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Actinobacterial 16S rRNA genes from freshwater habitats cluster in four distinct lineages

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Summary

We analysed the phylogenetic relatedness of 16S rRNA genes from freshwater bacteria affiliated with the class Actinobacteria. A polymerase chain reaction assay was developed to identify reliably rare Actinobacteria-related inserts within 16S rRNA gene clone libraries. In 18 libraries constructed from seven freshwater systems, altogether 63 actinobacterial sequence types were collected from a total of >1800 clones. Sixty of the newly obtained sequences grouped within four distinct phylogenetic lineages. They constitute approximately 75% of the nearly complete sequences within these clusters that are presently available. A comparison with >300 sequences from various soil habitats revealed that two of these monophyletic actinobacterial clades (acI and acII) almost exclusively harbour 16S rRNA sequence types from freshwaters and estuaries. This may indicate that such bacteria are not inoculated to freshwaters from terrestrial sources, but are autochthonous components of freshwater microbial assemblages. In contrast, sequence types from freshwaters, marine sediments and soils were clearly mixed in another of the actinobacterial lineages (acIV). Sequence divergence within acIV was the highest of all four lineages (88% minimum similarity), which potentially reflects its radiation across several habitat types. Within the freshwater lineages, groups of essentially identical sequence types were retrieved from geographically distant aquatic systems with strikingly different hydrological and limnological characteristics. This points to the necessity to investigate genotypic variability, *in situ* abundances and activities of these Actinobacteria in freshwater plankton in greater detail by cultivation-independent techniques.

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Introduction

Evidence is accumulating that there are fundamental differences in the composition of marine and freshwater pelagic microbial communities (Methé *et al*., 1998; Glöckner *et al*., 1999; Zwart *et al*., 2002). One conspicuous group within the freshwater picoplankton are the Actinobacteria. This class of Gram-positive bacteria with a high genomic G+C content comprises a great variety of validly described species and environmental isolates (Iizuka *et al*., 1998; Hahn *et al*., 2003), including biotechnologically important metabolite and antibiotic producers (e.g. *Corynebacterium glutamicum*, *Streptomyces griseus*). Traditionally, the Actinobacteria have been associated with soil (Goodfellow and Williams, 1983; Rheims *et al*., 1999). However, the class also accommodates several lineages of 16S rRNA sequences of uncultivated bacteria from various aquatic environments (Hiorns *et al*., 1997; Methé *et al*., 1998; Crump *et al*., 1999; Rappe *et al*., 1999; Glöckner *et al*., 2000; Donachie *et al*., 2002; Zwart *et al*., 2002; Humayoun *et al*., 2003).

Actinobacteria are probably one of the most abundant groups of freshwater bacterioplankton (Glöckner *et al*., 2000; Sekar *et al*., 2003), yet almost nothing is known about their role in the aquatic environment. Until recently, a direct visualization of these bacteria in environmental samples by fluorescence *in situ* hybridization (FISH) with oligonucleotide probes was hampered by their small cell size and supposedly Gram-positive cell wall (Glöckner *et al*., 2000; Sekar *et al*., 2003). Some Actinobacteria are possibly common in the plankton because they resist protistan predation (Pernthaler *et al*., 2001; Hahn *et al*., 2003). However, bacteria from this lineage were also abundant in waters of an ultra-oligotrophic high mountain lake where grazing plays a minor role (Glöckner *et al*., 2000).

Such unresolved contradictions point to the necessity for further differentiating between individual actinobacterial groups in field studies, e.g. by the design of more specific rRNA-targeted FISH probes. Presently, this is hampered by the small number of available full-length 16S rRNA sequences from this lineage. Most previous investigations of microbial diversity of freshwater picoplankton have predominantly produced partial sequence data (Methé *et al*., 1998; Urbach *et al*., 2001; Humay-

