

Hyalosphenia papilio paynei subsp. nov. – a highly conspicuous and localized *Sphagnum* peatland testate amoeba

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Abstract. Most eukaryotic microbial biodiversity is undescribed, and most species might be morphologically indistinguishable. Notable exceptions are so-called flagship species which are highly conspicuous and can therefore be used to address biogeographical questions. Here we describe *Hyalosphenia papilio* subsp. *paynei*, an arcellinid testate amoeba (Amoebozoa; Arcellinida; Hyalospheniidae) from wet hollows in two *Sphagnum* peatlands, one in Wales and one in Ireland. Phylogenetic analysis based on Cytochrome C oxidase subunit I (COI) sequencing places it within the lineage A of the *H. papilio* complex, but it differs from all 13 known *H. papilio* genetic lineages by its very distinctive, wider than long, morphology. The fact that such a conspicuous taxon was never reported in hundreds of studies published on Holarctic *Sphagnum* peatlands since Leidy's description of *H. papilio* in 1874 suggests that this subspecies has not dispersed and survived beyond Britain and Ireland. Furthermore its genetic similarity to *H. papilio* s. str. suggests that it has recently evolved. The discovery of this new taxon calls for a more detailed analysis of the morphological, ecological and molecular diversity of the *H. papilio* species complex.

Keywords: Testate amoebae, taxonomy, *Hyalosphenia*, dispersal limitations, microbial biogeography

INTRODUCTION

Testate amoebae are single-celled amoeboid organisms that build a test (shell). Because of their relatively

small size and high abundance, it was presumed that they had high dispersal abilities resulting in an absence of biogeographic patterns and a low diversity. However, morphological and molecular evidence have revealed a high diversity, including cryptic diversity (Kosakyan et al. 2012), and geographical distribution patterns within morphospecies in both euglyphids and arcellinids, the two major groups of testate amoebae (Heger et al. 2013; Singer et al. 2019; Lara et al. 2011). One

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of the best studied examples to date is *Hyalosphenia papilio* (Arcellinida, Hyalospheniidae), a common testate amoeba from Holarctic *Sphagnum* peatlands. Mitochondrial COI barcoding revealed the existence of at least 13 lineages that could each represent a distinct but morphologically indistinguishable species (Heger *et al.* 2013; Singer *et al.* 2019). These genetic lineages show contrasted distribution patterns, some being endemic to certain regions while others are found across the Holarctic. It has been suggested that both differences in ecological tolerances (Heger *et al.* 2013) and dispersal limitations (Singer *et al.* 2019) have shaped the current distribution of those lineages but the exact drivers of this speciation are not known.

Here, based on morphology and DNA barcoding, we describe *Hyalosphenia papilio* subsp. *paynei*, a new subspecies that is phylogenetically nested within one of the previously documented lineages of *H. papilio* but is morphologically very distinct. Furthermore, in contrast to the Pan Holarctic lineage to which it is associated, *H. papilio* subsp. *paynei* has currently only been found in two peatlands, one in Wales and one in Ireland. We discuss its validity as a subspecies and argue that it possibly evolved due to dispersal limitation rather than environmental adaptation.

METHODS

Study sites, single cell isolation, and morphometry

Testate amoebae were extracted from *Sphagnum* mosses collected in lawn microforms of two peatlands for which water table depth data were available. The first in the Cors Fochno peatland (Andrews *et al.* 2022), a raised estuarine *Sphagnum* bog on the coast of North Wales (coordinates 52.501944, -4.010833) which overlays tidal mud flats and formed during the mid-Holocene (Hughes and Schulz 2001). The second in Annaghbeg peatland, a well-preserved raised *Sphagnum* bog in the center of Ireland (coordinates 53.385278, -8.272500) that formed during the early Holocene (Meehan *et al.* 2019; Stastney and Black 2020). As we found only

three dead specimens of *H. papilio* subsp. *paynei* in a single sample from Annaghbeg bog, all analyses presented here were based on specimens found in Cors Fochno. Specimens of *H. papilio* subsp. *paynei* were spotted and isolated from three out of 36 sampled sites in Cors Fochno and observed under an Olympus IX81 inverted microscope. Morphometric measurements (length and width of the test, width of pseudostome) were taken on 87 individuals. Some individuals were rinsed in alcohol and deionized water, transferred to a scanning electron microscopy (SEM) stub, sputter coated with gold. SEM pictures were taken with a Hitachi TM 3030 at 10 kV.

