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Morphological, Ecological and Molecular Studies of *Vannella simplex* Wohlfarth-Bottermann 1960 (Lobosea, Gymnamoebia), with a new Diagnosis of this Species

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***Vannella simplex* (Gymnamoebia, Vannellidae) is one of the most common amoebae species, recorded from a variety of regions. It was originally described as a freshwater species, but has also been reported from shallow-water regions of the Baltic Sea. In the present work, we investigated the morphology and biology of three *V. simplex* isolates, originating from geographically distant regions. Among them is one brackish water strain, isolated from artificial cyanobacterial mats, which were originally sampled in Nivå Bay (Baltic Sea, The Sound). The strain is cyst-forming and can thrive at salinity ranges from 0–50 ppt. Phylogenetic relationships were investigated by sequencing partial SSU rDNA of the cultured *V. simplex* isolates. Additional sequences were obtained from four environmental DNA extractions of sediment samples collected from different localities in Switzerland. Analysis of all obtained sequences revealed a monophyletic group. Based on the analysis and comparison of morphological, ecological and molecular data sets we compiled a distribution map of *V. simplex* and propose an emendation of this species.**

Introduction

Species identification of naked lobose amoebae (Lobosea, Gymnamoebia) remains nearly exclusively morphological (Page 1991). The lack of distinctive morphological characters and poorly defined type descriptions frequently impede taxo-

nomic identification (Anderson 1996; Smirnov 1999). Many well-known amoeba species were established without proper diagnosis and type material. As a result, the identification of an amoeba or distinction of similar amoeba species requires serious comparative and literary study, and none the less sometimes remains speculative (Michel and Smirnov 1999; Smirnov and Goodkov 1998).

Molecular techniques, especially the analysis of DNA sequences, offer a powerful tool to examine or-

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ganism relationships at different taxonomic levels. Molecular data are independent of morphological and ecological ones, allowing investigation of controversial taxonomic issues from a different angle. Application of molecular methods for distinction between heterolobosean species looks promising (e.g. Brown and Jonckheere 1999) however, it is not widely used for lobose amoebae, except for the medically important genus *Acanthamoeba* (e.g. Stothard et al. 1998).

The naked lobose amoeba *Vannella simplex* Wohlfarth-Bottermann 1960 is one of the best-known amoebae species, originally described from a freshwater environment (Wohlfarth-Bottermann 1960) and reported from a variety of locations in Europe (Finlay and Maberly 2000; Smirnov and Goodkov 1995). Reliably identified isolates of *V. simplex* have also been reported from brackish water regions of the Baltic Sea (Garstetcki and Arndt 2000; Smirnov 2001a; Vørs 1992). It is generally considered that the same amoeba species cannot appear in both marine and freshwater habitats (Page 1988), but freshwater and marine species may co-occur in the brackish-water Baltic Sea (Garstetcki and Arndt 2000; Schmöller 1961; 1964) and several "marine" amoebae species can tolerate freshwater conditions (Hauer et al. 2001; Page 1983).

In the present work, we used a combination of morphological, ecological and molecular methods to examine the diversity and geographical distribution of *V. simplex*. Based on the analysis and comparison of the different data sets, we propose an emendation of the species *Vannella simplex* Wohlfarth-Bottermann 1960 and describe a new cyst-forming brackish-water strain of this species.

