## Protist

#### **ORIGINAL PAPER**

# Phylogeny, Evolution, and Taxonomy of Vannellid Amoebae

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We sequenced 18S rRNA genes from 21 vannellid amoebae (Amoebozoa; Vannellidae), including nearly all available type cultures, and performed a comprehensive phylogenetic analysis for 57 Vannellidae sequences. The results show that species of Vannella and Platyamoeba are completely mixed and do not form distinct clades. Several very closely related species pairs exist, each with a Vannella and a Platyamoeba species differing in only a few nucleotides. Therefore, presence (Vannella) or absence (Platyamoeba) of glycostyles in the cell surface coat is an invalid generic distinction; the genera must be merged. As Vannella has priority, we formally transferred Platyamoeba species into Vannella, except for the non-vannellid P. stenopodia, here renamed Stenamoeba stenopodia gen. n. comb. n. and transferred to the family Thecamoebidae. Our trees show that Vannella glycostyles were probably easily and repeatedly evolutionarily lost. We have established a new genus Ripella, with distinct morphology and sequence signatures for Vannella platypodia and morphologically similar species that form a clearly separate clade, very distant from other Vannellidae. Vannellids form four well-separated single-genus clades: Vannella sensu stricto, Ripella, Clydonella, and Lingulamoeba. Species of the revised genus Vannella comprise four closely related, well-supported subclades: one marine and three freshwater. Here, we provide an illustrated checklist for all 40 known Vannellidae species.

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#### Introduction

Flattened, fan-shaped amoebae of the family Vannellidae have only recently been accepted as an important major amoebozoan taxon. Once classified by Page (1987) in the class Lobosea subclass Gymnamoebia, they are now considered as members of the phylum Amoebozoa, class

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Discosea (Cavalier-Smith et al. 2004). Initially, they were dispersed among various genera and families. Probably the first reliably described vannellid was '*Amoeba platypodia*' (Gläser 1912). Schaeffer (1926) included vannellid amoebae then known in three genera, all in different families: *Flabellula*, where he placed '*F. mira*' (now *Vannella mira*); *Rugipes*, where he placed *R. vivax* (now *Clydonella vivax*), and *Unda* with a not yet

redescribed species *U. maris*. Wohlfarth-Bottermann (1960) added a fourth genus to this collection: '*Hyalodiscus simplex*' (now Vannella *simplex*). Bovee (1965) established the genus Vannella, for three of these species — 'Flabellula mira', 'Amoeba platypodia', and 'Hyalodiscus simplex'; he redefined the genus *Flabellula*, restricting it to amoebae producing trailing uroidal filaments and short conical subpseudopodia, neither character being present in Vannella. However, he considered the genus Vannella as closely related to flabellulid amoebae, so placed it in the family Flabellulidae; later he created a separate subfamily Vannellinae within Flabellulidae (Bovee 1970).

F.C. Page initially did not accept the genus Vannella and provided a new diagnosis for Schaeffer's species 'Flabellula mira' (Page 1968). The same paper described a new species, Rugipes placidus, thus accepting the validity of Schaeffer's genus Rugipes. However, a year later Page accepted Bovee's revision of Flabellula and noted that species redescribed by him as Flabellula must be identified as Vannella; he also established the genus Platvamoeba to accommodate Rugipes placidus and a newly described species, P. stenopodia (Page 1969). He believed these amoebae to be the closest relatives of thecamoebids, not flabellulids, so placed Platvamoeba in the family Thecamoebidae. Pussard (1973) created a genus Pessonella for a single species of Vannella-like amoeba - Pessonella marginata, possessing characteristic lobes on the surface of the frontal hyaloplasm, placing it in the family Mayorellidae; later Page (1976) transferred all these genera into Thecamoebidae.

Sawyer (1975a,b) established two more genera of vannellid amoebae - Clydonella and Lingulamoeba - to accommodate Vannella-like amoebae differing from both Vannella and Platyamoeba in locomotive morphology and floating form. The type species of Clydonella was Schaeffer's Rugipes vivax. Differentiation of these two genera from Vannella and Platyamoeba was difficult, since no ultrastructure was provided at the time of description. Page (1983) considered Lingulamoeba a junior synonym of Platyamoeba (the combined name Platyamoeba leei was provided), and listed Sawyer's Clydonella among 'other genera of Thecamoebidae'. Later Page (1987) started to consider all these genera (and Pessonella) as possible synonyms of Vannella or Platyamoeba (Page 1988). His revised classification of naked amoebae raised the rank of Bovee's subfamily Vannellinae to family Vannellidae, which included only two genera: *Vannella* and *Platya-moeba* (Page 1987). Later Rogerson and Patterson (2002) included *Clydonella* and *Pessonella* in the family Vannellidae together with the little-known genera *Discamoeba* Jahn, Bovee and Griffith 1979 and *Unda* Schaeffer 1926, but omitted the genus *Lingulamoeba*, thus accepting Page's suggestion that it is a synonym of *Platyamoeba* (Page 1983).

These taxonomic perturbations well illustrate the difficulty of morphological systematics of vannellid amoebae. The primary reason for this is that all vannellids are flattened, fan-shaped organisms with a very limited number of lightmicroscopic (LM) morphological characters. Page (1983) noted that a vannellid amoeba with slender, tapering pseudopodia on the floating form can be safely classified as Vannella; however, further studies show that some vannellas do not form such pseudopodia when afloat (Smirnov 2001), while some platyamoebian floating forms may be very similar to those of vannellas (Smirnov 1999). Therefore, when electron-microscopic (EM) studies revealed clear differences in cell surface structure between the then known species of Vannella (having glycostyles) and Platvamoeba (lacking glycostyles) (Page and Blakey 1979), this cell surface structure immediately became the key, and virtually only, character used to distinguish these genera and to classify all newly discovered vannellids into one of them. Page (1979) provided a new diagnosis of the genus Vannella that included cell surface structures. However, this character was not always congruent with LM features of a species. For example, Page (1980b, p. 939) wrote that it was a 'considerable surprise' to find that a *Platyamoeba*-like strain of a vannellid amoeba nowadays known as Vannella anglica possesses glycostyles.

Molecular phylogenies showed vannellid amoebae as a distinct monophyletic lineage of Amoebozoa, well separated from the other groups (Bolivar et al. 2001; Fahrni et al. 2003; Sims et al. 1999). Comprehensive analyses of amoebozoan SSU rRNA trees by Cavalier-Smith et al. (2004), Kudryavtsev et al. (2005), and Smirnov et al. (2005) strongly confirmed that all vannellids form a distinct clade on the SSU tree, consisting of four genera: Vannella, Platyamoeba, Clydonella, and Lingulamoeba. Peglar et al. (2003) provided SSU rRNA sequences and ultrastructure of Clydonella and Lingulamoeba, which confirmed the validity of both genera and the monophyly and distinctiveness of vannellid amoebae as a whole. However, relationships between the genera *Vannella* and *Platyamoeba* remain ill-resolved. It was shown that *Platyamoeba stenopodia* is not a vannellid, and is closely related with thecamoebids (Fahrni et al. 2003). Sims et al. (2002) using partial SSU rDNA sequences found that *Vannella* and *Platyamoeba* are mixed in the tree and suggested that their phylogeny is related more to LM features and to habitat (freshwater or marine) than to cell surface structure. Finally, Dyková et al. (2005a) obtained sequences of fish-associated vannellids identified only to genus, confirming that strains with a surface structure formerly attributed to *Vannella* and *Platyamoeba* do not form distinct clades in the tree.

GenBank now has a good number of 18S rRNA gene sequences of vannellids, but most belong to unnamed isolates. The number of complete sequences belonging to named species remains small and limits further progress in molecular identification of these amoebae. We now provide 22 new SSU rRNA gene sequences of vannellid amoebae, including all available type cultures, and a comprehensive phylogenetic analysis of all available near-complete 18S rRNA gene sequences of Vannellidae. We taxonomically revise vannellid amoebae and provide an illustrated checklist in order to clarify the number and status of all species in this group of widely distributed and often mentioned amoeboid protists.

#### **Results and Discussion**

### 1. Overall Configuration of the Phylogenetic Tree

All Bayesian and ML trees that included outgroups to vannellids revealed a large and strongly supported monophyletic clade corresponding to the family Vannellidae, i.e. all members of the genera Vannella, Clydonella, and Lingulamoeba, and all Platyamoeba except for Platyamoeba stenopodia, which consistently grouped with Thecamoebidae instead (Fig. 1) and is clearly not a vannellid (see below). The BioNJ distance trees also showed a monophyletic Vannellidae but this had no bootstrap support because the bootstapped consensus tree did not show Vannellidae as monophyletic because of the intrusion of Cochliopodium within Vannellidae. The order Dactylopodida was also consistently revealed as a clade by Bayesian and ML trees but only with moderate support; distance trees did not show their monophyly, as the very long-branch Vexillifera minutissima went elsewhere (probably a long-

branch attraction artefact). The bipartition between Glycostylida/Himatismenida ('core Discosea') and all the other taxa was reproducible but had only moderate to low support. Some trees suggest that Cochliopodium is sister to Dactylopodida plus Vannellidae, but others put it as sister of Vannellidae alone, but all trees grouped these three taxa together, albeit with only weak to moderate support. Figure 1 strongly confirms that Multicilia belongs to Varipodida (into which we now transfer it from Glycostylida), making the class Flabellinea (Smirnov et al. 2005) and the order Glycostylida (Cavalier-Smith et al. 2004) identical in composition.

Our trees also support earlier indications from SSU rDNA trees (Kudryavtsev et al. 2005; Smirnov et al. 2005) that *Dermamoeba* is not closely related to *Thecamoeba*, contrary to its inclusion in Thecamoebidae by Page (1988). In Bayesian trees, *Dermamoeba* groups with *Mayorella*, but this is weakly supported and, though consistently found with different taxon samples, is not seen on all distance or ML trees. Our trees show Thecamoebidae as sisters to Acanthopodida; however, this affiliation has almost no morphological support from either LM or EM.

### 2. *Platyamoeba stenopodia* Page, 1969 is not a Vannellid, but a Thecamoebid

Figure 1 confirms previous phylogenetic evidence that *Platyamoeba stenopodia* Page, 1969 is not a vannellid (Cavalier-Smith et al. 2004; Fahrni et al. 2003; Kudryavtsev et al. 2005) and clearly supports the idea that it should be classified in Thecamoebidae (Smirnov et al. 2005), not Vannellidae. Bootstrap support for *P. stenopodia* being sister to other Thecamoebidae is stronger with PHYML than BioNJ, but it was consistently clearly excluded from Vannellidae — and core Discosea — by all methods. By contrast, all 20 other *Platyamoeba* strains are robustly nested within the genus *Vannella*.

*Platyamoeba stenopodia* was included in this genus when few vannellid species were known (Page 1969). Further accumulation of data indicated that *P. stenopodia* differs in LM characters from all other platyamoebians, including the type species of the genus — *P. placida*. Its narrow, oblong, linguliform (not fan-like) shape, a tendency to acquire longitudinal ridges on the dorsal surface, and the complete absence of hexagonal structures in the glycocalyx were previous reasons for suggesting that it may be related to *Thecamoeba*, not *Platyamoeba* (Page and Blakey 1979; Smirnov and Goodkov 1999). Its earlier grouping



**Figure 1**. Bayesian tree for 57 vannellid SSU sequences plus 35 close outgroups from Discosea and Variosea (Amoebozoa). The 29 new sequences are in bold. The GTR model allowing for covarions and autocorrelation among nearby sites was used for 1577 alignment positions. In a few strains showing microheterogeneity among clones, more than one clone sequence is included (except for *V. plurinucleolus* CCAP 1565/7, these all robustly group together). Where the tree is especially crowded, support values (posterior probability on left, then BioNJ bootstrap, and then ML approximate bootstrap percentages using the LRSH edge support tool of Treefinder (Jobb 2006)) are not shown within the tree but by marker lines after the names comprising a clade (for clarity omitted within the terminal trivially divergent *V. plurinucleolus* clade). Molecular clones are indicated; the remaining new sequences were obtained directly from the PCR products. Sequences obtained from type strains are recognized as 'Type'. 'Variant 1' and 'Variant 2' are used to recognize two slightly different versions of *V. danica* sequences obtained with two different sets of selective primers.

with *Thecamoeba similis* in SSU rDNA phylogenetic trees (Cavalier-Smith et al. 2004; Fahrni et al. 2003; Kudryavtsev et al. 2005; Smirnov et al. 2005) and its present well-supported grouping as sister to the better sampled thecamoebid clade (*Thecamoeba*, *Sappinia*) and total failure to group

with recognized vannellids support this and decisively show that it is not closely related to other *Platyamoeba* strains or to *Vannella* or any other vannellid genus.

In LM characters P. stenopodia most resembles some striate Thecamoeba (Smirnov and Goodkov 1999). However, the anterior hyaline area in P. stenopodia is often larger than in most thecamoebids, and this species has a strong tendency to lack dorsal folds in locomotion. In contrast to most thecamoebids, the floating form of P. stenopodia has relatively long, tapering pseudopodia, which is not characteristic for Thecamoeba. P. stenopodia forms cysts, but no cyst-forming species of Thecamoeba are known yet, although cysts occur in Sappinia diploidea, closely phylogenetically related with Thecamoeba (Goodfellow et al. 1974). Thus, morphology is consistent with the molecular evidence that P. stenopodia is unrelated to vannellids but is most closely related to thecamoebids, although not belonging to Thecamoeba itself. Given this congruence of molecular and morphological evidence, we formally transfer Platyamoeba stenopodia to the family Thecamoebidae. Because this species demonstrates a unique combination of LM characters and fails to group specifically with Thecamoeba in molecular trees, we establish the new genus Stenamoeba for it.