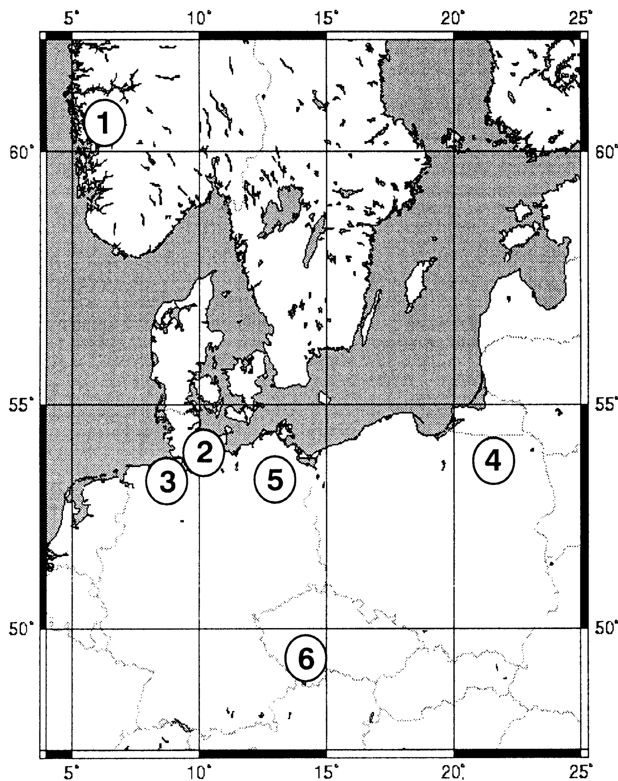

Fig. 1. Map of central Europe indicating the locations of the study sites. 1, Lake Sælenvannet; 2, Lake Schöhsee; 3. Wümmewiesen; 4, Great Masurian Lakes; 5, Lake Fuchskuhle; 6, Římov Reservoir.

oun *et al.*, 2003). Moreover, many studies have unselectively collected environmental rRNA gene sequences irrespective of their phylogenetic affiliation (Hiorns *et al.*, 1997; Methé *et al.*, 1998; Crump *et al.*, 1999; Glöckner *et al.*, 2000; Urbach *et al.*, 2001). Although the Actinobacteria are common in various lakes (Sekar *et al.*, 2003), they are, like the marine Bacteroidetes (Cottrell and Kirchman, 2000), apparently under-represented in 16S rRNA clone libraries generated with general bacterial primers.

Consequently, current phylogenies of freshwater Actinobacteria are contradictory, and the 16S rRNA diversity within this ecologically defined group is probably underestimated (Glöckner *et al.*, 2000; Zwart *et al.*, 2002; Humayoun *et al.*, 2003). We therefore selectively collected sequence information of freshwater Actinobacteria from a set of systems that is already in the focus of limnological and microbiological research (Babenzien and Babenzien, 1990; Šimek and Straskrabova, 1992; Jürgens *et al.*, 1994; Tuomi *et al.*, 1997; Kufel and Kufel, 1999) (Fig. 1). In particular, we aimed at providing a more solid phylogenetic framework for this group of abundant environmental bacteria by producing a substantially larger data set of almost full-length 16S rRNA sequences.

Results

Actinobacterial 16S rRNA sequence types in environmental clone libraries

Clones carrying actinobacterial 16S rRNA inserts could be readily distinguished from those with inserts from other phylogenetic lineages by our polymerase chain reaction (PCR) screening assay (Fig. 2). Fluorescence values after 30 PCR cycles ranged from 0.39 to 1.04 (mean 0.88) for the positive clones, from 0.02 to 0.15 (mean 0.04) for the negative control clones and from 0.00 to 0.02 (mean 0.01) for the no-template controls. Sequence analysis revealed a varying number of mismatches at the target sites of the primers HGC236F and HGC664R. One positive clone had 2 + 4 mismatches to these primers, which was reflected in its low fluorescence signal (0.39). The number of mismatches of the negative control clones at the primer sites varied between 0 + 8 and 6 + 8.

Altogether 18 environmental 16S rRNA gene clone libraries were established from seven different habitats (see Table 1). More than 1800 environmental clones were analysed using the described PCR assay. This led to the identification of 72 clones harbouring Actinobacteria-related inserts (see Table 1). The relative abundances of positive clones within the different clone libraries ranged from 0% (e.g. Lake Sælenvannet, 6 m depth) to 31% (Lake Fuchskuhle south-western basin). Sixty-three nearly full-length 16S rRNA sequences related to the class Actinobacteria were produced. Based on partial sequences, the remaining nine positive clones (Lake Sælenvannet, 1 m and 2 m depth) were found to be identical to already fully sequenced clones from the same libraries and were thus excluded from further analysis.

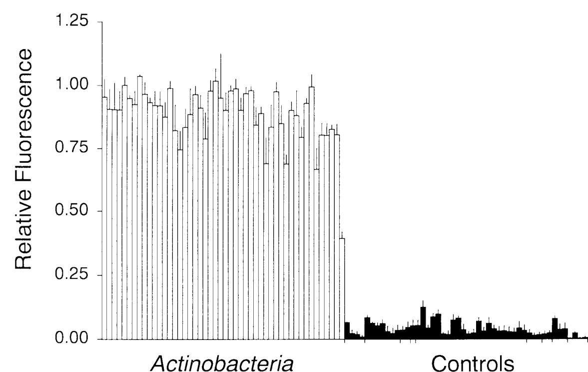

Fig. 2. Discrimination of actinobacterial and non-actinobacterial sequence types in freshwater 16S rRNA clone libraries by PCR screening (relative fluorescence ± 1 standard error, $n = 4$). Negative controls were clones from freshwater libraries related to other phylogenetic lineages. These were (from left to right): alpha- (4 clones), beta- (7), gamma- (2) and delta-Proteobacteria (1), Bacteroidetes (22), Acidobacteria (3), Gram positive bacteria with low genomic G+C content (2) and Verrucomicrobia (3). The last four data points represent no-template controls.