Results

1. Morphological Studies

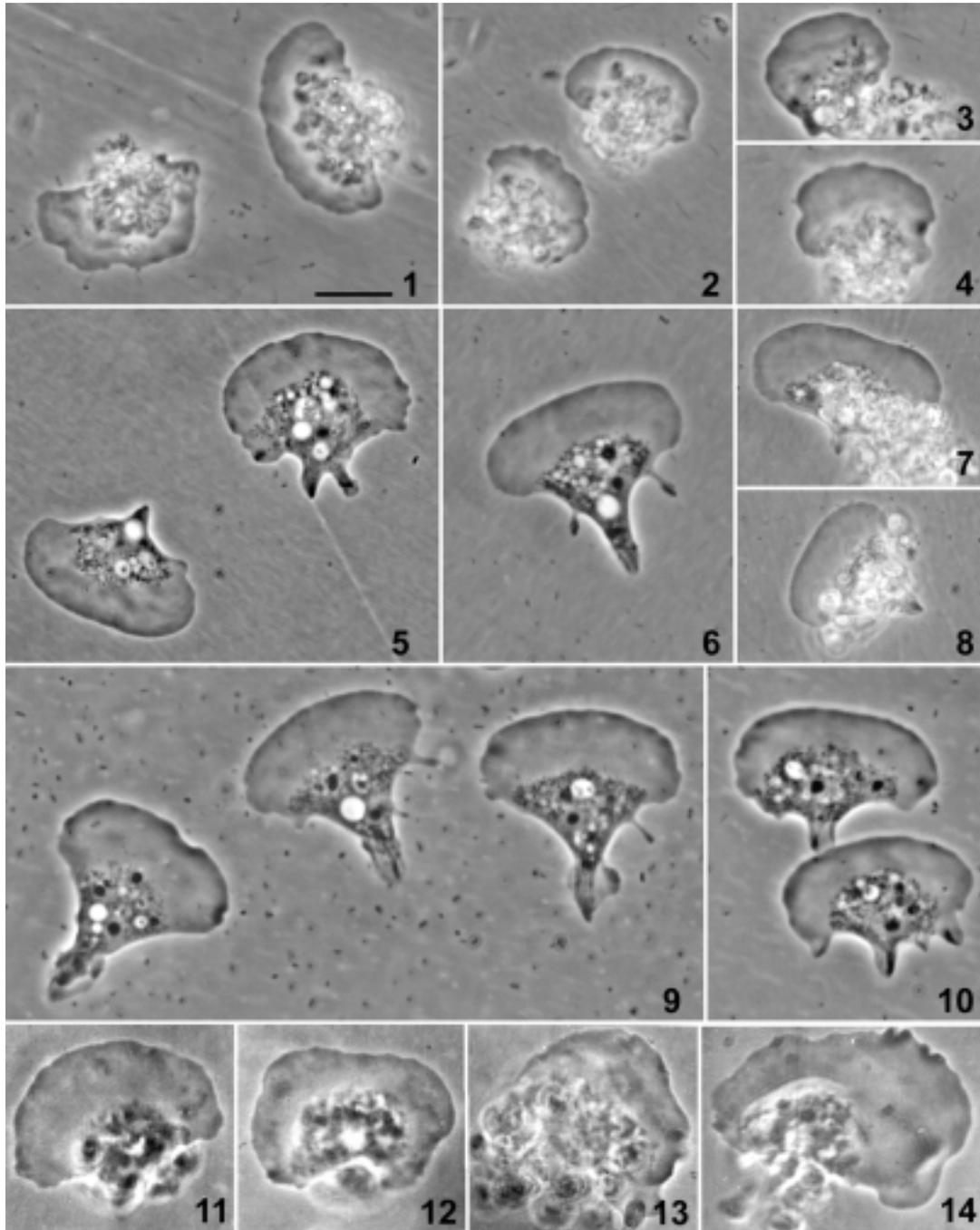
Locomotive morphology (Figs 1–4), floating form (Fig. 15) and nuclear structure (Fig. 16) of the *Vannella simplex* Nivå isolate were identical to published descriptions of *V. simplex* (Page 1976a; 1988; 1991; Wohlfarth-Bottermann 1960). The length of locomotive forms in Nivå amoebae as measured on plastic surfaces in culture ranged from 25–50 µm (average 32 µm) and the breadth varied between 25–50 µm (average 39 µm). Length/breadth ratio was 0.5–1.25 (average 0.82). The vesicular nucleus was about 7 µm in diameter; the central or slightly eccentric rounded nucleolus was about 4 µm in diameter. Cultured cells were always densely covered with rounded faecal pellets, defecation of these pellets was often seen. No contractile vacuoles were ob-

served in the cytoplasm of individuals kept in seawater (17 ppt), whereas such vacuoles were clearly visible in individuals living in freshwater media (PJ). No axial core of microfilaments was found in the pseudopodia of the floating form (Fig. 17). The cytoplasm in these pseudopodia was differentiated into less dense, homogenous cortical cytoplasm and a denser axial one.