Diagnosis: *Stenamoeba* gen. nov. Narrow, oblong, linguliform amoebae with large anterior hyaline area, occupying about half of the cell. Several parallel longitudinal ridges and few lateral folds are common, but ridges are never stable for a long time. In locomotive cells, the single nucleus usually maintains its position at the border between the granuloplasm and the hyaloplasm. Single-walled cysts; glycocalyx thin, amorphous.

Type species: *Stenamoeba stenopodia* (Page 1969) comb. nov.

Etymology: Gk stenos narrow + amoeba.

#### 3. Phylogeny of Vannellid Amoebae

All analyses, both with (Fig. 1) and without outgroups (Fig. 2) and independently of the method used, show that vannellids fall into four very distinct robust clades. Our analysis confirms the independent status of the genera *Clydonella* and *Lingulamoeba* shown by Peglar et al. (2003). However, for *Vannella* and *Platyamoeba* the SSU rRNA tree of vannellid amoebae clearly contradicts classical generic distinctions. Strains belonging by cell surface structure to the genera *Vannella* and *Platyamoeba* are intimately mixed in the tree, yet form two clearly separate clades, both 'generically' mixed: a small clade consisting of the smallest Vannella and Platvamoeba species with unusually short SSU rRNA genes (about 1700 bp), which is robustly sister to Clvdonella (Figs 1, 2) and a large clade comprising the rest of the species possessing markedly longer SSU rRNA genes (~2000 bp), which is robustly sister to the other three clades combined (we assume that sequence AY929908 is in reality from a Lingulamoeba not a Vannella). The absence of a Vannella/Platyamoeba dichotomy on the trees is congruent with previous findings (Dyková et al. 2005a; Kudryavtsev et al. 2005; Peglar et al. 2003; Sims et al. 2002) and shows that the traditional distinction between Vannella and Platyamoeba by surface structure is invalid. The genus Platyamoeba must be regarded as a junior synonym of Vannella, and all Platyamoeba species except the former P. stenopodia (see above) are here transferred to Vannella (Section 5.2). We establish a new genus Ripella for the smallest vannellid amoebae with an unusually short SSU rRNA gene (Section 5.3) and use the new names below for clarity: the name Platyamoeba is now used only to identify former members of the genus when appropriate.

By all methods, amoebae of the genus Vannella (in our new definition) form four distinct clades, which are robust and consistent among methods and are the same for both data sets. The largest of these clades includes all marine species and unnamed isolates (Figs 1 and 2), the other three consist of only freshwater and brackish-water species. Unfortunately, the branching order of these four clades is not robust, making the ancestral state somewhat uncertain. With the larger taxonomic sample including outgroups, but excluding the most rapidly evolving sequence regions, the Vannella simplex/persistens/danica freshwater/brackish clade is sister to the other three with all methods used (Fig. 1 and other trees not shown). For the vannellid-only data set, including more rapidly evolving regions, this is true for the Bayesian trees (Fig. 2) and PHYML using a Bayesian tree as starter, but BioNJ and PHYML using BioNJ trees as starter, weakly group all three freshwater clades together as sister to the marine clade. Bayesian methods are less likely to be biased by long-branch attraction effects than BioNJ, and such effects should be less serious for Figure 2 data set as the fastest regions were excluded.

We also used Treefinder to calculate ML trees for the vannellids only data set. Irrespective of whether we started with trees produced by



**Figure 2**. Bayesian tree for 57 vannellid sequences. The 29 new sequences are in bold. The GTR model allowing for covarions and autocorrelation among nearby sites was used for 1906 alignment positions. In a few strains showing microheterogeneity among clones, more than one clone sequence is included (except for *Vannella plurinucleolus*, these all robustly group together). Where the tree is especially crowded, support values (posterior probability on left, then BioNJ bootstrap, and then ML approximate bootstrap percentages using the LRSH edge support tool of Treefinder (Jobb 2006)) are not shown within the tree but by marker lines after the names comprising a clade (omitted within the terminal *V. plurinucleolus* clade). Molecular clones are indicated with 'cl'; the remaining new sequences were obtained directly from the PCR products. Sequences obtained form type strains are recognized as 'Type'. 'Variant 1' and 'Variant 2' are used to recognize two slightly different versions of *V. danica* sequences obtained with two different sets of selective primers.

neighbor joining (NJ), the best PHYML tree, or the best Bayesian tree, this produced trees of much lower likelihood than PHYML, in which the three freshwater clades were a single joint branch nested weakly within the marine group. We are unsure why Treefinder yielded such lower likelihood trees than PHYML, but it might be because only four gamma rate categories were used, which would model intrasite variation less well for this data set (with the highly aberrant, very fast evolving *Ripella* sequences) than the nine gamma rate categories used for PHYML and Bayesian analyses. Treefinder also showed that *Rippella* 18S rDNAs all have a very different base composition, with markedly fewer Ts and markedly more Gs than all other vannellids.

There is a striking segregation of marine and freshwater strains also between the other three genera. Ripella strains are exclusively from freshwater and Clvdonella and Lingulamoeba are marine. If the Bayesian topologies are correct (and ignoring the freshwater/brackish distinction in the V. simplex/ persistens/danica clade), only three switches between marine and freshwater/brackish, or vice versa, need be inferred during the vannellid evolutionary history. If the ancestor was in freshwater, there were three independent marine colonizations; if the ancestor was marine, two switches to freshwater, plus a reversion of the freshwater Vannella subclade to marine must have occurred. If the vannellid BioNJ tree were correct (less likely) and the vannellid ancestor was marine, only two switches to freshwater must have occurred; if the ancestor was freshwater, there were three independent switches to marine. A third or fourth switch to brackish by V. danica is needed for both scenarios, but such a switch was probably adaptive and related to the specificity of its particular habitat (Smirnov et al. 2002). The number of switches is so similar on the different scenarios that they cannot be used to decide by parsimony which tree is correct or whether the vannellid ancestor was marine or freshwater. What they do indicate is rather striking: probably only two or three major habitat switches between marine and freshwater occurred during the approximately 600 My of vannellid history (this age is based on the proportions of the amoebozoan Bayesian tree (Kudryavtsev et al. 2005) and the evidence that Amoebozoa are at least 760 My old (Porter and Knoll 2000; see also Cavalier-Smith 2006), assuming a simple molecular clock. This confirms the hint from the studies of Sims et al. (2002) that gross habitat preference is more conservative than the presence or absence of glycostyles. It also contradicts the widespread assumption that protozoa move readily between marine and freshwater, and thus extends the evidence for evolutionarily stable ancient marine and freshwater clades that have been found in several zooflagellate groups (von der Heyden and Cavalier-Smith 2005; von der Heyden et al. 2004) and in microsporidia (Vossbrinck and Vossbrinck 2005).

### 4. Molecular Identification of Species and Species Groups within the Genus *Vannella*

Initially, we hoped that sequence data from type and accurately named cultures would allow us to identify most unnamed vannellid sequences in GenBank. However, our results indicate that molecular distinction of species is difficult among vannellid amoebae. There are two primary reasons for this: (1) very slight divergence of SSU rRNA genes between some morphospecies and (2) microheterogeneity among clones obtained from the same PCR products. Anyway, in our trees Vannella species (in our new definition of this genus) form a number of subclades, most of which are well supported. All these species groups include species with Platyamoeba-type and with Vannella-type cell surface structures, except for Vannella simplex - V. persistens -V. danica clade, consisting only of amoebae with Vannella-type surface.

### 4.1. Vannella aberdonica – V. devonica Species Group

This group of four very closely related sequences, with 100% support by all three methods, consists of Vannella aberdonica, V. devonica, and two Platyamoeba strains sequenced by Dyková et al. (2005a). All these strains are morphologically clearly different. especially V. devonica and V. aberdonica, which have different size, locomotive, and floating form as well as nuclear structure. Yet, these two species differ only by 10 bp in their SSU rDNA sequences. Likewise sequences of the two Platyamoeba strains have a very high level of mutual sequence identity to V. devonica, varying from 0.966 to 0.982 (see Table S1 of the Supplementary Materials). Again, these two strains, despite their similarity in size, look rather different in LM and have cell coats differing in appearance (Dyková et al. 2005a). Both are smaller than V. devonica but generally larger than V. aberdonica. The similarity of direct sequences within this species group exceeds that of clones from the same PCR product in some other species (See Sections 4.2 and 4.4).

### 4.2. Vannella anglica — V. plurinucleolus Group of Sequences

This group is very tight indeed on the trees and has 100% support from all three methods, yet there is an important discrepancy with previous data. The sequence of *Platyamoeba plurinucleolus* GenBank AF464921 (partial sequence, not included in our trees) obtained by Sims et al. (2002) differs radically from all our sequences of *P. plurinucleolus* CCAP 1565/11 and 1565/7 strains. This is surprising, suggesting a data error, because Sims et al. (2002) supposedly sequenced the *P. plurinucleolus* CCAP 1565/11 strain. We performed direct sequencing of both P. plurinucleolus strains and cloning of the initial PCR product from both strains. Clonal sequences of 1565/7 (the type strain) had only 6 bp difference between each other, with no structure difference, but were rather different from the direct sequence, showing even some structure divergence in the variable region 1499-1528. It looks as if the direct sequence had a few 2-3bp deletions in this region. Clones of 1565/11 strain show 8 bp difference between each other. In general, all our sequences of P. plurinucleolus are very similar to each other and to the sequence of V. anglica CCAP 1589/8, confirming Page's statement based on LM that it is a very 'Platyamoeba-like' Vannella (Page 1980b). A sequence identity matrix for this group of species is in Table S2 (see Supplementary Materials).

The strain of *P. plurinucleolus* ATCC 50745 sequenced by Peglar et al. (2003) is very distant from both *P. plurinucleolus* marine strains from CCAP. It robustly groups among freshwater vannellids. The site of isolation of this strain is given as 'Patuxent river (USA)', and we suggest that it is another vannellid species, freshwater in origin and with peripheral nucleoli, thus superficially resembling *P. plurinucleolus*. It must be described as a new species. Another new species within this species group but more distant from other species is an unnamed strain with platyamoebian cell coat, isolated from the salt channels in Ebro Delta (Spain), here recognized as *Vannella* sp. ED40.

## 4.3. Platyamoeba bursella — Platyamoeba calycinucleolus — Vannella arabica Group of Species

This species group also has 100% support by all three methods and is of special interest, because its core are two morphologically distinct species with nearly identical sequences: Vannella arabica 1589/7 and 'Platyamoeba' bursella 1565/ 10 (Table S3 of the Supplementary Materials). Only 5 bases differ, one at the beginning of the sequence, the rest in the most variable parts of the molecule. These differences are much smaller than between clones of the same PCR product from some other species (e.g. strains of Platyamoeba plurinucleolus, see Table S3). To avoid any errors, both sequences were double checked by obtaining DNA and sequencing it from a freshly ordered culture; the results were identical. The similarity of the Vannella sp. AY929906 sequence of Dyková et al. (2005a) with all species of this group is also very high. Yet all these species are rather different morphologically. The largest,

calvcinucleolus, is  $22-56 \,\mu m$  in maximal Р. dimension (average  $32 - 39 \mu m$ ) and has a peculiar nuclear structure. Vannella arabica is much smaller (17-37 µm in maximal dimension, average 27 µm). Platyamoeba bursella has similar size (14-37 µm, average 19-25 µm), but its locomotive forms are rather different from those of V. arabica (clearly spatulate or semicircular versus generally fan-shaped in V. arabica). All these species have relatively short, blunt and curved pseudopodia in the floating form. There are no detailed LM data on the Dyková et al. (2005a) strain, but from their photographs it appears somewhat smaller than P. bursella, to which it is sister, and to have a peculiar locomotive morphology. These differences exclude possible morphological misidentification of either strain.

#### 4.4. Vannella simplex — V. persistens — V. danica Species Group

Our results invariably show this group 100% supported and probably the most divergent species group, consisting of all freshwater V. simplex strains, the soil species V. persistens described by Smirnov and Brown (2000), and a brackish-water V. simplex Nivå Bav strain. described by Smirnov et al. (2002). Here, we redescribe it as a new species Vannella danica, and for consistency use this name only henceforth. The phylogenetic distances among all freshwater strains of V. simplex are negligible, while V. danica and V. persistens are relatively distant from them. The soil species V. persistens is distinctly more closely related to the freshwater V. simplex strains (they are sisters with 99% support by PHYML (Fig. 2) and 100% (Fig. 2) or 78% (Fig. 1) support by BioNJ) than to the brackish-water V. danica (Table S4 of the Supplementary Materials).

Although these data show that *V. persistens* is nested within the former *V. simplex*, and might therefore be regarded as simply an unusual *V. simplex* strain, there is such great molecular divergence between the freshwater and brackish water *V. simplex* clades, each of which has 99% bootstrap support by PHYML, that we now consider them separate species. *V. danica* shows some unique sequence characters, not present in other strains (Table S5 in Supplementary Materials). Again, from this table it is evidently more divergent from freshwater strains of *V. simplex* than from *V. persistens*.

All strains of the *V. simplex* species group are similar in shape, size, overall appearance, and morphology of the locomotive form, except for

V. persistens which is smaller and more pronouncedly fan-shaped than all V. simplex strains (Smirnov and Brown 2000). Vannella danica is smaller in average size than freshwater V. simplex strains and measures maximally about 32 µm (Smirnov et al. 2002) against 42-52 µm in freshwater isolates of V. simplex (Page 1988). No known freshwater strains of V. simplex form cysts, while both V. persistens and V. danica are cyst-forming, which from the tree must be the ancestral state for this species group. Their cysts differ in size and wall structure (see Smirnov and Brown 2000; Smirnov et al. 2002). Both V. persistens and V. danica lack simple filaments among glycostyles, while freshwater strains of V. simplex possess them under the same conditions of fixation and observation (Smirnov et al. 2002). Consistently with this dichotomy, the Vørs (1992) strain of 'V. simplex' from the Gulf of Finland, also lacked simple filaments among glycostyles, judging from her illustrations.