Table 1. Origin of environmental 16S rRNA clone libraries analysed in this study.

Location	Sampling site/habitat	Sampling date	No. of clones Screened	No. of clones Positive
Řimov Reservoir	River (near inflow)	May 1999	48	5
	Station 3A (half-way of total length)	May 1999	48	1
	Dam (near outflow)	May 1999	48	1
Lake Schöhsee	Parallel library no. 1	July + August 2000[a]	40	3
Lake Schöhsee	Parallel library no. 2	July + August 2000[a]	36	4
Lake Schöhsee	Parallel library no. 3	July + August 2000[a]	43	4
Lake Sælenvannet	1 m water depth	June 2000	184	15
	2 m water depth	June 2000	184	7
	6 m water depth	June 2000	184	0
Masurian Lakes	Niegocin	November 2001	150	6
	Dargin	November 2001	122	3
	North Mamry	November 2001	184	3
Wümmewiesen		February 2002	184	4
Acidic hot spring		April 2002	184	2
Lake Fuchskuhle	South-western basin	January 2002	29	9
	SW basin experimental enrichment		48	0
	North-eastern basin	January 2002	48	5
	NE basin experimental enrichment		48	0
Sum			1812	72

a. Samples from July and August 2000 were pooled.

Phylogenetic affiliation of freshwater Actinobacteria

Figure 3 depicts an overview consensus tree that summarizes the 16S rRNA-based phylogeny of more than 400 partial and full-length sequences. The backbone (topology) of this tree was reconstructed using almost complete sequences only (longer than 1400 nucleotides). Sixty of 63 actinobacterial sequences obtained in this study grouped into four distinct phylogenetic clusters (Table 2). These clusters, termed acI, acII, acIII and acIV, remained stable in different types of tree reconstructions (neighbour joining, maximum parsimony, maximum likelihood). They were clearly separated from actinobacterial lineages from soil and marine habitats. Two clones from the Wümmewiesen flood plains were not affiliated with any of the proposed ac clusters, and one clone from Lake Fuchskuhle was affiliated with sequence types obtained from soils.

The molar G+C content of the 16S gene of the ac clusters varied from 53% to 57% (Table 2). The minimal similarities within the individual lineages (sequences longer than 1400 nucleotides) ranged from 88% to 96%. Detailed phylogenetic consensus trees of the proposed freshwater actinobacterial clusters acI–IV are presented in Figs 5–7. In these trees, solid lines depict almost complete sequences (longer than 1400 nucleotides) that were included in the topology-relevant calculations. Dotted lines represent selected partial sequences that were subsequently added to the consensus tree to depict the phylogenetic diversity within clusters.

Actinobacterial sequences from six of the studied habitats were affiliated with cluster acI (none from Lake Sælenvannet) (Fig. 4). Presently, more than 200 environmental clone sequences are positioned within this lineage (Table 2, Figs 3 and 5). Most of these sequences (>95%) originate from lakes, rivers and estuaries (Table 3). Twenty-one of the available 24 nearly complete sequences in cluster acI were produced during this study

Table 2. Comparison of the proposed freshwater actinobacterial clusters.

Cluster	Total	No. of sequences >1400 nt total	No. of sequences >1400 nt this study	Minimum sequence similarity (%) within each cluster[a]	16S rRNA gene mol% G+C within each cluster[a]
acI	202	24	21	90	53–55
acII	48	34	23	94	55–56
acIII	13	7	6	96	55
acIV	152	14	10	88	54–57
Outside clusters			3		
Sum			63		

a. Determined using nearly full-length sequences.