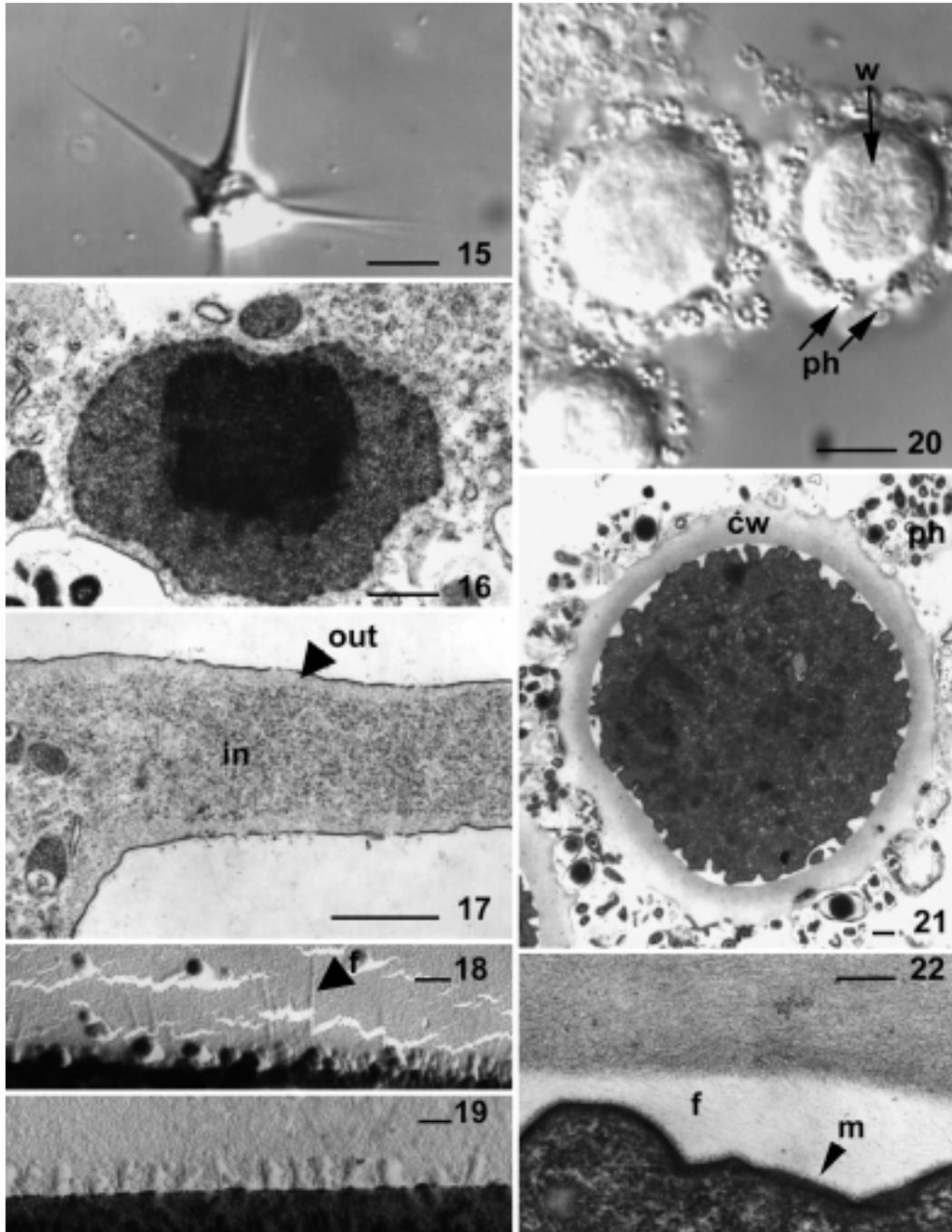
Vannella simplex Nivå isolate readily formed cysts (Figs 20–21) in 2–3 week-old cultures. Cysts were single walled, rounded and reached 16–26 µm in diameter. Each cyst was always surrounded by a layer of faecal pellets. The cyst wall was of variable thickness (1.1–1.8 µm) and consisted of very fine filamentous material, slightly more dense in the inner part of the wall and forming a very thin, dense "cortical" outer layer (Figs 21–22). The cell surface inside the cyst was wrinkled (Fig. 21). It is interesting to note that within cysts, the cell membrane did not possess glycostyles. It would be hard to believe that they had been destroyed due to fixation, as an empty "halo" of the respective width normally marks the place where glycostyles have been located. The membrane was covered with a layer of filamentous material, apparently the same as found in the cyst wall (Fig. 22). The space between the cell surface and the cyst wall was loosely filled with the same filaments.

According to our experiments, amoebae of the *V. simplex* Nivå isolate can live at salinity ranges from 0 (PJ medium) to 50 ppt. At extreme salinities (40–50 ppt) and in freshwater, amoebae grow slowly, most cells were non-locomotive, and the few locomotive forms found so far were fan-shaped, with uneven frontal hyaline area. At all other salinity ranges that were tested (6, 10, 17, 30 ppt), they showed normal locomotion. The fastest growth of cultures was observed at salinity values of 10 ppt.