These clear differences taken together with the molecular data indicate that V. danica, despite a general similarity of the trophozoite with freshwater V. simplex must be recognized as an independent species. If instead we unified all these strains (including V. persistens) under the name V. simplex with a very broad diagnosis, much broader than for any other Vannella species, that 'species' would have a molecular distance between strains greatly exceeding most of those between other vannellid species and the important differences in habitat and cysts would be ignored, which would be inadvisable. Therefore, based on both morphological and molecular evidence, we establish a new species, Vannella danica for the brackish strain, described by Smirnov et al. (2002) as V. simplex Nivå Bay isolate. We emend the diagnosis of V. simplex by Smirnov et al. (2002) to eliminate characters of V. danica and clarify this species distinction (Section 8).

The analysis of relationships in this species group is slightly complicated by the relatively large divergence between the direct sequence of *V. simplex* 1589/3 and the sequence of one of the clones from the same PCR product. Data suggest that this clone most resembles the direct sequence of 1589/3 strain (which is logical), but the sequence identity of this clone and 1589/3 strain is lower than between, e.g. *V. persistens* and *V. danica* (Table S4 of the Supplementary Materials).

### 4.5. Vannella lata — V. miroides Group of Sequences

This 100% bootstrap-supported clade includes five relatively distant species and isolates, and a

sequence identity matrix for this species group is provided in Table S6 (see Supplementary Materials). Most closely related are Vannella lata CCAP 1589/12 strain and V. miroides ATCC 30945 (GenBank sequence AY183888A) (100% support as sisters). However, they show remarkable sequence differences (e.g. in positions 1189 - 12031909 - 1923and relative to V. anglica, confirming their independent status. V. lata is remarkable, normally showing a widely fan-shaped or even crescent-shaped locomotive form, with the breadth nearly twice its length, while V. miroides is semicircular or narrowly fanshaped. Vannella strain CH88/I (sequence number AY929912) and Platyamoeba VV/I (sequence AY929923) were isolated by Dyková et al. (2005a) and are similar in size to V. lata; Vannella CH88/I also resembles V. lata in locomotive morphology, but the relatively large sequence divergence, much exceeding interspecific sequence divergence, in e.g. V. simplex, does not allow us to recognize it as a strain of this species. The strain Vannella cf. miroides (Geneva) isolated by Smirnov in 2002 shows clearly different sequence from that of V. miroides ATCC 30945: because the Geneva strain was lost and the accumulated data are insufficient for species description, we leave it unnamed.

### 4.6. Unnamed Sequences by Dyková et al. (2005a) and other *Vannella* Groups

There are two independent clades of unnamed sequences obtained by Dyková et al. (2005a), each forming a very tight cluster with 100% support. Most different in sequence from other vannellids is that comprising three species of Vannella freshwater (GenBank AY929909: AY929910; AY929911). These sequences are mutually rather similar (Tables S7 and S8 of the Supplementary Materials), yet the strains are distinguishable by LM, especially the Vannella sp. 4362/VII strain (sequence AY929909). Here again, the sequence similarity of morphologically different strains exceeds that among clones of the same PCR product (see Sections 2.2 and 2.4). Similarly, the four Platyamoeba sequences (Gen-Bank numbers AY929915AY929916AY929918 and AY929919) form a distinct clade of marine 'Platyamoeba' (Table S8, Supplementary Materials), all differing in LM appearance and size (see Dyková et al. 2005a), despite remarkably similar sequences.

Other Vannella groups are represented by two Vannella strains by Dyková et al. (2005a), closely related to *Platyamoeba australis* and a strain of

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*Vannella* (GenBank AY 929904) related with *V. septentrionalis* CCAP 1589/10. In all these cases, sequence divergence is relatively high compared with that between different isolates of the same species (e.g. *V. simplex*) and does not allow us to recognize them as isolates of a single species.

#### 5. Demarcating Vannellid Genera

#### 5.1. Four Genera of Vannellid Amoebae

The molecular data show that neither the presence nor absence of glycostyles nor floating form structure can be used for grouping vannellids into genera. These characters seem useful only for species distinctions. The same is true for size, nuclear structure, and other characters. Although no single morphological character is simply congruent with the molecular phylogeny of vannellid amoebae, every group revealed in our phylogenetic analysis can be described by a combination of morphological characters, and is thus reasonable from a morphological standpoint. Easiest to outline is the genus Lingulamoeba: oblong, lingulate amoebae with an expanded frontal hyaline area but without wrinkles on the dorsal surface and possessing a uniform, fuzzy glycocalyx about 50 nm thick. The genus Clydonella may be outlined as comprising ovoid or ellipsoid vannellid amoebae with long but never helical pseudopodia in the floating form and possessing a glycocalyx of a thin irregular fibrous layer with tower-like glycostyles about 100 nm in length. Vannella includes fan-shaped, spatulate, crescent-shaped or semicircular amoebae with variable floating form or radial type. The cell surface of these amoebae includes either pentagonal glycostyles 90-140 nm in length (sometimes with simple filaments up to 300 nm long) or a layer of densely packed hexagonal elements 20-40 nm in thickness. The same characteristics are applicable to the newly established genus Ripella, which is most difficult to delimit morphologically; probably the only distinctive characters of these amoebae are relatively small size, fast locomotion, and tendency to adopt a disc-like or drop-like shape in rapid movement. As too few ripellas are morphologically characterized, more unifying characters may be apparent in future. At present, because of the general similarity of all vannellid amoebae, we have to use molecular characters even to discriminate some genera.

Clear molecular signatures are obvious for each of the four genera. All species of *Vannella* have a characteristic region of 649–857 bp (all positions are relative to *V. anglica*), not present or very

different in other protists. This region may be used for distinction of groups of species but differs among species. It is completely absent in the genus Ripella and is highly modified in Clydonella and Lingulamoeba. The latter three genera each have their own characteristic, unique regions, e.g. 56-85 bp and 112-335 bp in *Ripella*, 109-151 bp and 229-271 bp in Lingulamoeba, and 126-147 bp and 461-518 bp in Clydonella. The SSU rDNA of Ripella is unusually short (about 1700 bp instead of average 1960 bp) due to large deletions in region 660-836 bp. These very marked molecular differences among the four genera, as now redefined, mean that despite the relatively long branch leading to vannellid amoebae in the amoebozoan phylogenetic tree (Kudryavtsev et al. 2005), it was hard to find more or less continuous shared molecular signatures valid for all vannellid amoebae. It may be very hard to make a reliable universal PCR primer system specific for all vannellid amoebae, but should be relatively easy to construct primers specific for genera and some species groups.

#### 5.2. Suppression of the Genus Platyamoeba

Page (1969) considered his newly established genus Platyamoeba a close relative of the genus Thecamoeba, and only briefly mentioned the "Flabellula-Vannella" group of species, despite the fact that Bovee (1965) had already established the genus Vannella with very similar morphological characters. Sawyer (1975a, b) established the genus Clydonella to accommodate Schaeffer's species Rugipes vivax and three newly described species - C. rosenfieldi, C. sindermani, and C. wardi. He clarified the light-microscopic differences between the genera Vannella, Clydonella, and Platvamoeba, stressing the differences in the morphology of the locomotive and floating form. Page (1971, 1974) described more species of Platyamoeba and noted that they all fitted the characteristics of this genus that he had stated earlier (Page 1969). However, LM differentiation of genera remained difficult. Some marine Platyamoeba may temporarily adopt a characteristic Vannella-like locomotive form with pronounced tail while some vannellas may become pronouncedly spatulate and adopt temporary ridges and folds on the dorsal surface, as Platyamoeba does (Page 1980a; Smirnov 2001). Differences in the floating form noted by Page (1983) may also be doubted. Floating forms of some platyamoebans may have long tapering pseudopodia, while the floating form of some vannellas may have short, blunt pseudopodia or not have them at all (Page 1974, 1980a; Smirnov 1999, 2001). Probably, only the floating form of *V. simplex* is really remarkable and cannot be confused with any other. We have studied several confusing isolates, where only EM allowed us to classify them as *Vannella* or *Platyamoeba*. The same was noted by Page (1980b, 1983); he wrote: 'some marine isolates of *Vannella* seem undistinguishable from *Platyamoeba* with the light microscope' (Page 1983, p. 29).

The difficulty of the LM distinction among these genera, the mixed distribution of characteristics among Vannella and Platyamoeba, and the overlapping variability of the morphology of the locomotive and (sometimes) the floating form forced investigators to rely only on cell surface structure as the generic criterion, ignoring other characters or suggesting them to be unusual, as with the remarkable species Platyamoeba pseudovannellida, strongly resembling Vannella in all LM characters (Hauger et al. 2001) or Vannella anglica, strongly resembling Platyamoeba in LM (Page 1980b). This contradiction between LM and EM over the demarcation between Vannella and Platyamoeba is now clearly resolved by our new molecular data. These unambiguously show that even extremely closely related strains of vannellid amoebae may differ with respect to the presence or the absence of glycostyles. Thus, this character is evolutionarily very flexible and cannot be used to separate genera. Because of the absence of other characters allowing a clear morphological differentiation between Vannella and Platyamoeba, we now suppress the genus Platyamoeba and transfer all its species to the older genus Vannella, as having priority (except, of course, for P. stenopodia transferred above to Stenamoeba). All 30 species now included in the genus Vannella are listed in Table 1; the name Platyamoeba should not be used any more.

Emended Diagnosis of the Genus Vannella Bovee, 1965

Flattened, fan-shaped, spatulate, crescentshaped, or semicircular amoebae with a large frontal hyaline area. Locomotive cells form neither pseudopodia nor subpseudopodia and are smooth in outline. Cell coat includes pentagonal glycostyles or a layer of densely packed prismatic elements 20–40 nm in thickness.

Type species: Vannella mira (Schaeffer, 1926) Smirnov, 2002

#### 5.3. New Vannellid Genus Ripella

Our study clearly indicated the existence of a separate clade of vannellid amoebae possessing unusually short SSU rRNA genes (about 1700 bp

instead of the usual  $\sim$ 2000). The only named species in this clade is Vannella platypodia. Sims et al. (2002) noted the unusually short length of the SSU rRNA gene in this species in the gel but did not perform sequencing. SSU rDNA length is not the only difference; many sequence characters in this species differ from those in other vannellids. This clade comprises Vannella platypodia, and several unnamed species: a similar vannellid strain isolated in 1999 from Priest Pot (Lake District, UK) and serving as a food source in a Paradermamoeba levis CCAP 1555/2 culture and two unnamed strains isolated by Dyková et al. (2005a) from the gills of fish. Interestingly, one of them has a Vannella-like cell coat, while another is Platyamoeba-like, so again both types of cell coat are mixed among very closely related species. Molecular data clearly support establishing a separate new genus for these strains, and warrant re-estimation of their morphological similarities and dissimilarities with other vannellids.

#### Ripella n.g.

Diagnosis: Fan-shaped, spatulate, or discoid amoebae, usually small and rapidly moving, without any ridges or waves on the dorsal surface and always with smooth outlines. 18S rRNA gene is markedly shorter than in *Vannella* and most other Amoebozoa (approximately 1700 bp long) due to a number of deletions at the region 660–836 bp (relative to *Vannella anglica*). A characteristic signature occurs in the 130–189 bp region of 18S rRNA gene.

Type species: *Ripella platypodia* (Glaeser, 1912) comb. n.

Type strain: CCAP 1589/2

Etymology: Combination of the stem rip- of ripis (Gk - a fan for raising a flame) and -ella (*Latin* diminutive suffix).

#### 6. Glycostyles and Glycocalyx Evolution

We have found that many named species, whether identical or different in surface structure, show only minor differences in SSU rDNA sequences. Some morphologically different species show nearly identical SSU rDNA sequences. Our result, as well as those by other authors who studied the phylogeny of vannellid amoebae, clearly indicate that the surface structures of the *Vannella*-type and *Platyamoeba*-type are evolutionarily very close.

Analysis of the evolution of glycocalyx structure is complicated by the fact that it may be easily damaged or destroyed with inadequate EM fixation; therefore, published data must be interpreted 306 A.V. Smirnov et al.

**Table 1.** New composition of the family Vannellidae Bovee, 1970, with references to the most representative illustrations.

