary Alignment S4.

The data set was analyzed using Bayesian inference method with posterior probability implemented in Beast ver. 1.10.1. [35] to construct phylogeny. For each of the alignment partitions, the most appropriate substitution model was estimated using the Bayesian information criterion (BIC) as implemented in jModelTest 2.1.10[46]. This BIC-based model selection procedure selected the following models, shape parameter α and a proportion of invariable sites (pinvar): TrNef + I and pinvar = 0.8330 for 28S rDNA; F81 + I and pinvar = 0.9030 for the first codon position of the rbcL gene; JC for the second codon position of the rbcL gene; TPM1uf + G and α = 0.8100 for the third codon position of the rbcL gene. We used the HKY model of nucleotide substitution instead of TrNef, F81, JC and TPM1uf given that they were the best matching model available for Bayesian inference method. A Yule process tree prior was used as a speciation model. The analysis ran for 10 million generations with chain sampling every 1000 generations. The parameters–estimated convergence, effective sample size (ESS) and burn-in period were checked using the software Tracer ver. 1.7.1. [36] The initial 25% of the trees were removed, the rest retained to reconstruct a final phylogeny. The phylogenetic tree and posterior probabilities of its branching were obtained on the basis of the remaining trees, having stable estimates of the parameter models of nucleotide substitutions and likelihood. ML analysis was performed using the program RAXML[37]. The nonparametric LB analysis
with 1000 replicas was used. As there are no outgroup taxa included in our analysis, we chose an unrooted tree design. The statistical support values were visualized in FigTree ver. 1.4.4 and Adobe Photoshop CC (19.0).

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Author contributions

Y.M. and M.K. designed the research; Y.M. isolated the strains of Hantzschia, made morphological and molecular descriptions, wrote the manuscript; S.M. cultured, performed the DNA extraction and PCR reactions; J.P.K. and R.J. edited the manuscript.

Competing interests

The authors declare no competing interests.

Additional information

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