Amoebae of other studied strains: *V. simplex* CCAP 1589/2 (not illustrated), Gurre (Figs 5–8), Malagnou 1–3 (Figs 9–10) and Valamo (Figs 11–14) show basically identical morphology and sizes, corresponding to the above description and other published descriptions of *V. simplex* (op. cit.). The only variable character was the presence of faecal pellets covering amoebae. It was nearly permanent in Valamo and Gurre lake strains (few non-covered specimens were found during the whole period of observation). Concerning the Malagnou isolates, nearly all amoebae were covered in young cultures, while most amoebae were free from pellets in mature cultures, but a lot of pellets and patches of these pellets appeared floating in the culture medium. All amoebae of *V. simplex* CCAP1589/2, cultivated in PJ medium exhibited faecal pellets on the dorsal surface.



Figures 1–14. Various isolates of *Vannella simplex*. **1–4:** *V. simplex* Nivå (brackish-water). Cells are covered with faecal pellets, note the variety of locomotive form. **5–8:** *V. simplex* Gurre Lake (freshwater). Note pronounced “tail” in 6. Few cells (7–8) are covered with faecal pellets. **9–10:** *V. simplex* Malagnou_1 (freshwater). Note pronounced tails in all cells. No cells are covered with faecal pellets. **11–14:** *V. simplex* Valamo (freshwater). Nearly all cells are covered with faecal pellets. Scale bar is 10 μm throughout; visible size difference in figures is due to the choice of appropriate specimens for photographing and does not mean the overall size difference between isolates.



Figures 12–22. *Vannella simplex* Nivå (brackish-water). **15:** Floating form. **16:** Nucleus. **17:** Pseudopod of the floating form with noticeable difference in the density and appearance of the outer (out) and inner (in) cytoplasm. **18:** Cell coat of *V. simplex* Valamo (freshwater, control) with well-visible simple filaments (f). **19:** Cell coat of *V. simplex* Nivå (brackish-water) without simple filaments. **20:** Cysts of *V. simplex* Nivå, note wrinkled cell surface (w) and surrounding faecal pellets (ph). **21:** Same, TEM. Note cyst wall (cw), faecal pellets (ph) and wrinkled cell surface inside the cysts. **22:** Cyst wall, closer. Note fibrous structure of the cyst wall and same fibrous material (f) between the cell surface and the cyst wall. There is no glycostyles on the cell membrane (m). Scale bar is 10 μm in Figs 15 and 20; 1 μm in Figs 16, 17 and 21; and 100 nm in the Figs 18, 19 and 22.

Transmission electron microscopical (TEM) studies revealed an identical pattern of glycostyles in *V. simplex* Gurre, Valamo, CCAP1589/2 and Nivå strains (Malagnou isolates were not examined by EM). Simple filaments among the glycostyles were seen in all studied isolates except for *V. simplex* Nivå strain (Fig. 19), where they were also absent in CH preparations. Although we cannot neglect possible fixation artefacts, CH preparations gave rather clear pictures of glycostyles and filaments in other investigated strains (e.g. Fig. 18, illustrating cell coat of Valamo strain). Therefore, it might be possible that filaments are absent in the *V. simplex* Nivå strain.

2. Sequence Data

Ten partial SSU rDNA sequences were obtained for *Vannella simplex* and two sequences were acquired for *Vannella ebro* and *Vannella anglica* respectively. Sequence variations within all studied sequences range from 0% (Grande Caricaie_44 a+b) to 3% (Malagnou_3, Grande Caricaie_43). The investigated sequences contain between 433 to 455 base pairs (bp), their G+C content ranges from 37.7% (*V.*

ebro) to 42.2% (Grande Caricaie 44_a+b). They correspond to the 3' terminal region of *Mus musculus* (M-X00686), starting at position 1267 and ending at position 1709. The examined fragment includes three eukaryotic expansion segments according to Van de Peer et al. (1998).

Analysis of the partial SSU rDNA gene conducted by Neighbor Joining (NJ) and Maximum Likelihood (ML) methods applying Kimura's two parameters model (Kimura 1980) yielded identical phylogenetic trees (Fig. 23). Additionally, Tajima and Nei's six-parameter model (Tajima and Nei 1984) was used to test NJ and ML methods, which resulted in trees identical to the one in Figure 23 (data not shown).