1	Vannella aberdonica Page, 1980 (illustrations: Page 1980b)
2	Vannella anglica Page, 1980 (illustrations: Page 1980b)
3	Vannella arabica Page, 1980 (illustrations: Page 1980b; Figs 5–9)
4	Vannella australis (Page 1983) comb. nov. (illustrations: Page 1983; Figs 10-12 as Platyamoeba)
5	Vannella bursella (Page 1974) comb. nov. (illustrations: Page 1974 (as Platyamoeba); Figs 13-17)
6	Vannella caledonica Page, 1979 (illustratons: Page 1979)
7	Vannella calycinucleolus (Page, 1974) comb. nov. (illustrations: Page 1974 (as Platyamoeba);
	Figs 18–22)
8	Vannella cirifera (Frenzel, 1892) Page, 1988 (illustrations: Page 1976; 1988)
9	Vannella crassa (Schaeffer, 1926) (illustrations: Schaeffer 1926)
10	Vannella danica n.sp. (illustrations: Smirnov et al. 2002; Figs 23–27)
11	Vannella devonica Page, 19/9 (illustrations: Page 19/9; Figs 32–38)
12	Vannella douvresi (Sawyer, 19/5a) comb. nov. (illustrations: Sawyer 19/5a, as Platyamoeba)
13	Vannella ebro Smirnov, 2001 (illustrations: Smirnov 2001; Figs 39–41)
14	Vannella flabellata (Page, 1974) comb. nov. (Illustrations: Page 1974 as Platyamoeba)
15	Vannella langae (Sawyer, 1975) (illustrations: Sawyer 1975a as Platyamoeba)
16	Vannella lata Page, 1988 (illustrations: Page 1988; Figs 42–46)
1/	Vannella mainensis (Page, 1971) comb. nov. (illustrations: Page 1983 as Platyamoeba)
18	Vannella mira (Schaeffer, 1926) Smirnov, 2002 (illustrations: Smirnov 2002; Figs 47-50)
19	Vannella miroides Bovee, 1965 (illustrations: Bovee 1965)
20	Vannella murchelanol (Sawyer, 1975) comb. nov. (Illustrations: Sawyer 1975a as Platyamoeba)
21	Vannella nucleolilateralis (Anderson, Nerad et Cole, 2003) comb. nov. (illustrations: Anderson et al. 2003 as Platyamoeba)
22	Vannella peregrinia Smirnov et Fenchel 1996 (illustrations: Smirnov and Fenchel 1996)
23	Vannella persistens Smirnov et Brown, 2000 (illustrations: Smirnov and Brown 2000; Figs 51-53)
24	Vannella placida (Page, 1968) comb. nov. (illustrations: Page 1988 as Platyamoeba; Figs 54-57)
25	<i>Vannella plurinucleolus</i> (Page, 1974) comb. nov. (illustrations: Page 1974 as <i>Platyamoeba</i> ; Figs 58-60)
26	Vannella pseudovannellida (Hauger, Rogerson et Anderson, 2001) comb. nov. (illustrations: Hauer et al. (2001) as <i>Platvamoeba</i> )
27	Vannella sensilis Boyee, 1953 (illustrations: Boyee and Sawyer 1979)
28	Vannella septentrionalis Page, 1980 (illustrations: Page 1980b; Figs 61-62)
29	Vannella simplex (Wohlfarth-Bottermann, 1960) (illustrations: Haberev and Hülsmann (1975): 1Smirnov
	et al. (2002): Figs 28–31)
30	Vannella weinsteini (Sawver, 1975) comb. nov. (illustrations: Sawver 1975a as Platvamoeba)
31	Ripella platypodia (Gläser, 1912) comb. nov. (illustrations: Page 1988; 1991 as Vannella; Figs 63-68)
	The genus contains at least five more species, known as isolates, which are unnamed yet.
32	Clydonella rosenfieldi Sawyer, 1975 (illustrations: Sawyer 1975a)
33	Clydonella sindermanni Sawyer, 1975 (illustrations: Sawyer 1975a)
34	Clydonella vivax (Schaeffer, 1926) Sawyer, 1975 (illustrations: Sawyer 1975b)
35	Clydonella wardi Sawyer, 1975 (illustrations: Sawyer 1975a)
	The genus contains at least two more species, known as isolates, which are unnamed yet
36	Lingulamoeba leei Sawyer, 1975 (illustrations: Sawyer 1975a)
	The genus contains at least one more species, known as yet unnamed isolate
37	Pessonella marginata Pussard, 1973 (illustrations: Pussard 1973)

accurately (Smirnov 1999). However, summarizing available materials, it is possible to conclude that the vannellid cell surface consists of an amorphous basal layer, 10–20 nm in thickness followed by a layer of glycostyles (Fig. 3A). Thickness and density of the basal layer vary among species,

while glycostyles always have an identical organization, but differ in length, varying from 90 to 130 nm. Some species have hair-like filaments among glycostyles, while others do not (Page 1980b, 1983, 1988; Page and Blakey 1979). The platyamoebian glycocalyx consists of



**Figure 3**. Cell surface structures of vannellid amoebae. **A**. Cell surface of *Vannella* sp. Arrows indicate glycostyles. **B**. Cell surface of *Platyamoeba* sp. Scale bar: 100 nm.

an amorphous dense basal layer, 7-10 nm in thickness, followed by a loose outer layer, often appearing as densely packed prismatic elements, hexagonal in cross-section (Fig. 3B). The total thickness of the 'platyamoebian' cell coats varies from 20 to 40 nm and details of the EM appearance differ in virtually every Platyamoeba species. In some isolates, fine, hair-like filaments radiating from the cell surface were noted (Page 1980b, 1983). The only data on the cell surfaces of Clydonella and Lingulamoeba were provided by Peglar et al. (2003); the EM fixation looks imperfect, but from available micrographs, the cell surface of Lingulamoeba consists of a fuzzy amorphous layer about 50 nm thick. In Clydonella, the surface includes tower-like glycostyles about 100 nm in length, with hair-like simple filaments among them. The shape of glycostyles in crosssection is unclear. A fibrous layer of cross-linking filaments, about 20 nm in thickness, is attached to the plasma membrane. It is organized in patches of unclear structure and is 'interposed' between the glycostyles and filaments (Peglar et al. 2003, p. 228). From the available micrographs, it seems that glycostyles and filaments are probably attached to this layer, as in *Vannella*.

The presence of glycostyles in both Vannella and *Ripella* (in the two major vannellid branches) means that pentagonal glycostyles were probably present in the vannellid common ancestor; pentagonal glycostyles similar to those of Vannella and Ripella have been reported in Multicilia marina (Mikrjukov and Mylnikov 1998), but it is yet unknown whether they are endogenous, because this species feeds on Vannella. More broadly, pentagonal cup-shaped structures are also known in the cell surface on Paradermamoeba - on the tips of spiral glycostyles, but they are very different from those of Vannella (Smirnov and Goodkov 1999). Hexagonal glycostyles are known outside Vannellidae - in Vexillifera and in Pseudoparamoeba (Page 1979, 1983); hexagonal cupshaped elements were reported from the cell surface of Saccamoeba (Page 1988).

The simplest interpretation, assuming that true glycostyle homologs are lacking in Multicilia, is that the ancestral cell coat of Glycostylida consisted of an amorphous basal layer underlying an outer laver of prismatic structures. In Vannellidae. these structures were separated from each other and modified into pentagonal glycostyles, which were later lost in Lingulamoeba and further modified in Clydonella (Fig. 4). In Vannella and *Ripella*, there were multiple independent reversals to the initial structure of the cell coat. This frequent polyphyletic loss indicates that the loss of pentagonal glycostyles is not a difficult evolutionary step. In principle, it could occur by a single mutation. Thus, we should now probably regard the presence or absence of glycostyles simply as a variable character useful for distinguishing some sibling species of species clusters, but not of fundamentally greater significance than LM characters.

A theoretical alternative to multiple losses would be that the *Vannella*-like and *Platyamoeba*-like surface morphologies might reflect alternate phases in a dimorphic life cycle. On that hypothesis, either phase of the cycle might be independently evolutionary lost relatively recently, leaving 'vannellid' and 'platyamoebian' phases intermingled on the tree. For that to be true, each phase would have to be stable for many years in culture, and we would expect to find several examples of both types with identical SSU



**Figure 4**. Possible pattern of glycocalyx evolution among Glycostylida. \* hexagonal glycostyles were modified to scales.

sequences and ITS sequences. Although we have found very similar sequences for both types, none are yet identical. Thus, we think this a less likely explanation than simple mutational loss.

Outside Vannellida, the putatively hexagonal structures of the initial cell coats were probably separated from each other and enlarged, forming the cell coat of Vexillifera, and further modified to the tower-like hexagonal glycostyles of Pseudoparamoeba, which is a derived group in our trees (Fig. 4). Our trees suggest that the Vexillifera -Pseudoparamoeba grouping is paraphyletic and basal for Dactylopodida and that Paramoeba, Korotnevella, and Neoparamoeba are derived from them. Probably therefore, the hexagonal glycostyles were lost by their common ancestor; Paramoeba and Korotnevella evolved scales instead, while Neoparamoeba retained only the basal amorphous layer of the glycocalyx. Some Neoparamoeba species have hair-like filaments radiating from this layer (Page 1983). Possibly such filaments are easily acquired and convergent even inside Glycostylida. Scales of Cochliopodium vary greatly among species and have at least three basically different symmetry types (circular, consisting of plate-like base, a column of 4-5 legs and a capital in most species; cubical in Cochliopodium larifelly; and plate-like in Cochliopodium gallicum) (Bark 1973; Kudryavtsev 1999, 2004). We suggest that they probably evolved independently of the glycostylid structures shown in Figure 4. Independently of the evolutionary scheme, our results suggest that some cell surface structures of amoebae can easily be lost or modified among low-level taxa; thus, within Glycostylida, they are not as good phylogenetic markers as was believed by Page (1983, 1987, 1988), despite some structures being confined to certain groups of genera.

#### 7. What is a Vannella Species?

We have sampled 15 named vannellid amoebae (with multiple strains of several) and included 30 other vannellid sequences (some named) in the analysis. In no case were we able to identify any unnamed sequences by complete sequence identity with a named species. Furthermore, sequences of different strains of V. simplex isolated by A. Smirnov and Platyamoeba plurinucleolus isolated by F. Page, despite the evident morphological relatedness show certain differences, thus suggesting that even within a single, reliably identified morphospecies there is genetic diversity in SSU rRNA genes. It is not really surprising that in some parts of the tree, strains with very closely related sequences can be distinquished as classical morphospecies, whereas in others strains differing genetically to the same degree cannot be distinguished by LM. There is no reason whatsoever why rRNA and morphology should evolve at the same rate. In the absence of knowledge about amoeba sexuality and applicability or otherwise of the biological species concept, defining amoeba species is at present a matter of convenience. Our experience here indicates that a combination of microscopic and sequence data is more powerful than either alone, but it remains a matter of opinion just where to place species boundaries. Molecular signatures probably need to be combined with morphology in defining amoeba species.

Recently, there have been two very divergent approaches in amoeba systematics. At one extreme in the Heterolobosea, it is becoming customary to separate species even with identical 18S rRNA differing only in the more rapidly evolving ITS regions (De Jonckheere and Brown 2005). At the other extreme, Dyková et al. (2005b) investigated 33 sequences of 32 different Neoparamoeba strains and found typically 15-30 bp difference between the strains previously regarded as the same named species, P. pemaguidensis (maximally with 53 different bases). Their much larger and probably more representative data set contradicted conclusions by Elliot et al. (2001) and Wong et al. (2004) for four *Neoparamoeba* strains, finding virtual identity among sequences from the same species. That degree of lumping of divergent sequences into one *Neoparamoeba* species, would not be acceptable for vannellids, where more closely related strains are clearly morphologically distinguishable. Our morphological analysis suggests that it is not possible to unify all amoebae comprising a subclade of *Vannella* into a single species, as was done by Dykova and co-workers with *Neoparamoeba*.

Division as fine as that now practised for Heterolobosea might one day prove desirable also for vannellids. However, there is a practical difficulty in doing this when direct sequencing of PCR products does not give clear traces. This is the level of microheterogeneity observed here among cloned SSU rRNA genes even in Vannella strains that originally were clonal when first isolated. Only for some vannellid species was direct sequencing of PCR products satisfactory; most required cloning the PCR products (see Methods section). Comparison of clones obtained from the same PCR product revealed short indels in different versions of gene, which made direct sequence of the PCR product non-readable (first we suspected possible contamination of the culture, but cloning revealed no contaminants). Some strains, e.g. V. simplex, show considerable variation among clones in some regions of the molecule. It is interesting that 3 years ago, we did not experience difficulties when directly sequenced PCR products of the same V. simplex strains soon after their isolation by Smirnov et al. (2002), while in the present study, 3 years later, we have found that all of them required cloning due to the presence of paralogs harboring deletions. Is this an accumulation of paralogs during continuous cultivation? On the other hand, some CCAP species were easily sequenced directly, despite having been in culture much longer.

This microheterogeneity seems greater than in some other protist groups (for example making a single PCR library from a pure culture of *Cercomonas* typically generates only one sequence type when several clones are sequenced: D. Bass pers. comm.). Microheterogeneity within a cellular clone necessarily occurs at least temporarily in multicopy genes such as rDNA as they diverge. In some species, its level is sufficiently high that it could prove hard to assign clones from environmental gene libraries correctly to cultured species, as one would not know for such culture-free methods whether a given clone was the dominant version used by the cell or a possibly insignificant minor paralog of no long-term evolutionary significance. Thus, molecular ecology of Amoebozoa using 18S rDNA of environmental DNA libraries may prove more difficult than for more tractable protist groups such as the Cercozoa (Bass and Cavalier-Smith 2004). Possibly single copy genes would be more reliable for low-level molecular systematics of amoebae, but technically more difficult for environmental PCR, unless more abundant mitochondrial genes were used.

This problem may be further investigated by studying SSU rDNA polymorphism in molecular clones obtained from fresh clonal strains of vannellids and by isolating and sequencing more independent strains of morphologically distinct species, e.g. *V. simplex*. However, SSU rDNA sequences, despite their availability, do not yet provide a practical tool for identification and species distinction in vannellids.

Our discovery of clonal microheterogeneity raises a potential problem with some unnamed vannellid sequences of Dyková et al. (2005a). There are groups of very similar sequences. differing by few bases: they might be different species, but careful analysis of the mismatches suggests that they may potentially be the result of sequence polymorphism within one species or even errors of sequencing, because some are located in normally very conservative regions. This especially concerns deletions and inserts of a single nucleotide in such regions. Since Dyková et al. (2005a) cloned all PCR products, strains Vannella sp. AY929909, Vannella sp. AY929910, and Vannella sp. AY929911 might all be the same species, especially the first two strains which look morphologically rather similar. The same applies to Platyamoeba spp. AY929916, AY929918, and AY929915. Each of these species groups has very characteristic molecular signatures, like GGGGT-YAAACCC in the position 240-251, and GCAT-TAAATTGGTTGCACGTTTGTG (pos. 662-686) and CGCAAGGAAAAGTCTTTGACG (pos. 1860-1881) in Vannella sp. AY929909 or TAATGTTTAA-TTGCATT (pos. 1191-1207) and TTGTAAAGTTT-TYTGTT (pos. 1866-1882) in Platyamoeba sp. AY929916. However, since no detailed LM description is available for these strains, the question is open.