Molecular analyses and phylogenetic reconstruction

Living cells were isolated individually and DNA extraction was performed following the protocol of Duckert *et al.* (2018). The PCR amplifications were conducted following the protocol of Singer *et al.* (2019) using *Hyalosphenia*-specific primers to obtain partial mitochondrial COI gene sequences. A maximum-likelihood phylogenetic tree including the DNA sequences gathered by Heger *et al.* (2013) and Singer *et al.* (2019) was built using RaxML 8.2.10 (Stamatakis 2014) with GTR + GAMMA model and automatic bootstrapping halt as implemented on the CIPRES Science Gateway v.3.3 (Miller *et al.* 2010). Sequences from genera *Planocarina*, *Cornutheca*, *Quadrulella*, *Alabasta*, *Longinebela* and *Nebela* were used as the outgroup.

RESULTS

Morphology

Hyalosphenia papilio subsp. *paynei* has a highly conspicuous shape (Fig. 1), being broadly ovate, almost triangular, about twice as wide as the typical *H. papilio* s.str. morphotype. We recorded the following measurements (Table 1, full table in Supplementary Material 1) based on 87 individuals from Cors Fochno: length: 87.5–132.5 μm (mean 108 μm), breadth: 101–160 μm (mean 127 μm), width of pseudostome: 27.5–65 μm (mean 47 μm). *Hyalosphenia papilio* subsp. *paynei* and *H. papilio* s.str. have distinct length-breadth ratios with no overlap: *H. papilio* subsp. *paynei* 0.72–1.12, mean 0.85; *H. papilio* s.str. 1.29–1.67 (Kosakyan *et al.*

Table 1. Summary of the measurements of 87 specimens of *Hyalosphenia papilio* subsp. *paynei*, showing the mean, minimum, maximum, standard deviation and coefficient of variation of the length and width of the test, the width of the pseudostome, and the length-breadth ratio.

	Mean	Min	Max	SD	CV (%)
Length	107.8	87.5	132.3	9.7	9
Breadth	127.0	100.7	160	12.3	9.7
Pseudostome Width	46.9	27.5	65	7.1	15
Length-Breadth Ratio	0.85	0.72	1.12	0.07	8.8

Supplementary material 1. Full measurement table.