Figure 23 represents the ML tree with *Vannella ebro* and *Vannella anglica* used as an outgroup. All obtained *Vannella simplex* sequences form a very well supported monophyletic clade (100% bootstrap) that is divided in two sister groups (54% and 82% bootstrap, respectively). Both groups contain sequences obtained from environmental DNA samples (Seymaz_33, Neuchâtel Lake_51, Grande Caricaie_43, 44 a+b) and from cultured isolates (Malagnou pond_1–3, Gurre Lake, Nivå Bay). All obtained sequences of *V. simplex* share a characteristic pattern of nucleotides: 5' CTA GTT ATA T(AG)T ATA CTT (TA)TG TAT ATA TAG ATG AA 3', (pos. 1491–1525 in *Vannella anglica*). The indicated transition and transversion occur only in two sequences (Grande Caricaie 44_a and 44_b, clones from the same PCR product), which were obtained from environmental DNA extractions.

Discussion

1. Species Identification and Distinction

All investigated isolates, including the brackish-water strain from the Nivå Bay were identified as *Vannella simplex* Wohlfarth-Bottermann 1960, based on the comparison of appearance and size of locomotive and floating forms, nuclei and ultrastructure with published data on *V. simplex* (Hausmann 1975; Hausmann and Hülsmann 1996 (p.30); Hausmann and Stockem 1972; Hausmann et al. 1972; Hülsmann and Habery 1973; Page 1988; 1991; Vørs 1992; Wohlfarth-Bottermann 1960).

There are two formally existing species, resembling *V. simplex*, namely *Vannella cirifera* (Frenzel 1892) and *Vannella mira* (Schaeffer 1926). The taxonomical problems related with this species complex were discussed by Smirnov (2002) and it was shown that *V. mira* is a distinct marine amoeba species, whereas *V. cirifera* has no morphological characters allowing a reliable distinction from *V. simplex* and is

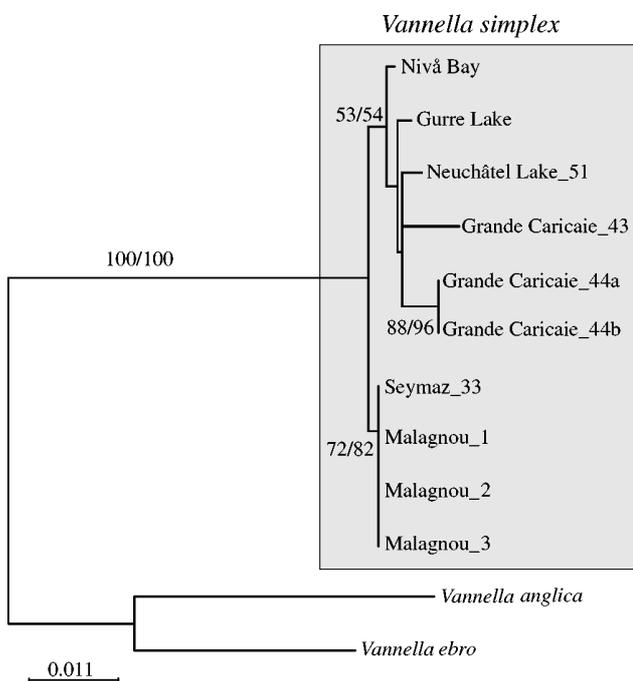


Figure 23. Phylogenetic tree based on 12 partial SSU rDNA sequences of *Vannella* spp. Using maximum likelihood analysis. The tree is rooted in *V. anglica* and *V. ebro*. Bootstrap values are based on 1000 resamplings for the NJ and on 500 resamplings for the ML tree (first and second number respectively). Sequences Grande Caricaie_44a and _44b are clones from the same PCR product.

therefore announced to be *nomen dubium*. Even the absence of simple filaments in the glycocalyx of “*V. cirifera*” – the only seemingly clear difference (Page 1988) between this species and *V. simplex* – is shown to be not reliable in the present study.

Culture experiments have shown that *V. simplex* Nivå is not only able to grow at different salinities but also forms cysts, a fact that has never been documented before. The potential of cyst formation might be a prerequisite for life in fluctuating environments with serious shifts in salinity and oxygen concentrations, as is the case for Nivå Bay (Fenchel 1969). Recent observations on *Vannella persistens* (Smirnov and Brown 2000) and *Vannella ebro* (Smirnov 2001b) have shown that the formation of resting stages is not an outstanding feature for this genus.