Finally, despite potentially considerable sequence heterogeneity within a morphospecies, there are many sequences of unnamed vannellid amoebae clearly different from any named species. Thus, the biodiversity of vannellid amoebae is still heavily undersampled and the number of unknown species is still high.

### 8. Diagnoses of New or Revised Vannella Species

Vannella danica n. sp. – isolate, first described as *V. simplex* Nivå Bay strain by Smirnov et al. (2002)

Locomotive form semicircular or fan-shaped. Length of the locomotive form  $25-50 \mu m$  (average  $32 \mu m$ ); breadth  $25-50 \mu m$  (average  $39 \mu m$ ). Length/breadth ratio 0.5-1.25 (average 0.82). Single vesicular nucleus ca.  $7 \mu m$  in diameter; the central or slightly eccentric rounded nucleolus about  $4 \mu m$  in diameter. Cultured cells always densely covered with rounded fecal pellets. Cysts rounded 16-26  $\mu m$  in diameter, always surrounded with a layer of fecal pellets. Single cyst wall, 1.1-1.8  $\mu m$  in thickness, consists of a filamentous material. Cell surface consists of glycostyles about 115 nm in length, without simple filaments among them.

Observed habitat: brackish-water: Nivå Bay (Baltic Sea, The Sound, 15 km South of Helsingør, Denmark); probably also Baltic Sea, Gulf of Finland. Maybe Garstecki and Arndt (2000) observed this species in the Southern Baltic Sea.

Differential diagnosis: trophozoites resemble *V. simplex*, but are somewhat smaller. Cyst-forming, in contrast with the known strains of *V. simplex*. No simple filaments among glycostyles. Characteristic signatures in 18S rRNA gene sequence, illustrated in Table S5.

Type material: CCAP 1589/17 (culture) (formerly *V. simplex* strain). Sequences: EF051203— EF051206. We also provide a revised diagnosis of *V. simplex* to eliminate the characters of *V. danica* from it.

Vannella simplex (Wohlfarth-Bottermann, 1960) emend.

Locomotive form varies from crescent-shaped or semicircular to fan-shaped with pronounced "tail"; maximum dimension  $60 \,\mu$ m; usual size  $42-52 \,\mu$ m in length and breadth. Floating form of radial type, non-symmetrical, with 1-9pointed, tapering hyaline pseudopodia without basal thickening. Vesicular nucleus  $6-11 \,\mu$ m in diameter with one central or slightly eccentric nucleolus  $7.4-8.6 \,\mu$ m in diameter. No cysts known.

Habitat: freshwater. Known from numerous locations in Europe, probably from Asia and North America. Type material (neotype): *Vannella simplex* CCAP1589/3 (culture). Sequences: EF051208–EF051211.

#### 9. Illustrated Checklist of Vannellid Amoebae

Data on vannellid amoebae have become more and more dispersed. To aid recognition of species and further progress in their study, we provide a complete checklist with illustrations, synonymy, and relevant references. We include all species documented well enough to be recognized, as well as poorly known species that possess some specific characters potentially recognizable in case of re-isolation (Table 1). Probably, the best way to handle the latter will be to establish neotypes when a strain, sufficiently well resembling the original description, is re-isolated and studied, as was done for Vannella mira (Smirnov 2002). Species descriptions are compiled from all available sources and our own observations, but are not intended to replace their formal diagnoses, which can be found in the cited literature. When size and other morhophometric data differ among sources, the maximal known variation is given.

1. Vannella aberdonica Page, 1980

Locomotive form semi-circular to fan-shaped or drop-shaped; posterior edge straight or concave; some cells may acquire drop-shaped form with pronounced 'tail' (see Page 1983, fig. 67). The frontal hvaloplasm occupies about half the cell. Greatest dimension  $6.5 - 13 \,\mu m$  (average  $8.8 \,\mu m$ ), length/breadth ratio 0.8-1.5 (average 1.0). Single vesicular nucleus 1.9-3.2 µm in diameter (average 2.6  $\mu$ m) with central nucleolus 0.9-1.4  $\mu$ m in diameter (average 1.1 µm). Floating form thickly flattened, twisted, never rounded up; never with pseudopodia. Locomotive rate at 23 °C on a glass surface is  $14.8-28.6 \,\mu$ m/min (1.5-3.7 times the length of amoebae) (Page 1980b). Cell surface includes glycostyles about 120 nm long and simple filaments up to 280 nm long. Description: Page (1980b). Illustrations: Page (1980b, 1983). Because of the general similarity of the smallest vannellid amoebae, later records of this species (e.g. Butler and Rogerson 2000; Garstecki and Arndt 2000; Vørs 1993a, b) require molecular and TEM checks to prove that mentioned strains really are V. aberdonica, and not some other small vannellid amoebae. Type slides by F.C. Page: British Museum of Natural History (holotype 1980:1:24:1; paratype 1980:1:24:2). Marine. Available cultures: ATCC 50815 isolated by M. Peglar; there is no published LM or EM data on this isolate; its complete 18S rRNA gene sequence was obtained by Peglar et al. (2003; GenBank AY121853).

2. Vannella anglica Page, 1980

Locomotive form semicircular, spatulate or fanshaped. The granuloplasmic mass is thick and rounded; frontal hyaloplasm occupies 0.3–0.5 of its total length: posterior edge rounded or irregularly triangular, never straight or drawn out into a tail. Greatest dimension 15-37 µm (average  $21-24 \,\mu\text{m}$ ): length/breadth ratio 0.7-2.0 (average 1.0–1.2). Single vesicular nucleus  $3.8-6.2 \,\mu m$  in diameter (average  $4.9-5.3 \,\mu$ m) with central nucleolus  $2.3-3.7 \,\mu m$  in diameter (average 2.5-3.0 um). Floating form with blunt hvaline pseudopodia, irregularly distributed and with length not greater than the diameter of central cell mass. Rate of locomotion 13-32 um/min (0.5-1.7 length of an amoeba) (Page 1980b). Cell surface includes glycostyles 117-126 nm long and simple filaments up to 240 nm in length. Description: Page (1980b). Illustrations: Page (1980b, 1983). Type slides by F.C. Page: British Museum of Natural History (holotype 1980:1:24:3; paratype 1980:1:24:4). Marine. Available cultures: CCAP 1589/8 (type culture) and 1589/11by F.C. Page. Partial 18S rRNA gene sequence of 1589/8 strain (GenBank AF464913) by Sims et al. (2002); complete 18S rRNA gene sequence of this strain (GenBank AF099101) by Sims et al. (1999).

3. Vannella arabica Page, 1980

Locomotive form flabellate or semi-circular. Frontal hvaloplasm occupies about half the body length; posterior end widely rounded, straight or (rarely) concave. Greatest dimension 17-37 µm (average 27 μm); length/breadth ratio 0.5-1.2 0.8). Sinale vesicular (average nucleus  $4.2-6.5\,\mu\text{m}$  in diameter (average  $5.4\,\mu\text{m}$ ) with central nucleolus 1.9-3.7 µm in diameter (average 2.7 µm). Floating form a rounded mass, without pseudopodia or with few (not more than three) blunt hyaline pseudopodia, rarely exceeding in length the diameter of the central mass. Locomotive rate 20-48 µm /min (1.0-2.1 times the cell length) (Page 1980b). Cell surface includes glycostyles about 114 nm long and simple filaments up to 215 nm in length. Description: Page (1980b). Illustrations: Page (1980b, 1983; Figs 5-9). Type slides by F.C. Page: British Museum of Natural History (holotype 1980:1:24:5; paratype 1980:1:24:6). Marine. Available culture: CCAP 1589/7 (type culture). Complete 18S rRNA gene sequence (GenBank EF051194) obtained in this study.

4. Vannella australis (Page, 1983) comb. nov. (formerly *Platyamoeba australis*)

Spatulate or fan-shaped in locomotion. Posterior end often triangular; frontal hyaline zone normally occupies entire anterior half of the cell. The posterior granuloplasmic region is often thick and very refractile. Greatest dimension  $22-44 \,\mu\text{m}$ (average  $32.3 \,\mu\text{m}$ ). Length/breadth ratio 0.6-2.5 (average 1.0). Floating form with thick, blunt, sometimes curved radiate pseudopodia, in length exceeding the diameter of the central cytoplasmic mass by up to two-fold. Single vesicular nucleus  $5.6-8.4 \,\mu\text{m}$  in diameter (average  $6.7 \,\mu\text{m}$ ) with central nucleolus  $2.8-4.7 \,\mu\text{m}$  in diameter (average  $3.8 \,\mu\text{m}$ ). Glycocalyx about 30 nm in thickness; consists of densely packed hexagonal elements. Marine. Description and illustrations : Page (1983); Figures 10-12. Available culture: CCAP 1565/9 (type culture). Complete 18S rRNA gene sequence (GenBank EF051199) was obtained in the present study.

5. Vannella bursella (Page, 1974) comb. nov. (formerly *Platyamoeba bursella*)

Locomotive form flattened ovoid, semi-circular or spatulate; thickness of the granular region often shaped as a transverse spindle. Posterior edge rounded or convex; surface wrinkles often occur parallel and near to edges. Greatest dimension of locomotive form  $12-37 \,\mu m$  (average  $18-25\,\mu$ m). Single vesicular nucleus  $3.7-7.4\,\mu$ m in diameter with rounded central nucleolus. Locomotive form with several blunt hyaline pseudopodia, often asymmetrically disposed relative to the central cytoplasmic mass. Cell coat of densely packed hexagonal elements is about 26 nm thick. Description: Page (1974). Illustrations: Page (1974, 1983); Figures 13-17. Type slides by F.C. Page: British Museum of Natural History (holotype 1974:1:9:3; paratype 1974:1:9:4). Marine and brackish-water. Available culture: CCAP 1565/10 by F.C. Page. Complete 18S rRNA gene sequenced (GenBank EF051195) in the present studv.

#### 6. Vannella caledonica Page, 1979

Locomotive form fan-shaped or flabellate, often with uneven anterior edge. Frontal hyaline zone arc-shaped; occupies about half the total cell area. Greatest dimension 10-25 µm (average 16 µm); length/breadth ratio about 1.2. Single nucleus, 2.8-4.7 µm in diameter, commonly vesicular, with single central nucleolus, but other configurations of the nucleolar material also were noted (Page 1979). Floating form without radiating pseudopodia. Cell coat includes glycostyles about 94 nm long and simple filaments up to 281 nm long. Rate of locomotion 22-47 µm/min (1.3-2.3 times the length per minute). Description: Page (1979). Illustrations: Page (1979, 1983). Type slides by F.C. Page: British Museum of Natural History (holotype 1978:6:28:3; paratype 1978:6:28:4). Marine. No available cultures.

7. *Vannella calycinucleolus* (Page, 1974) comb. nov. (formerly *Platyamoeba calycinucleolus*)



**Figures 5–31**. Diversity of vannellid amoebae. **Figures 5–9**. *Vannella arabica* CCAP 1589/7. Locomotive (5-8) and floating form with short, blunt pseudopodia (9). **Figures 10–12**. *Vannella australis* CCAP 1565/9. Locomotive (10-11) and floating (12) form. **Figures 13–17**. *Vannella bursella* CCAP 1565/10. Locomotive forms (13-15) and floating (16-17) form. **Figures 18–22**. *Vannella calycinucleolus* CCAP 1565/6. Locomotive (18-20) and floating (21-22) forms. **Figures 23–27**. *Vannella danica* CCAP 1589/17. Locomotive forms (23-25), floating form (26), and cysts (27). **Figures 28–30**. *Vannella simplex* (Geneva strain). Locomotive form. **Figure 31**. *Vannella simplex* CCAP 1589/3. Floating form. Scale bar is 10  $\mu$ m except in Figure 17 (200  $\mu$ m).

Locomotive form flattened, ovoid, spatulate or semi-circle. The hyaloplasm occupies half or more of the total body area; posterior edge commonly is straight or convex. Some wrinkles may occur parallel to the edge of an amoeba. Greatest dimension in locomotion  $20-56 \,\mu\text{m}$  (average  $21-29 \,\mu\text{m}$ ); length/breadth ratio 0.6-1.3 (average 0.8-0.9). Floating form with several uneven

blunt hyaline pseudopodia. Single nucleus 3.7–7.5 μm in diameter with peculiar central "calyciform" nucleolus, cup-shaped, with depression on one side. Cell coat consists of densely packed hexagonal elements and is about 40 nm in thickness. Description: Page (1974). Illustrations: Page (1974, 1983); Figures 18–22. A strain identified as *P. calycinucleolus* was illustrated by

Smirnov by LM and EM characters (1999), but not sequenced so it is a preliminary identification. Type slides by F.C. Page: British Museum of Natural History (holotype 1974:1:9:5; paratype 1974:1:9:6). Marine, possibly brackish-water. Available culture: CCAP 1565/6 (type culture). Complete 18S rRNA gene sequence (GenBank EF051193) was obtained in the present study.

#### 8. Vannella cirifera (Frenzel 1892) Page 1988

Doubtful species, never reliably re-isolated. The name was arbitrary applied by Page (1976) to recognize a Vannella simplex-like species, possessing minor differences from V. simplex in locomotive and floating form. This strain was described by Page (1976) as V. mira Schaeffer, 1926; later he accepted that V. mira must be marine, so used the name V. cirifera as substitute (see Smirnov 2002 for nomenclatural history). No argument for its similarity with Saccamoeba cirifera Frenzel, 1892 beyond "great resembles of figures" was given; since we now know how polymorphous vannellas can be, this argument can hardly be accepted. This strain was lost long ago. It would be best to establish a neotype of V. cirifera if one finds a freshwater vannellid strain resembling that described by Page (1976, 1988). Formal description of this strain after Page (1976, 1988): 'usually flabellate, seldom somewhat spatulate, greatest dimension 25-55 µm; nucleus 4.8-8.3 µm; floating from often symmetrical but somewhat irregular, with most of cytoplasm in 1-3 long pseudopodia; slow to settle from floating form; attaches poorly. Cell surface includes glycostyles about 110 nm in length; no simple filaments among glycostyles. It seems that the latter character and the low number of pseudopodia in the floating form differentiates it from V. simplex. Description: Page (1976 – as V. mira; 1988). No type material. ATCC strain 50924 (isolator T. Nerad) designated V. cirifera lacks published data, hence we cannot comment on its identification.