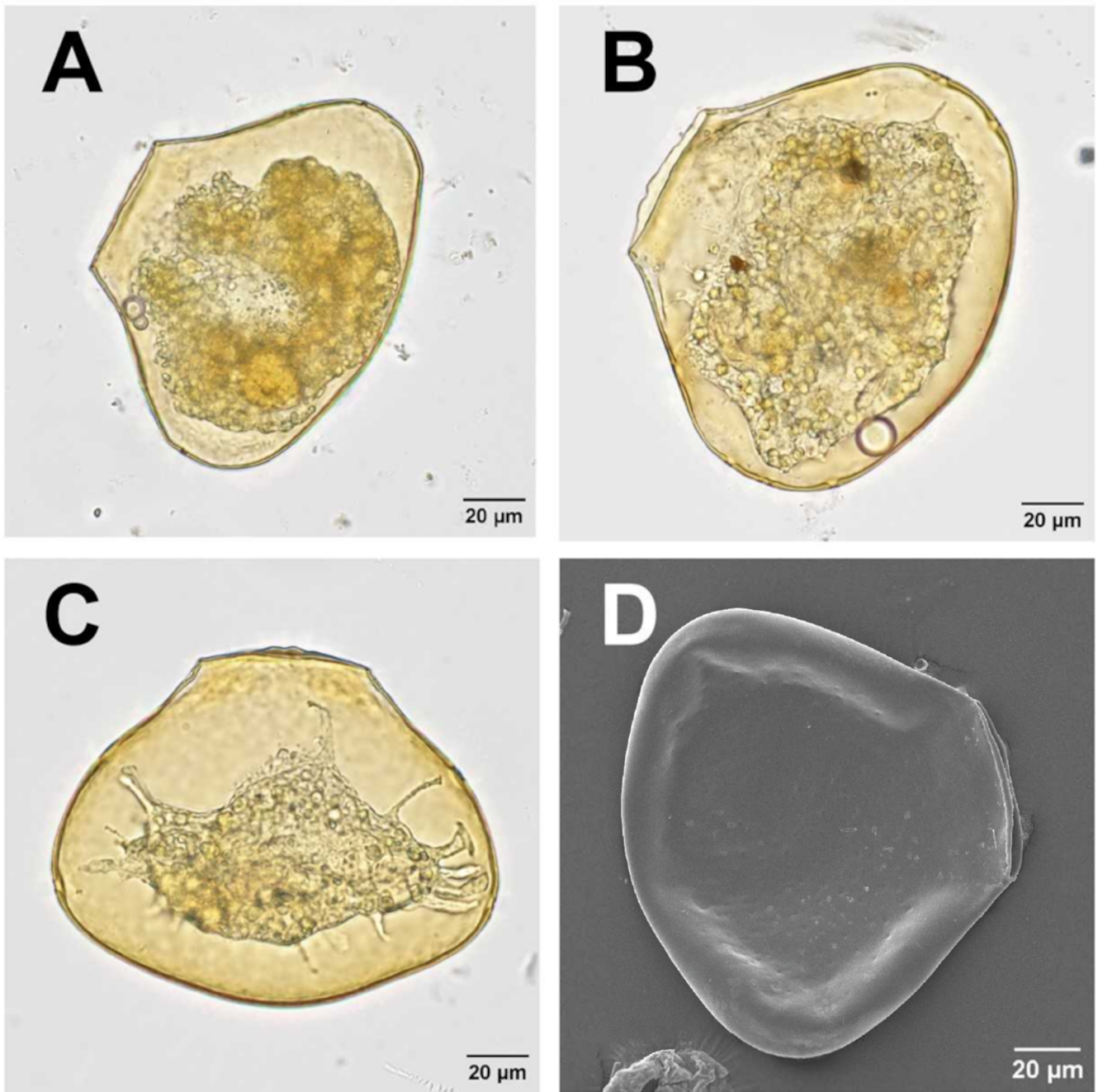


Figure 1. Pictures of four different specimens of *Hyalosphenia papilio* subsp. *paynei*, A–C with Light Microscopy (LM) corresponding to barcoded cells 2a4, 2b4, 2b5 respectively and D with Scanning Electron Microscopy (SEM).

in press). Except for the difference in width, *H. papilio* subsp. *paynei* is very similar to *H. papilio* s.str.

Ecology

In both Cors Fochno and Annaghbeg peatlands, *H. papilio* subsp. *paynei* specimens were found in the wettest sites, this taxon might thus be limited to the

wettest parts of the peatlands (i.e., bog hollows and pools). Interestingly, we found only few individuals of the typical *H. papilio* morphotype in our samples from Cors Fochno and Annaghbeg peatlands despite it being widespread and usually abundant in Holarctic peatlands. We did not find intermediate forms between *H. papilio* subsp. *paynei* and the regular morphotype.

Molecular data and phylogenetic reconstruction

We obtained partial COI gene sequences ca. 450 nucleotides long from eight individuals (Genbank accession numbers OR964076-OR964083). Four of these sequences were identical and the four others differed only by one nucleotide. In our phylogenetic tree (Fig. 2), all sequences of *H. papilio* subsp. *paynei* clustered in the same subclade within lineage A of *H. papilio* as defined by Heger *et al.* (2013) and Singer *et al.* (2019). The monophyly of *H. papilio* subsp. *paynei* is however not supported as sequences of *H. papilio* s. str. are also placed within this subclade.

DISCUSSION

H. papilio subsp. *paynei* is genetically very similar to other members of lineage A of *H. papilio* s. str.

However, clear morphological differences and the absence of intermediate morphotypes between *H. papilio* subsp. *paynei* and *H. papilio* s. str. suggest it is a valid taxon. The fact that the DNA sequences of *H. papilio paynei* all clustered in the same clade, albeit being not monophyletic, further suggests that *H. papilio* subsp. *paynei* has, from a certain point, evolved independently from *H. papilio* s. str. which justifies considering it as a distinct subspecies. Nevertheless, the use of a genetic marker with a faster mutation rate is needed to clearly evaluate the evolution of this taxon.

The morphological differences between *H. papilio* s. str. and *H. papilio* subsp. *paynei* could be interpreted as an adaptation to local environmental conditions, or possibly a result of phenotypic plasticity. Morphological changes correlated to the intensity of environmental factors have been observed for *H. papilio* s. str. in experimental mesocosm studies (Mulot *et al.* 2017) or along ecological gradients (water table depth) in