2. Sequence Data

Molecular analysis indicates that all studied sequences, obtained both from cultivated *V. simplex* strains and from total DNA extracts, are very similar and form a well-supported clade (100% bootstrap value), which is distant from other sequenced *Vannella* species. The monophyletic *V. simplex* clade consists of two sister groups, which might represent different varieties of the same species. The two groups, however, are only moderately well supported (53 and 72% for NJ, and 54 and 82% bootstrap value for ML analysis, respectively). Each group includes both sequences of morphologically identified strains and those obtained from environmental DNA extractions. Genetic distances are more pronounced between sequences from different Swiss sampling localities than between sequences from Danish and Swiss sampling stations.

3. Freshwater vs Marine Occurrence of *V. simplex*

Vannella simplex has originally been described as a freshwater species (Wohlfarth-Bottermann 1960), but our results and published data clearly indicate its occurrence in marginal marine environments. This is contradictory to the opinion that amoeba species are adapted either to freshwater or to marine environments and that a particular species cannot occur in both environments (Page 1983; 1988). Another example of a brackish water *V. simplex* strain, isolated from the Gulf of Finland at salinities of 5 ppt, has been described by Vørs (1992). Schmöller (1961; 1964), Page (1983) and Garstecki and Arndt (2000) have also reported the co-occurrence of freshwater and marine amoeba in some areas of the Baltic Sea. Coastal water regions undergo serious shifts of

salinity, depending on the amount of rain, presence of freshwater streams running into the sea and income of marine water. Freshwater amoebae species might repeatedly invade such regions and survive if conditions are favourable. They might gradually adapt to a new habitat and develop an elaborate resistance against changing salinities. The close genetic relationship of the brackish water strain from Nivå Bay to several freshwater strains (Fig. 23) also supports the idea that freshwater amoeba might be able to colonize oligohaline environments, and a similar suggestion was proposed by Hauer et al. (2001) to explain the wide salinity tolerance of *Platyamoeba pseudovannellida*. Salinity tolerance tests, which have been performed for some amoeba strains (e.g. Sawyer 1975a,b), may not reveal the whole potential of the exposed organism, because adaptation might require more time than is available within a set of experiments.

4. Geographic Distribution of *Vannella simplex*

So far, the first and only attempt to investigate the geographical distribution of naked amoebae has been performed by F. C. Page (1976b). *Vannella simplex* is one of the best-documented and relatively easy recognisable amoebae species, and based on published records as well as our own data, we compiled a map of its geographic distribution (Fig. 24). This species is known from both freshwater and brackish-water habitats and seems to be widely distributed.

Recent molecular studies have revealed cryptic diversity in several species of planktonic and benthic Foraminifera. The cryptic species are adapted to different ecological conditions and most of them show a restricted geographical distribution (Darling et al. 2000; De Vargas et al. 2001; Holzmann 2000). Similar patterns may exist in naked amoebae. Our molecular results suggest that *V. simplex* consists of two molecular varieties. Further molecular studies should reveal the level of genetic heterogeneity in this morphospecies. Molecular subtypes might be used in future studies to create a precise map about the regional distribution of *V. simplex* varieties.

5. *Vannella simplex*: Emendation

Vannella simplex is one of the best known amoebae species and has been reported by many authors from all over the world. The species was well described by Wohlfarth-Bottermann (1960), but was established without formal diagnosis, type material and respective taxonomical formalities. Our new data on cyst formation, euryhalinity and possible



Figure 24. Records of *V. simplex* from various regions. References are in the reference list; absence of the reference or any other note mean “present study”. Finding of *V. simplex* in Spain (Fluvia et al. 1983) is questionable, as the cited paper does not contain any material allowing to verify the identification.

variations in cell coat structure expand the recorded characters and warrant an emendation of the species *Vannella simplex* Wohlfarth-Bottermann 1960. To avoid taxonomical problems, we have es-

tablish new type material for this species – a freshwater strain originating from the university pond in Bonn (Germany) held in the CCAP collection under reference number 1589/2.

Diagnosis: *Vannella simplex* Wohlfarth-Bottermann, 1960, emend.

The locomotive form varies from crescent-shaped or semicircular to fan-shaped with pronounced "tail". The locomotive form may reach a maximum dimension of 60 µm; usual sizes vary from 42–50 µm in length and breadth. The floating form is of radial type, non – symmetrical, with one to nine pointed, tapering hyaline pseudopodia without basal thickening. Vesicular nucleus is 6–11 µm in diameter with one central or slightly eccentric nucleolus. Cyst formation is known from a brackish-water strain.