#### 9. Vannella crassa (Schaeffer, 1926)

This was never re-isolated since the initial description, but its remarkable size and good description by Schaeffer (1926) would probably allow recognition, if found. A description compiled from Schaeffer's observations follows.

Locomotive form triangular or fan-shaped. Frontal hyaloplasm forms a wide anterior margin and occupies less than half the total cell area. The anterior edge is smooth, but its margin is frequently deeply incised; the posterior edge is irregularly triangular or drawn out into a characteristic tail. Length in locomotion  $40-50 \,\mu$ m. Rate of

locomotion  $\sim 25 \,\mu$ m/min. Single vesicular nucleus  $\sim 12 \,\mu$ m in diameter with spherical central nucleolus  $\sim 5 \,\mu$ m in diameter. Floating form with numerous irregular blunt hyaline pseudopodia. Marine. Description: Schaeffer (1926). No available cultures or type material.

10. Vannella danica n. sp

The diagnosis is provided above (Section 8). Description and illustrations: Smirnov et al. (2002); Figures 23–27. Brackish-water; thrives at marine salinity. Available culture: CCAP 1589/17 (type culture). Complete 18S rRNA gene sequences (GenBank EF051203–EF051206) were obtained in the present study (Figs 28–31).

11. Vannella devonica Page,1979

Broad, flabellate, or fan-shaped locomotive form. Frontal hyaloplasm occupies from 1/2 to 2/3 of the total body area and has smooth anterior edge. The thickness of the amoeba increases gradually to the posterior end, which is convex to pyramidal. Locomotive form 14-33 µm in greatest dimension (average 22 µm), length/breadth ratio 0.5-1.4 (average 0.9). Single nucleus  $3.7-5.6\,\mu\text{m}$  in diameter with 1-5 peripheral nucleoli (may appear to have vesicular nucleus under the LM because the nucleoli often superimpose). Floating form of radial type, with long, slender, tapering hyaline pseudopodia, which may become helical. Locomotive rate 11-33 um/min (0.9-1.9 time the cell length) (Page 1979). Cell surface coat includes glycostyles about 94 nm in length without simple filaments among them. Description: Page (1979). Illustrations: Page (1979, 1983); Figures 32-38. Type slides by F.C. Page: British Museum of Natural History (holotype 1978:6:28:5; paratype 1978:6:28:6). Marine. Available culture: CCAP 1589/5 (type culture). ATCC strain PRA-137 isolated by T. Nerad from salt marsh sediment and identified as V. devonica lacks published data. Complete 18S rRNA gene sequence of CCAP 1589/5 strain (GenBank number EF051196) obtained in the present study.

12. Vannella douvresi Sawyer, 1975 comb. nov. (formerly Platyamoeba douvresi)

In locomotion, discoid to wide ovoid with smooth anterior margin. Narrow transverse ripples or waves flow anteriorly, often one after the other, and quickly disappear on reaching the anterior margin; lateral rides or ripples absent. Length in locomotion  $12-15\,\mu\text{m}$  (average  $13\,\mu\text{m}$ ), width  $10-16\,\mu\text{m}$  (average  $12\,\mu\text{m}$ ). Single vesicular nucleus about  $3\,\mu\text{m}$  in diameter, nucleolus about  $2\,\mu\text{m}$  in diameter. Floating form with multiple blunt pseudopodia not exceeding in length the diameter of the central mass of cytoplasm. Ultrastructure

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unknown. Rate of locomotion  $40-45\,\mu$ m/min. Marine, can survive up to 7.5 ppt salinity but without multiplication. Description and illustrations: Sawyer (1975a). Neither type material nor culture available.

#### 13. Vannella ebro Smirnov, 2001

Fan-shaped or semicircular amoeba, with a tendency to form waves or even short longitudinal ridges on the frontal hyaloplasm. Length of locomotive form  $25-40 \,\mu m$  (average  $30 \,\mu m$ ); breadth 35-60 µm (average 41 µm). Length/ breadth ratio 0.5-1.0. Single vesicular nucleus about 8 µm in diameter with rounded central nucleolus about 4 um in diameter. Floating form variable, with 4-8 long, thick, tapering hyaline pseudopodia in fresh isolates, and without pseudopodia in older cultures. Cell coat consisting of glycostyles about 140 nm long without associated simple filaments. Marine; survives 6-90 ppt salinity. Description and illustrations: Smirnov (2001); Figures 39-41. Available culture: CCAP 1589/14 (type culture). Partial 18S rRNA gene sequenced by Smirnov et al. (2002) and in the present study (GenBank EF051198).

14. Vannella flabellata Page, 1974 comb. nov. (formerly *Platyamoeba flabellata*)

Locomotive form flabellate or ovoid with rounded or convex posterior edge. Surface wrinkles often parallel to or near the edges. The hyaloplasm occupies about 2/3 of the total cell area. Greatest dimension of the locomotive form is  $20-42 \,\mu\text{m}$  (average  $26-30 \,\mu\text{m}$ ). Length/breadth ratio 0.7-1.5 (average 1.0). Single vesicular nucleus 3.7-7.0 µm in diameter. Floating form asymmetrical, with numerous blunt, uneven hyaline pseudopodia not exceeding the diameter of the central cytoplasmic mass. Cell coat of densely packed hexagonal elements is 26-28 nm thick. Description: Page (1974). Illustrations: Page (1974, 1983). Type slides by F.C. Page: British Museum of Natural History (holotype 1974:1:9:1; paratype 1974:1:9:2). Marine, possibly brackish; thrives in freshwater media. No available cultures.

15. Vannella langae Sawyer, 1975 comb. nov. (formerly Platyamoeba langae)

In locomotion, broadly triangular to ovoid with a slightly convex posterior margin. Frontal hvaloplasm from 1/3 to 1/2 of the total body area. Length of the locomotive form  $7-12 \,\mu m$  (average  $9 \mu m$ ), breadth  $5 - 10 \mu m$  (average  $8 \mu m$ ). Single vesicular nucleus about 2.5 µm in diameter, nucleolus 1.5-2 µm in diameter. Floating form without radial pseudopodia, rod- or peg-shaped with one end slightly wider than the other. Rate of locomotion 15-20 µm/min. Ultrastructure unknown. Marine, can survive in 7.5 ppt salinity. Description and illustrations: Sawyer (1975a). No formally designated type material. Available culture: ATCC 50816 by T.K. Sawyer.

16. Vannella lata Page, 1988

Locomotive form semicircular or widely fanshaped; width of a moving cell usually is much greater than the breadth. The frontal hyaline area occupies about 2/3 of the total cell area and commonly extends along sides. Maximal dimension of the locomotive form 24-46 µm (average  $33 \,\mu\text{m}$ ; length/breadth ratio 0.5 - 1.0 (average 0.6). Single vesicular nucleus  $3.7-6.5 \,\mu m$  in diameter (average 5.1 um). Floating form with 3-14 more or less symmetrically distributed long, tapering pseudopodia. Cell surface coat includes glycostyles with simple filaments among them. Freshwater. Description and illustrations: Page (1988); Figures 42–46. Type slides by F.C. Page: British Museum of Natural History (holotype 1986:8:11:1; paratype 1986:8:11:2). Available culture: CCAP 1589/12 by F.C. Page. Complete 18S sequenced (GenBank number rRNA gene EF051201) in the present study.

17. Vannella mainensis Page, 1971 comb. nov. (formerly Platyamoeba mainensis)

Locomotive form fan-shaped, oblong, or spatulate. Frontal hyaloplasm occupies up to 2/3 of the total body area; amoebae often form fine longitudinal lines or wrinkles near sides. Greatest dimension of locomotive form  $19-37 \,\mu m$  (average

**Figures 32–68.** Diversity of vannellid amoebae. **Figures 32–38.** Vannella devonica CCAP 1589/5. Locomotive (32-36) and floating (37-38) forms. **Figures 39–41** Vannella ebro CCAP 1589/14. Locomotive forms (39); small lobes on the ventral surface of the hyaloplasm (40) and temporary dorsal ridges (41), characteristic for this species. **Figures 42–46**. Vannella lata CCAP 1589/12. Locomotive (42-44) and floating (45-46) forms. **Figures 47–50**. Vannella mira CCAP 1589/15. Locomotive (47-49) and floating (50) form. **Figures 51–53**. Vannella persistens CCAP 1589/13. Locomotive (51-52) and floating (53) form. **Figures 54–57**. Vannella placida CCAP 1565/2. Locomotive forms (54-56) and cysts (57). **Figures 58–60**. Vannella plurinucleolus CCAP 1565/7. Locomotive (58-59) and floating (60) form. **Figures 61–62**. Vannella septentrionalis CCAP 1589/10. Locomotive form. **Figures 63–68**. Ripella platypodia CCAP 1589/2. Locomotive (63-66) and floating (67-68) form. Scale bar is 10 µm throughout.

 $26 \,\mu$ m); length/breadth ratio 1.0 - 1.4 (average 1.1) usually greater than breadth. Single vesicular nucleus  $3.7 - 6.5 \,\mu$ m in diameter (average  $5.6 \,\mu$ m) with central nucleolus  $2 - 4 \,\mu$ m in diameter. Rate of locomotion  $44 - 62 \,\mu$ m/min. Floating form usually irregularly rounded up, sometimes with several slender, blunt pseudopodia. Cell coat of densely packed hexagonal elements is  $20 - 22 \,\mu$ m thick. Description: Page (1971). Illustrations: Page (1971, 1983). Marine, thrives in freshwater media. Neither type material nor available cultures.

18. Vannella mira (Schaeffer,1926) Smirnov, 2002

Locomotive form fan-shaped, semicircular, or (rarely) spatulate. Prominent folds or lobes on the frontal hyaloplasm. Length and breadth of locomotive form  $15-35 \,\mu$ m; (average length  $28.5 \,\mu$ m; average breadth 26.5 µm) length/breadth ratio 0.5-1.8 (average 1.08). Single vesicular nucleus,  $\sim$ 6  $\mu$ m in diameter with central spherical nucleolus  $\sim 3 \,\mu m$  in diameter. Floating form non-symmetrical, with up to 11 straight or coiled pseudopodia, often with distinct conical thickening of the basement. Pseudopodia of the floating form have rounded, not pointed ends and are relatively thick. Cell coat consisting of glycostyles, about 130 nm in length without associated simple filaments. Marine. Description and illustrations: Smirnov (2002); Figs 47-50. Available culture: CCAP 1589/15 (type culture; neotype).

19. Vannella miroides Bovee, 1965

This species is known only from line drawings by Bovee (1965). It is desirable to establish a neotype if a similar strain is re-isolated. A description compiled from Bovee's observations follows: Locomotive form flabellate or semicircular. Frontal hyaloplasm occupies more than half of the total body area and often is so extended laterally that it nearly surrounds the granuloplasmic mass. Frontal edge often wavy. Greatest dimension of locomotive form 25-35 µm. Single vesicular nucleus 4-4.5 µm in diameter. Floating form very regular, with tapered pseudopodia of almost equal length. Few bipyramidal crystals up to 1.5 µm in the cytoplasm are reported (unusual for Vannella). Freshwater. Description and illustrations: Bovee (1965), Page (1976, 1988 - both use the same picture by Bovee). Ariza et al. (1989) illustrated a strain that fits well with the above characters. No type material. ATCC 30945 culture isolated by J.L. Griffin and designated Vannella miroides lacks LM/EM data, but its complete 18S r RNA gene was sequenced by Peglar et al (2003) (GenBank AY183888).

20. Vannella murchelanoi Sawyer, 1975 comb. nov. (formerly Platyamoeba murchelanoi)

Locomotive form ovoid or rounded, sometimes spatulate, with flattened posterior margin. Frontal hyaloplsm occupies about half the total body area. Length in locomotion  $8-13 \,\mu\text{m}$  (average  $11 \,\mu\text{m}$ ), breadth  $6-11 \,\mu\text{m}$  (average  $9 \,\mu\text{m}$ ). Single vesicular nucleus ca.  $2-2.5 \,\mu\text{m}$  in diameter, nucleolus  $\sim 1.2-2 \,\mu\text{m}$ . Floating form without pseudopodia, with crumpled or wrinkled surface. Rate of locomotion  $20-30 \,\mu\text{m}/\text{min}$ . Ultrastructure unknown. Marine, can survive 7.5 ppt salinity. Description and illustrations: Sawyer (1975a). Neither type material nor culture available.

21. Vannella nucleolilateralis (Anderson, Nerad et Cole, 2003) comb. nov. (formerly *Platyamoeba nucleolilateralis*)

Locomotive form discoid to flabellate. Frontal hyaloplasm occupies more then half of the total body area. Length in locomotion is 12-24 µm (average  $15.6 \,\mu$ m), breadth  $7.5 - 15 \,\mu$ m (average 12.2  $\mu$ m). Single nucleus 2.5-3.5  $\mu$ m in diameter (average  $3\mu m$ ) with one or (rarely) two laterally located nucleoli. Floating form asymmetric and has 1 to 4 long, tapering pseudopodia with pointed tips. Rate of locomotion  $29-42 \,\mu\text{m}/$ Cell coat of densely packed hexamin. gonal elements, about 30 nm thick (measured in micrographs). Isolated from salt marches in Assateague Island, VA, but tolerates salinity down to 2.5 ppt. Description and illustrations: Anderson et al. (2003). Available culture ATCC 50987 by T. Nerad.