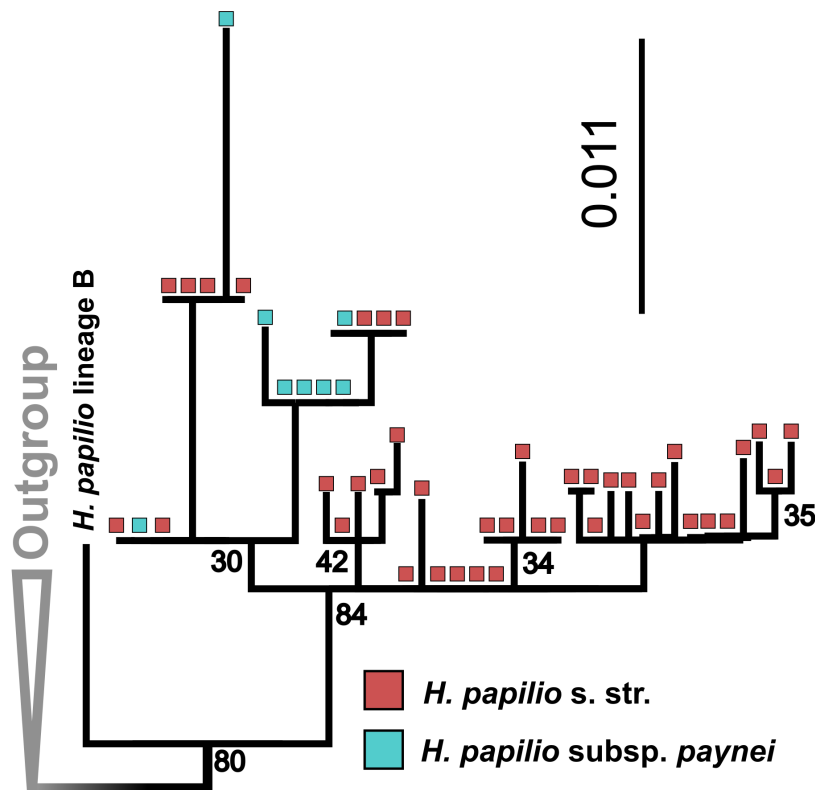


Figure 2. Maximum likelihood phylogenetic reconstruction of *Hyalosphenia papilio* based on 153 unique COI gene sequences of *H. papilio* available from Genbank (in red) and the eight sequences of *H. papilio* subsp. *paynei* obtained during this study (in blue), with a focus on the lineage A as defined by Heger *et al.* (2013) and Singer *et al.* (2019). Lineages C to M were collapsed together with the outgroup composed of seven other species within the Hyalospheniidae. Bootstrap values of 30 and above are indicated next to their respective nodes. This portion of the tree is highly magnified and long branches can be caused by single nucleotide differences.

natural settings (Booth and Meyers 2010). In addition, phenotypic plasticity has been demonstrated experimentally for other arcellinid testate amoeba genera (Wanner and Meisterfeld 1994; Porfirio-Sousa et al. 2017). However, we doubt this is the case here as we did not observe any intermediate forms between *H. papilio* s.str. and *H. papilio paynei*, in the same samples. Furthermore, the characteristic shape of *H. papilio* subsp. *paynei* is unlikely to be an on/off response to ecological factors present only in Cors Fochno and Annaghbeg peatlands, we thus rule out the hypothesis of phenotypic plasticity.

This leaves an open question: “why did we observe only so few *H. papilio* s.str. in Cors Fochno and Annaghbeg bogs?”, as these two peatlands do not seem to differ ecologically from other peatlands in Britain and Ireland where *H. papilio* is commonly reported (Amesbury et al. 2016) or from the other Holarctic sites sampled in Heger’s and Singer’s studies (Heger et al. 2013; Singer et al. 2019). The fact that typical morphotype of *H. papilio* was rare in our samples from Cors Fochno and Annaghbeg peatlands, despite having been found in abundance in other peatlands in Wales and other regions of the British Isles and Ireland (Cash and Hopkinson 1909; Heal 1961; Heal 1964) suggests either one of two things: 1) Hyalospheniid testate amoebae disperse frequently and several lineages of *H. papilio* s.str. could have reached either Cors Fochno or Annaghbeg peatland, however *H. papilio* subsp. *paynei* (or the ancestor of what will become *H. papilio* subsp. *paynei*) has displaced *H. papilio* s.str. or prevented subsequent colonization by competitive exclusion. 2) Successful dispersal events are rare, and since these peatlands formed, they were colonized by *H. papilio* subsp. *paynei* (or its ancestor) which was then genetically isolated from *H. papilio* s.str. which might have arrived later. As Heger et al. (2013) and Singer et al. (2019) found up to four lineages (each possibly a distinct species) of *H. papilio* in the same peatland, exclusion by competition seems unlikely. Additionally, as these studies showed that *H. papilio* is rather limited in its dispersal abilities, we thus favor the second hypothesis. A detailed exploration of peatlands in the region would be useful to clarify if this taxon is endemic to only two (relatively) distant sites, which would be truly remarkable or if, more likely, it occurs in other sites in Britain and Ireland or beyond (e.g. Brittany). Furthermore, analyzing subfossils test from peat cores in both sites and surrounding peatlands could reveal which of the two morphotypes was there first and if *H. papilio* subsp.

paynei had a previously wider distribution. This seems unlikely as there has been quite a large number of palaeoecological studies done in the British Islands over the last decades (Swindles et al. 2019) and this conspicuous taxon has never been reported.