Habitat: freshwater, brackish-water. Known from numerous locations in Europe, probably from Asia and North America.

Type material (neotype): *Vannella simplex* CCAP1589/2 (freshwater)

Description: The shape of locomotive cells is highly variable. Cells may be crescent-shaped, with a length/breadth ratio less than 0.5 or fan-shaped, with pronounced "tail" and l/b ratio up to 2.0. Some cultured strains are always covered with faecal pellets, whereas others may lose this tendency during cultivation. Normally mononucleate, but some strains tend to have one or two supernumerary nuclei (Page 1988). Contractile vacuoles always exist in freshwater strains, but were not found in brackish-water cultures of the *V. simplex* Nivå isolate. Simple filaments among the glycostyles of the cell coat may be absent or indistinguishable in some strains. Cyst formation was observed in the *V. simplex* Nivå strain under brackish water conditions, but not in freshwater cultures. Cysts are rounded, single-walled, with a diameter of 16–26 µm. The cell surface inside the cyst is characteristically wrinkled. The thickness of the cyst wall is 1.1–1.8 µm. Light microscopic (LM) or TEM illustrations of various strains may be found in Ariza et al. (1989); Haberey and Hülsmann (1973); Hausmann (1975); Hausmann and Hülsmann (1996, p.30); Hausmann and Stockem (1972); Page (1976a; 1988; 1991); Vørs, (1992); Wohlfarth-Bottermann (1960).

Differential diagnosis: *V. simplex* is significantly larger than any other known *Vannella* species to make it readily distinguishable. Cells in liquid cultures are often covered with faecal pellets, which is not a character of other known vannellids. The remarkable floating form of *V. simplex* resembles a star with very fine, narrow conical, pointed pseudopodia. In other *Vannella* species, pseudopodia tips are more blunt in the floating form.

Methods

Strains of *Vannella simplex*:

1. ***V. simplex* Nivå** (brackish-water, 17 ppt). It originates from artificial laminated cyanobacterial mats (Fenchel 1998), initially inoculated with sediments from Nivå Bay (Denmark, The Sound, 15 km South of Helsingør) and maintained at the Marine Biological Laboratory Helsingør under 17 ppt salinity (Smirnov 2001a). This isolate was deposited with the Culture Collection of Algae and Protozoa CEH Windermere, UK under the reference number CCAP1589/17.
2. ***V. simplex* Gurre** (freshwater). It originates from the freshwater Gurre Lake, 10 km North from Helsingør, Denmark.
3. ***V. simplex* Malagnou 1 – Malagnou 3** (freshwater, three independent clones originating from the same initial culture). The culture originates from the local pond of the Station de Zoologie Exp. Gen., University of Geneva, Route de Malagnou, 154, Geneva (Switzerland).
4. ***V. simplex* Valamo** (freshwater). It originates from freshwater Leshevoe Lake at Valamo Island, Lake Ladoga, North-Western Russia (Smirnov and Goodkov 1995).
5. ***V. simplex* CCAP 1589/2** (freshwater, originates from the university pond in Bonn, Germany).

All freshwater strains were maintained in 90 mm Petri dishes filled with PJ (Prescott and James 1955) medium and one wheat grain per dish. All strains were subcultured monthly. *Vannella simplex* Nivå was maintained in 17 ppt and *Vannella ebro* CCAP 1589/14 in 42 ppt artificial seawater (Wiegandt GmbH, Germany) in the same manner. All cultures were clonal. To determine salinity tolerance, amoebae of the *V. simplex* Nivå strain were inoculated in a media displaying different salinities – from freshwater PJ medium to 130 ppt salinity (artificial seawater). Growth was examined after 7 and 15 days of incubation.

Sediment sampling: Surface sediment samples were taken from the Seymaz River (Geneva, N46°11'14.8" E006°11.02.9'), the Neuchâtel Lake (Switzerland, N46°50'22.47" E6°48'41.39") and a natural reserve (Grande Caricaie, Switzerland, N46°12'28.7" E006°12'06.7") that encompasses the southern border of the Neuchâtel Lake.

Electron microscopical (EM) studies: For EM examination, *V. simplex* strain from Nivå Bay was fixed as described by Smirnov (2001b); *V. simplex* strains from Gurre Lake and Valamo Island – as de-

scribed by Smirnov and Brown (2000). Chromium shadowed (CH) preparations of all three strains were done by Ken Clarke (CEH Windermere).