22. Vannella peregrinia Smirnov et Fenchel, 1996

Locomotive form broad fan-shaped to flabellate with a long, spatulate posterior, but usually irregularly semi-circular, often with a transverse, spindle-shaped thickening of the posterior part of the granular region. Frontal hyaloplasm occupies 1/3 - 2/3 of the total body length. The length of the locomotive form is  $4.5-14\,\mu\text{m}$  (average:  $9\,\mu\text{m}$ ) and the breadth is  $3-13 \,\mu m$  (average:  $8 \,\mu m$ ). The length/breadth ratio varies between 0.7 and 1.7 (average 1.1). Floating form twisted and wrinkled, without pseudopodia. Vesicular nucleus 2-3 µm in diameter with a single, spherical central nucleolus up to 0.6-0.9 µm in diameter. Cell coat includes glycostyles only 42-60 nm long and less regularly organized than in other vannellas. Simple filaments among glycostyles up to 300 nm in length are very abundant. Brackish-water, can thrive in anerobic conditions. Description and illustrations: Smirnov and Fenchel (1996); Smirnov (1999). Type slides deposited at the British Museum of Natural History (holotype 1995:9:6:3; paratype 1995:9:6:4).

23. Vannella persistens Smirnov et Brown, 2000

Locomotive form rather regular, semi-circular, or flabellate; more rarely oval or spatulate. Frontal hyaloplasm occupies about half of the total body area. The posterior end of the body slightly drawn back in some specimens. Length in locomotion  $23-35\,\mu\text{m}$  (average  $29\,\mu\text{m}$ ); breadth  $23-40\,\mu\text{m}$ (average  $34 \,\mu\text{m}$ ), length/breadth ratio 0.65 - 1.1. Vesicular nucleus 3.5-5 µm in diameter with a single central nucleolus, 2.6-3.3 µm in diameter. Floating form rather polymorphic, generally asymmetric, with up to 8 tapering pseudopodia if well-developed, but may have only 2-3 long, sometimes helical pseudopodia, or only several short, blunt pseudopodia with rounded ends. Cysts 13-16 µm in diameter, with thick "gelatinous" outer coat, clearly visible using light microscope. They are double walled, with clearly separated walls. Inner wall very fine, outer wall wrinkled, about 100 nm in thickness. The cell coat consists of a layer of glycostyles, about 110 nm in height, without simple filaments among them. Soil. Description and illustrations: Smirnov and Brown (2000): Figures 51-53. Available culture: CCAP 1589/13 (type culture). Partial 18S rRNA gene sequence (GenBank number EF051207) was obtained in the present study.

24. Vannella placida (Page, 1968) comb. nov. (formerly Platyamoeba placida)

Locomotive form spatulate, oval, or broadly elongate; anterior edge arched or truncate, posterior edge straight or irregularly triangular. Frontal hyaloplasm occupies from 1/3 to 2/3 of the total body area. Surface wrinkles sometimes occur parallel to or near edges. Maximal dimension in locomotion is 15-35 µm, length/breadth ratio 0.6-1.9 (average 1.2). Single vesicular nucleus  $3.4-5.5\,\mu m$  in diameter. Floating form with short, blunt hyaline pseudopodia in length not exceeding the diameter of the central mass. Cysts  $7-10.5 \,\mu\text{m}$  in diameter, double walled. Cyst walls closely apposed; inner wall thicker than outer. Cell coat includes apparently hexagonal prismatic elements and is about 15 nm thick. Soil. Description and illustrations: Page (1968 as Rugipes placidus; 1988, 1991; Page and Blakey 1979); Figs 54-57. Available culture: CCAP 1565/2 (type culture); CCAP 1565/14 isolated by S. Brown in 1999 and identified by LM and EM as P. placida (Brown and Smirnov 2004). Partial 18S rRNA gene sequence of 1565/2 strain by Fahrni et al. (2003) (GenBank AY294250); complete sequence EF051200.

### 25. Vannella plurinucleolus (Page, 1974) comb. nov. (formerly *Platyamoeba plurinucleolus*)

Locomotive form ovoid or spatulate; posterior edge rounded, straight, or slightly convex, Hvaloplasm occupies most of the total cell area; the granuloplasm is often a small ovoid mass along the posterior edge. Surface wrinkles sometimes occur parallel to or near edges. Maximal dimension in locomotion  $8-34 \,\mu\text{m}$  (average  $11-26 \,\mu\text{m}$ ); length/breadth ratio 0.9-1.2 (average 1.0-1.1). Single nucleus 2.4–6.5 µm in diameter. Nucleolar material in several parietal pieces (usually two or three). Rate of locomotion 15-47 µm/min. Floating form with short, blunt hyaline pseudopodia, in length not exceeding the diameter of the central mass. Marine, thrive up to 10 ppt salinity. Cell coat includes apparently hexagonal prismatic elements and is 25-27 nm thick. Description and illustrations: Page (1974, 1983); Figures 58-60. The strain called 'Platyamoeba plurinucleolus' by Smirnov (1999) has peculiar surface structure and floating form and may be a new species of Clydonella, not V. plurinucleolus. Type slides by F.C. Page: British Museum of Natural History (holotype 1974:1:9:7; paratype 1974:1:9:8). Available cultures: CCAP 1565/7 (type culture) and 1565/11. Complete 18S rRNA genes sequenced CCAP strains (GenBank from both EF051186-EF051190) in the present study. ATCC strain 50745 of T. Sawyer identified as P. plurinucleolus must be described as a new species.

26. Vannella pseudovannellida (Hauger, Rogerson et Anderson, 2001) comb. nov. (formerly *Platyamoeba pseudovannellida*)

Locomotive form ovoid or spatulate, sometimes with pronounced tail. Frontal hyaloplasm occupies about half the total body area; posterior end concave, flat or irregularly triangular. Length of the locomotive form  $8.5-22.5 \,\mu m$  (average  $14.2 \,\mu m$ ); length/breadth ratio about 1.02. Single vesicular nucleus about 2.1 µm in diameter with central nucleolus about 0.9 µm in diameter. Rate of locomotion 20.4 µm/min. Floating form with relatively long, uneven, blunt hyaline pseudopodia; some floating cells produce tapering pseudopodia. Cell coat does not include glycostyles and is a nearly amorphous layer  $\sim 10 \, \text{nm}$  thick, maybe because of fixation problems. Saltwater. Description and illustrations: Hauger et al. (2001). Type slides: British Museum of Natural History (holotype 2001:6:25:1). No culture available.

27. Vannella sensilis Bovee 1953

Locomotive form fan-shaped, semicircular or spatulate. The hyaloplasm occupies from 1/2 to 2/3 of the total body area. The anterior edge of the

cell is often uneven, finely dentate; the cell sometimes forms longitudinal ridges and transverse waves on the frontal hyaloplasm. Length of the locomotive form  $13-20 \, \text{um}$ : breadth 15-24 µm. Single vesicular nucleus about 3 µm in diameter with central endosome about 1.5 µm in diameter. Floating form of radial type, with long, tapering pseudopodia, tending to coil spirally (it is unclear why Page (1983, p.31) indicated that 'pseudopodia of floating form are short' for this species — see illustrations by Boyee and Sawyer). Ultrastructure unknown. Marine. Decription: Bovee (1953) (in some sources indicated 1950, but that was his Ph.D. thesis - not a formal publication). Description: Bovee and Sawyer (1979); Sawyer (1975b, includes photographs). Neither type material nor cultures available.

#### 28. Vannella septentrionalis Page, 1980

Locomotive form fan-shaped or spatulate; frontal hyaloplasm occupies about half the total body area. Posterior edge of cell concave, straight, or pyramidal. Greatest dimension in locomotion  $15-33 \,\mu\text{m}$  (average  $\sim 22 \,\mu\text{m}$ ); length/breadth ratio 0.5-1.3 (average 0.8-0.9). Locomotive rate about 3 cell lengths per minute. Single vesicular nucleus 3.7-5.6 um in diameter with central nucleolus 1.9-3.2 µm in diameter. Floating form with asymmetrically distributed short, thick hyaline pseudopodia not exceeding twice the diameter of the central cytoplasmic mass. Cell surface includes glycostyles with few simple filaments among them. Marine. Description and illustrations: Page (1980b, 1983); Figures 61 and 62. Type slides by F.C. Page: British Museum of Natural paratype (holotype 1980:1:24:7: History 1980:1:24:8). Available culture: CCAP 1565/10 (type culture). Complete 18S rRNA gene sequenced (GenBank EF051197) in the present study.

### 29. Vannella simplex (Wohlfarth-Botterman, 1960) Bovee, 1965

Locomotive form semicircular or fan-shaped, often with long 'tail'. Frontal hyaloplasm occupies about half of the cell; the front edge may be smooth or slightly wavy. Rapidly moving cells often have long 'tail'; in cultures amoebae are commonly covered with patches of faecal pellets. Locomotive forms vary in size; 25-80 µm in dimension (average 42—52 μm). greatest Rounded vesicular nucleus 6-11 µm in diameter with single spherical central nucleolus. Floating form of radial type, with a number of fine, tapering hyaline pseudopodia. Cell surface is covered with glycostyles about 100 nm in length; simple, filaments present among them. Freshwater. This species was illustrated in numerous papers; probably the most representative illustrations are in Wohlfarth-Bottermann (1960 as *Hyalodiscus simplex*); Haberey and Hülsmann (1973); Page (1976, 1988, 1991); Ariza et al. (1989); Smirnov et al. (2002); Figures 28–31. Available cultures: CCAP 1589/3 by N. Hülsmann (not the original strain, isolated by Wohlfarth-Bottermann in 1960, but originating from the same pond) and ATCC 50925 by T. Nerad (the latter lacks published data). Complete sequence of the 18S rRNA gene from CCAP 1589/3 obtained in the present study (Genbank EF051208; EF051209); sequences of other strains: EF051210–EF051211.

30. Vannella weinsteini (Sawyer, 1975) comb. nov. (formerly *Platyamoeba weinsteini*)

Locomotive form ovoid to discoid with smooth rounded anterior margin and flattened or slightly convex posterior margin. Frontal hyaloplasm occupies about 2/3 of the total cell area or even more; the cell tends to form longitudinal ridges and transverse ripples on the hyaloplsm. Length in locomotion  $11-14 \,\mu\text{m}$  (average  $12 \,\mu\text{m}$ ); breadth  $12-16 \,\mu\text{m}$  (average  $13 \,\mu\text{m}$ ). Single vesicular nucleus ca.  $3 \,\mu\text{m}$  in diameter with rounded central nucleolus. Rate of locomotion about  $40 \,\mu\text{m/min}$ . Floating form with short, blunt hyaline pseudopodia. Marine. Description and illustrations: Sawyer (1975a). Neither ultrastructure nor type material.

31. *Ripella platypodia* (Glaeser, 1912) comb. nov. (originally *Amoeba platypodia* and later *Vannella platypodia*)

Locomotive form fan-shaped, often with pronounced tail or discoid. Anterior margin smooth and rounded; posterior margin often triangular. Frontal hyaloplasm occupies about 2/3 of the total cell area or even more. Greatest dimension of the locomotive form  $10-30 \,\mu m$  (average  $16-21 \,\mu m$ ). Single vesicular nucleus 3.4-5.0 µm in diameter with rounded central nucleolus. Floating form with long tapering pseudopodia far exceeding the diameter of the central mass. Cell surface is covered with glycostyles about 110 nm in length; simple, filaments present among them. Freshwater. Description: Page (1968). Illustrations: Page (1976, 1988, 1991; Page and Blakey 1979 - cell surface); Figures 63-68. Available culture: CCAP 1589/2 by F.C. Page. Complete 18S rRNA gene sequences (GenBank numbers EF051184-EF051185EF051184EF051185) were obtained in the present study.

32. Clydonella rosenfieldi Sawyer, 1975

Locomotive form discoid with smooth anterior margin and shallow convex posterior margin. The hyaloplasm occupies half or more of the total body area. Length in locomotion  $14-19 \mu m$ (average  $17 \mu m$ ), breadth  $14-19 \mu m$  (average  $16 \mu m$ ). Single vesicular nucleus ca  $4.5 \mu m$  in diameter with central nucleolus  $2-2.5 \mu m$  in diameter. Rate of locomotion about  $40 \mu m/min$ . Floating form with numerous long blunt-tipped hyaline pseudopodia which may bend slightly along their axes to resemble rootlets; some may fork at the tip. Marine, tolerates up to 7.5 ppt salinity. Description and illustrations: Sawyer (1975a). Neither ultrastructure nor type material.

33. Clydonella sindermanni Sawyer, 1975

Locomotive form ovoid with slightly rippled or smooth anterior margin and flattened posterior margin. The hyaloplasm occupies half or more of the total body area. Length in locomotion  $21-40\,\mu$ m (average  $28\,\mu$ m), breadth  $18-37\,\mu$ m (average  $27\,\mu$ m). Single vesicular nucleus, ca.  $4.5\,\mu$ m in diameter with central nucleolus  $3-3.5\,\mu$ m in diameter. Rate of locomotion, ca.  $35-40\,\mu$ m/min. Floating form with numerous long blunt-tipped hyaline pseudopodia. Marine, tolerates up to 7.5 ppt salinity. Description and illustrations: Sawyer (1975a). Neither ultrastructure nor type material.

34. *Clydonella vivax* (Schaeffer, 1926) Sawyer, 1975

Locomotive form semicircular or fan-shaped with smooth anterior margin and slightly concave or triangular posterior. Hyaloplasm occupies half or more of the total body area. Length in locomotion  $12.6-18.0 \,\mu\text{m}$  (average  $14.2 \,\mu\text{m}$ ), breadth  $9.0-15.3 \,\mu\text{m}$  (average  $12.9 \,\mu\text{m}$ ). Single vesicular nucleus ~ $2.8 \,\mu\text{m}$  in diameter. Rate of locomotion ~ $46 \,\mu\text{m}/\text{min}$ . Floating form with numerous long blunt-tipped hyaline pseudopodia; some of these pseudopodia may fork distally. Marine, tolerates up to about 17.5 ppt salinity. Description and illustrations: Sawyer (1975b). Neither ultrastructure nor type material.