The case of *H. papilio* subsp. *paynei* is likely not unique and it is possible that a non-negligible fraction of the testate amoeba diversity is composed of hyper endemic taxa that diverged only recently. However, most of them might be morphologically or genetically indistinguishable from their parental lineage and formally describing these would not be useful. Such cryptic and pseudo-cryptic microspecies pose challenges for testate amoeba taxonomy as shown in the case of *H. papilio* (Heger et al. 2013; Singer et al. 2019) and the *Nebela tinctoria* complex (Singer et al. 2018). They are nevertheless useful models for the study of microbial biogeography and ecology.

CONCLUSION

Arcellinid testate amoebae are a relatively well-studied group of free-living microorganisms but nevertheless some highly conspicuous taxa remain to be discovered, as recently show with the examples of *Arcella peruviana* (Reczuga et al. 2015), *Arcella gandalfi* (Féres et al. 2016), *Apodera angatakere* (Duckert et al. 2021) and *Certesella larai* (Bobrov et al. 2022). While these examples may not be very surprising given that they were found in relatively poorly studied regions, *Hyalosphenia papilio* is however one of the best studied testate amoeba taxa and is very common in northern *Sphagnum* peatlands, arguably the most intensively studied ecosystem for testate amoebae since more than a century. The odds of finding a new taxon, especially a large and conspicuous one, were therefore low. This finding should motivate anyone studying testate amoebae to look for unusual morphotypes and report them. *Hyalosphenia papilio* subsp. *paynei* is so conspicuous that it should be easy to establish its geographical distribution and ecology accurately and we therefore call anyone studying peatland testate amoebae to report this new taxon, and ideally collect material for DNA sequencing. This is clearly a golden age for protistology and microbial biogeography.

TAXONOMIC ACTION

Description of new subspecies: *Hyalosphenia papilio* subsp. *paynei* DUCKERT, GREEVES, BLANDENIER, PAYNE, MITCHELL

Taxonomic summary:

Arcellinida Kent 1880.

Hyalospheniidae (Schultze 1877) Kosakyan *et al.* 2012.

Hyalosphenia (Stein 1857) Schultze 1877

Hyalosphenia papilio Leidy 1874

Hyalosphenia papilio paynei subsp. nov.

Description: Test proteinaceous, rigid, diaphanous, yellowish, lacking idiosomes or xenosomes, compressed, slightly wider than long, broadly ovate in broad view, almost triangular, with a maximal width at about one quarter from the fundus with concave sides tapering towards the aperture, sometimes with a prominent fundus. Pseudostome slightly convex in broad view, no distinct neck. Lateral pores usually present at the maximal width. Algae endosymbionts (presumably from genus *Chlorella*) present in the cytoplasm. Measurements: length = 87.5–132.5 μm (mean 108 μm); breadth = 100.5–160 μm (mean 127 μm); width of the pseudostome = 27.5–65 μm (mean 47 μm).

Comparison with related taxa: *Hyalosphenia papilio* subsp. *paynei* can be easily distinguished from other taxa within the *H. papilio* species complex by its length-breadth ratio: *H. papilio* subsp. *paynei* (0.72–1.16, mean 0.85), *H. papilio* (1.29–1.67).

Etymology: We dedicate this subspecies to our esteemed colleague Richard Payne who first reported this taxon and initiated this work. He tragically passed away in a mountaineering accident in the Indian Himalaya in May 2019 and could not witness the completion of this work, we thus consider appropriate to name this taxon after him.

Type: We declare the specimen in Figure 1. A as the type. Unfortunately, all type material has been lost.

Type locality: Cors Fochno peatland in Wales. Coordinates: 52.501944, -4.010833

The COI gene sequences of *H. papilio* subsp. *paynei* have been submitted to GenBank under the accession numbers: OR964076-OR964083

LSID number for the taxonomic action: urn:lsid:zoobank.org:act:3265AC2C-81F5-48EF-BFD9-E97EF719A536

LSID number for this publication: urn:lsid:zoobank.org:pub:51225923-554D-4DC7-B267-13E6AE9E-8A8B

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