DNA extraction, amplification, cloning and sequencing: DNA extraction of cultivated *Vannella simplex* strains from Nivå Bay, Gurre Lake and Malagnou pond as well as from *Vannella ebro* was carried out using guanidine thiocyanate and isopropyl alcohol fractionation following a protocol by Maniatis et al. (1982).

Total DNA was extracted from untreated sediment samples following a modified protocol by Zhou et al. (1996): 9 ml extraction buffer (100 mM Tris, 100 mM EDTA, 100 mM sodium phosphate, 1.5 M NaCl, 1% CTAB) and 100 µl Proteinase K (10 mg/ml) were added to 5 g sediment. After mixing the samples on a rotary platform at 225 rpm for 30 min at 37 °C, 1.5 ml SDS, (20%) were added and each sample was incubated at 65 °C for 2 h.

Following centrifugation (6000 rpm/10 min), the supernatant from each sample was transferred into 50 ml tubes. The remaining sediment pellet was re-extracted twice, by adding 4.5 ml extraction buffer and 0.5 ml SDS (20%) and heating at 65 °C for 10 min. The combined supernatants from 3 cycles of extraction were mixed with an equal volume of chloroform-isoamylalcohol (24:1) and centrifuged at 16,000 rpm for 20 min. The aqueous phase of each sample was recovered and an equal volume of ether was added to remove traces of chloroform. The ether and DNA sample were mixed and the two phases were separated by letting them stand for 5 min. The upper layer was discarded and the addition of ether and subsequent removal of the upper layer was repeated twice.

DNA was then precipitated by adding 2 volumes of 100% ethanol and leaving the samples overnight at -20 °C. After centrifugation (16,000 rpm/20 min), the resulting pellet was rinsed with 70% ethanol and dried at 37 °C for 30 min. The crude DNA extract was subsequently purified by following Qiagen's protocol for DNEasy Plant Mini Kit.

SSU rDNA was amplified by PCR using the primer pair AM-PV (5'-ACCAGGTCCAGACAKTAG, position 1267-1284 in mouse) and s20r (Pawlowski 2000) for environmental DNA extractions and the primer pair s6f and s20r (Pawlowski 2000) for DNA extractions of cultivated strains. The thermal cycle parameters consisted of 40 cycles of 30 s at 94 °C, 30 s at 50 °C and 60 s at 72 °C, followed by 5 min at 72 °C for final extension. Positive amplification products were purified using High Pure PCR Purification Kit (Roche Diagnostics) and either sequenced directly or ligated into pGEM-T Vector system (Promega) and cloned in XL-2

Ultracompetent Cells (Stratagene). One to two clones were sequenced for each amplified PCR product.

Sequencing reactions were prepared by using ABI-PRISM Big Dye Terminator Cycle Sequencing Kit and analysed with an ABI-377 DNA sequencer (Perkin-Elmer), all according to the manufacturer's instructions. The new sequences reported in this paper have been deposited in the EMBL/Gen Bank database under accession numbers AF486074 – AF486083. The sequence of *Vannella anglica* (AF099101) has been published by Sims et al. (1999).

Sequence analysis: Sequences were aligned using the GDE 2.2 software (Larsen et al. 1993). A Chi-square test was applied to all aligned sequences to test the homogeneity of base frequencies across taxa by using PAUP* 4.0b version (Swofford 2000), resulting in Chi square = 5.41, df = 33 and p = 0.999. According to the results of the Chi-square test which indicate an unbiased base composition, we chose Kimura's two-parameter model, applied to distances corrected for multiple hits and for unequal transition and transversion rates (Kimura 1980) for our analysis. Phylogenetic analysis was carried out by using the Neighbor joining (NJ) method (Saitou and Nei 1987) and the Maximum likelihood (ML) method as implemented in the fast DNAmI program (Olsen et al. 1994). NJ and ML method were additionally tested using Tajima and Nei's six-parameter model (Tajima and Nei 1984). Selected sites in homologous regions without gap were retained for the analysis. 27 out of 460 sites were informative. The reliability of internal branches was assessed by bootstrapping (Felsenstein 1988) with 1000 resamplings for the NJ and 500 resamplings for the ML trees. The PHYLO_WIN program (Galtier and Gouy 1996) was used for distance computations, NJ and ML tree-building and bootstrapping.

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