35. Clydonella wardi Sawyer, 1975

Locomotive form ovoid with nearly parallel lateral margins and flat or slightly concave posterior margin. The hyaloplasm occupies half or more of the total body area; in locomotion wide waves of the hyaloplasm flow anteriorly and disappear on reaching the advancing margin . Length in locomotion  $14-20 \,\mu\text{m}$  (average  $18 \,\mu\text{m}$ ), breadth  $13-19 \,\mu\text{m}$  (average  $16 \,\mu\text{m}$ ). Single vesicular nucleus, ca.  $3-3.5 \,\mu\text{m}$  in diameter with central nucleolus, ca.  $2.5 \,\mu\text{m}$  in diameter. Rate of locomotion, ca.  $35 \,\mu\text{m}/\text{min}$ . Floating form with numerous long, blunt-tipped, slightly bent hyaline pseudopodia, which may fork at their tips. Marine, tolerates up to  $22.5 \,\text{ppt}$  salinity. Description and

illustrations: Sawyer (1975a). Neither ultrastructure nor type material.

36. Lingulamoeba leei Sawyer, 1975

Locomotive form tongue-shaped with blunt. smooth anterior anterior margin and flattened or triangular posterior hyaloplasm occupies half or more of the total body length. Length in locomo-16-23 um (average 20 um). tion breadth 12-18 µm (average 14.5 µm). Single vesicular nucleus, spherical, or slightly ovoid, ca. 3.5 µm in diameter with central nucleolus, ca. 2.5 µm in diameter. Rate of locomotion  $\sim 20-30 \,\mu\text{m/min}$ . Floating form spherical, with wrinkled surface, without pseudopodia. Marine, tolerates up to ca. 7.5 ppt salinity. Description and illustrations: Sawyer (1975a); ultrastructure: Peglar et al. (2003). No type material. Available culture: ATCC 30734. Complete 18S rRNA gene sequenced by Peglar et al. (2003) (GenBank AY183886).

37. Pessonella marginata Pussard, 1973

Locomotive form fan-shaped or semicircular. Frontal hyaloplasm often forms deep and relatively narrow antero-lateral crescent, but can also produce usual Vannella-like frontal hyaline area occupying up to half of the cell. Anterior margin often uneven: numerous lobes on the anterior hyaline area; sometimes distinct longitudinal ridges. Posterior margin flattened or slightly convex. Length in locomotion  $\sim$ 35 µm. breadth  $\sim$ 45 µm. Single nucleus 4–8 µm in diameter with several peripheral pieces of nucleolar material. Rate of locomotion  $20-24 \,\mu$ m/min. Floating form with few short, thick, irregular and sometimes furcating hyaline pseudopodia. Isolated from compost in France. No ultrastructure or type material. Description: Pussard (1973).

#### **Methods**

Table 2 lists strains used for sequencing and LM study. They include all previously unsequenced strains of Vannellidae available from the Culture Collection of Algae and Protozoa (CCAP, UK), and all extant type strains, except for *Vannella mira* (where we failed to obtain a complete sequence). Strains which are not available through public collections can be obtained from the authors upon request. Amoebae were maintained on agar plates; for LM studies, cultures were transferred to liquid media [25 ppt seawater for marine species and PJ medium (Prescott and James 1955) for freshwater ones] and incubated for about 1 week. Observations, measurements, and

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Species and strain	Source	Habitat, isolator, and year of isolation	Notes
1. Vannella devonica	CCAP 1589/5	Marine, Kingsbridge Estuary, Devon, England, F.C. Page, 1977	Type culture
2. Vannella ebro	CCAP 1589/14	Marine, cyanobacterial mat in Ebro Delta, Spain. A. Smirnov, 2000	Type culture
3. Vannella arabica	CCAP 1589/7	Marine, seawater tank in Kuwait Inst. for Scientific Research. F.C. Page, 1977	Type culture
4. Vannella lata	CCAP 1589/12	Freshwater, Loch Morar, Scotland, isolator unknown, deposited by F.C. Page, 1988	
5. Vannella persistens	CCAP 1589/13	Soil, Sourhope Research Station, Scotland. S. Brown, 1999	Type culture
6. 'Vannella' platypodia	CCAP 1589/2	Freshwater, creek, Indiana, USA. F.C. Page, 1964	<b>-</b> 1
7. Vannella septentrionalis	CCAP 1589/10	Marine, estuary of River Don, Scotland. F. C. Page, 1979	Type culture
8. vannella simplex	CCAP 1589/3	University of Bonn, Germany. N. Hülsmann,	neotype
9. Vannella danica	CCAP 1589/17	Brackish-water, artificial microbial mats inoculated with sediment from Nivå Bay, Helsingør, Denmark, A. Smirnov, 2000	Type culture
10. <i>Vannella simplex</i> Geneva strain		Freshwater, pond at the Station de Zoologie Exp. Gen., University of Geneva, Route de Malagnou, 154, Geneva, Switzerland, A. Smirnov, 2001	
<ol> <li>Vannella simplex Gurre Lake strain</li> <li>Vannella sp.</li> <li>Geneva strain</li> <li><i>Ripella</i> sp. Priest Pot</li> </ol>		Freshwater, Gurre Lake, 10 km North from Helsingør, Denmark, A. Smirnov, 2001 Freshwater, Lake Neuchatel, Switzerland. A. Smirnov, 2001 Accompanying species in CCAP 1555/2 culture of <i>Paradermamoeba levis</i> , isolated from Priest Pot LIK A. Smirnov, 2002	
14. 'Platyamoeba' australis 15. 'Platyamoeba'	CCAP 1565/9 CCAP 1565/10	Marine, Marorchydore, near Brisbane, Australia. F.C. Page, 1977 Marine, River Deben, Woodbridge, Suffolk,	Type culture
bursella 16. 'Platyamoeba' calvoinucleolus	CCAP 1565/6	England. F.C. Page, 1973 Marine, beach West Mersea, Essex, England, F.C. Page, 1972	Type culture
17. 'Platyamoeba' plurinucleolus	CCAP 1565/7	Marine, beach West Mersea, Essex, England, F.C. Page, 1972	Type culture
18. 'Platyamoeba' plurinucleolus	CCAP 1565/11	Marine, beach West Mersea, Essex, England. F.C. Page, 1972	
19. <i>'Platyamoeba'</i> <i>placida</i> 20. <i>'Platyamoeba'</i> sp. ED40 strain 21. <i>'Platyamoeba'</i> sp. ED-AS strain	CCAP 1565/2	Freshwater; stream outflow, Madison, Wisconsin, USA. F.C. Page, 1964 Marine, cyanobacterial mat in Ebro Delta, Spain. A. Smirnov, 2000 Marine, cyanobacterial mat in Ebro Delta, Spain. A. Smirnov, 2000	Type culture

**Table 2.** Sources, status, and reference numbers of studied amoeba strains. Quotation marks indicate species and genera names that were revised after the present study.

photographs were done using inverted Olympus IX70 microscope.

DNA was extracted using guanidine thiocyanate followed by isopropyl alcohol precipitation (Man-

iatis et al. 1982). To collect cells, amoebae and food bacteria were gently scraped from the agar surface with a disposable plastic scraper to form an aggregate, to the top of which  $\sim$ 100 µl of

guanidine thiocyanate was added, mixed briefly, and immediately transferred by Pasteur pipette to an Eppendorf tube. DNA of *V. persistens* was isolated as in Cavalier-Smith et al. (1995).

We were unable to amplify the SSU rRNA gene in one piece in some "difficult" species; so specific custom-made primers were used to amplify it in several fragments. Figure S1 (see Supplementary Materials) shows the primer map and schemes of amplification for individual species; primer sequences are in Table S9 (see Supplementary Materials). Thermal cycle parameters varied among species; typically initial denaturation (5 min at 95 °C) followed by 40 cycles of 30 s at 94 °C, 90 s at 50–58 °C (depending on the primers), and 60 s at 72 °C, followed by 10 min at 72 °C for final extension. For some species, it helped to increase the initial denaturation time to 15 min; gradient PCR was used to find optimal annealing temperatures. Positive amplification products were purified using GFX PCR Purification Kit (Amersham Biosciences) and either sequenced directly or ligated into Topo TA Cloning<sup>®</sup> vector (Invitrogen) and cloned in One Shot<sup>®</sup> TOP10 *E. coli* ultracompetent cells (Invitrogen). Two or more clones were sequenced for each cloned PCR product. Sequencing reactions used the ABI-PRISM Big Dye Terminator Cycle Sequencing Kit. Table 3 lists Genbank accession numbers of all new sequences.

All 57 available nearly complete vannellid SSU rDNA sequences were aligned manually (the alignment is available upon request) and 1904 reliably aligned nucleotide positions were selected for phylogenetic analysis by Bayesian, distance (BioNJ: Gascuel 1997), and

**Table 3.** Genbank accession numbers of newly obtained 18S rRNA gene sequences. Molecular clones are indicated; absence of specific indication means direct sequence of the PCR product.

Ripella sp. from CCAP 1555/2 cultureEFGRipella sp. from CCAP 1555/2 culture, molecular clone 1EFGRipella platypodia (former Vannella) CCAP 1589/2, molecular clone 8EFGRipella platypodia (former Vannella) CCAP 1589/2, molecular clone 13EFGVannella platypodia (former Vannella) CCAP 1589/2, molecular clone 13EFGVannella platypodia (former Platyamoeba) CCAP 1565/7EFG	051182 051183 051184 051185 051186 051187
Vannella plurinucleolus (former Platyamoeba) CCAP 1565/7 molecular EF(	
Vannella plurinucleolus (former Platyamoeba) CCAP 1565/7 molecular EFC clone 2	51188
<i>Vannella plurinucleolus</i> (former <i>Platyamoeba</i> ) CCAP 1565/11 molecular EFC clone 8	051189
<i>Vannella plurinucleolus</i> (former <i>Platyamoeba</i> ) CCAP 1565/11 molecular EFC clone 2	051190
Vannellasp. ED40 strainEFGVannellasp.ED-AS strain, molecular clone 1EFGVannellacalycinucleolus (former Platyamoeba) CCAP 1565/6EFGVannellaarabica CCAP 1589/7EFGVannella bursella (former Platyamoeba) CCAP1565/10EFGVannella devonica CCAP 1589/5EFGVannella septentrionalis CCAP 1589/10EFGVannella australis (former Platyamoeba) CCAP 1565/9EFGVannella australis (former Platyamoeba) CCAP 1565/9EFGVannella placida (former Platyamoeba) CCAP 1565/2EFGVannella placida (former Platyamoeba) CCAP 1565/2EFGVannella lata CCAP 1589/12EFGVannella sp. Geneva strainEFGVannella danica CCAP 1589/17 variant 1EFGVannella danica CCAP 1589/17 variant 2EFGVannella danica CCAP 1589/17 molecular clone 1EFGVannella danica CCAP 1589/17 molecular clone 2EFGVannella persistens CCAP 1589/13EFGVannella simplex CCAP1589/3EFGVannella simplex CCAP1589/3 molecular clone 1EFGVannella simplex Gurre Lake strainEFG	J51191 J51192 J51193 J51194 J51195 J51196 J51196 J51197 J51198 J51199 J51200 D51201 D51202 J51204 J51205 J51205 J51206 J51207 J51208 J51209 J51209 J51210

maximum-likelihood (PHYML: Guindon and Gascuel 2003) methods. To verify the position of the root of the vannellid tree, preliminary trees were run with a variety of taxon samples chosen from a broader alignment of 107 sequences representing the amoebozoan subphylum Protamoebae, using GTR+ $\Gamma$ +I distance analyses only. For more thorough analysis, the closest outgroups to Vanellidae (35 sequences of other discosean and variosean amoebae: Kudryavtsev at al. 2005) were included with the 57 vannellids in an 87 sequence data set and 1577 well-aligned positions were analyzed by all three methods. For both data sets, Model Test (v. 3.7: Posada and Crandall 1998) showed that GTR with gamma intersite rate variation and invariant positions was the best substitution model, which was used for BioNJ and PHYML (using nine gamma rate categories, PHYML optimized the number of invariant sites to zero; for PHYML gamma alpha parameters and tree topology were also optimized). Bayesian analysis (Mr Bayes v. 3.1.2: Huelsenbeck and Ronguist 2001) used the GTR gamma model with allowance for covarions and rate correlations between adjacent sites: two independent sets of four simultaneous chains were run for 1.74-3.65 million generations, sampling trees every 250 generations; after discarding early trees for burn in, 5450-7006 trees were used for consensus analysis. For PHYML, the starting tree was the BIONJ tree, but if the Bayesian tree differed significantly, this was also used and the resulting consensus tree with the higher likelihood was chosen. Bootstrap analysis used 100 (PHYML) or 1000 (BIONJ) pseudoreplicates.

For the vannellid only data set, we also carried out base composition analysis and ML analysis using Treefinder, with starting trees produced by all three above methods, and the GTR gamma plus invariant sites model with four rate categories (Jobb 2006).

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#### Note in proof

We hereby transfer two newly described Platyamoeba species (Moran et al. 2007) to Vannella as new combinations: *Vannella oblongata* and *Vannella contorta*. In corroboration of our results the novel *V. epipetala* is most closely related to former Platyamoeba strains (Amaral-Zettler et al. 2006). These publications since our paper was submitted raise the vannellid species total to 40.

#### Appendix A. Supplementary materials

Supplementary data associated with this article can be found in the online version at doi:10.1016/ j.protis.2007.04.004

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