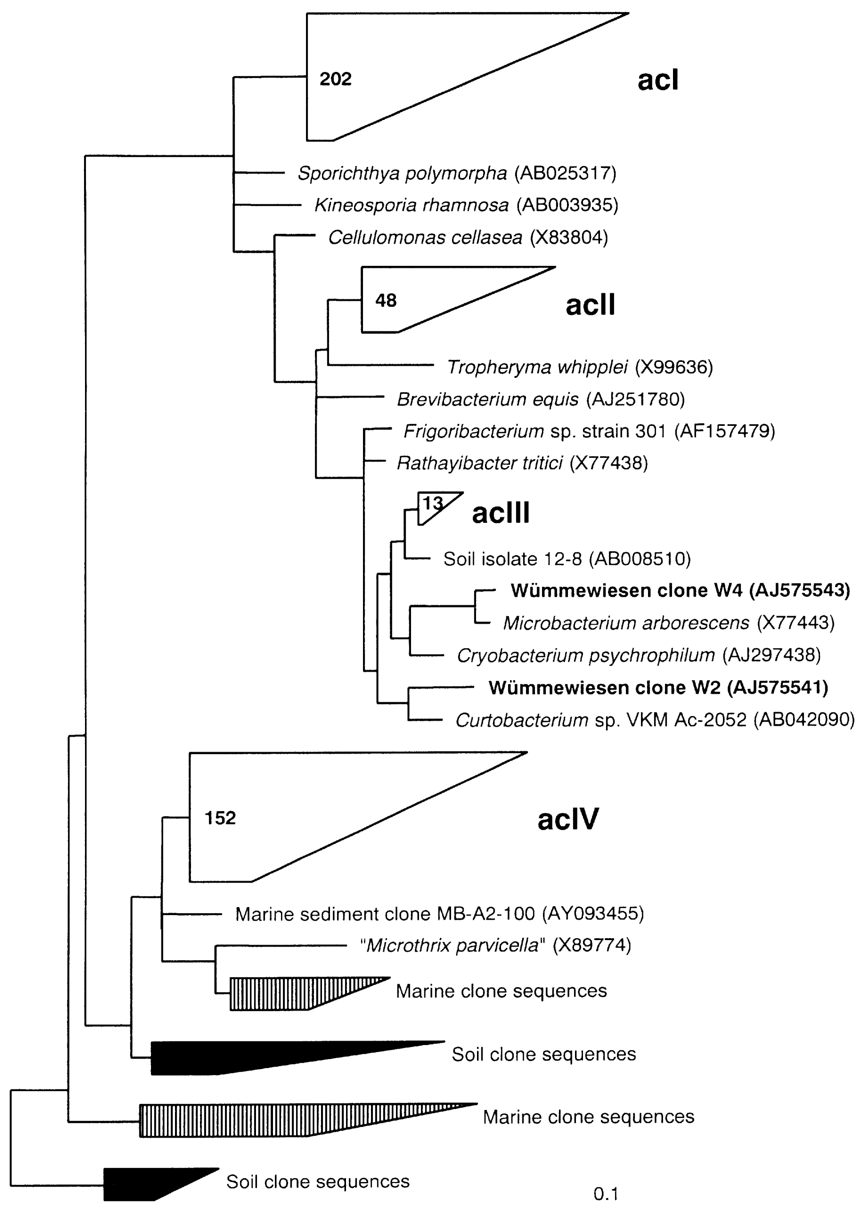

Fig. 3. Phylogenetic consensus tree of the proposed actinobacterial freshwater clusters. Bifurcations indicate branchings that appeared to be stable and well separated from neighbouring branchings in all calculations, whereas multifurcations indicate tree topologies that could not be resolved unambiguously. For clarity, only a subset of sequences used for calculations is depicted in the tree. Clone sequences obtained in this study are shown in bold. GenBank accession numbers are given in parentheses, and the scale bar indicates 10% estimated sequence divergence. Numbers in the group boxes refer to the total number of available full and partial sequences.

(Table 2). Cluster acI contains no cultured representative. Within this cluster, three separated lineages of at least two nearly complete sequences can be distinguished (Fig. 5). Subcluster acI-A is consistent with cluster ACK-M1 described by Zwart *et al.* (2002), whereas subclusters acI-B and acI-C represent novel lineages.

The acII and acIII clades contain 48 and 13 partial and full-length 16S rRNA gene sequences respectively (Fig. 6). Sequences within cluster acII were obtained from all studied habitats (Fig. 4). Most sequences in this lineage were produced during this investigation. In addition, the cluster harbours sequences of ultramicrobacterial strains that were isolated recently from different lakes in Germany and China (Hahn *et al.*, 2003). Altogether, 83% of all sequence types from this clade have been recovered from freshwater habitats. Two distinct lineages within acII are consistent with the previously described Luna-1 and Luna-2 clusters (Hahn *et al.*, 2003) (acII-B, acII-D). Additionally, two novel lineages of sequences from Lake Sælenvannet (acII-A) and Lake Fuchskuhle, the Řimov Reservoir and the acidic hot spring (acII-C) are proposed. The cluster acIII contains only sequences derived from Lake Sælenvannet, predominantly from the chemocline, and from a hypersaline soda lake in California (Humayoun *et al.*, 2003). It is consistent with the actinobacterial cluster 2 proposed by Humayoun *et al.* (2003). The most closely related cultured representatives of acIII are a soil isolate (Iizuka *et al.*, 1998) and *Cryobacterium psychrophilum* (sequence similarities of 97.2% and 96.6% respectively).

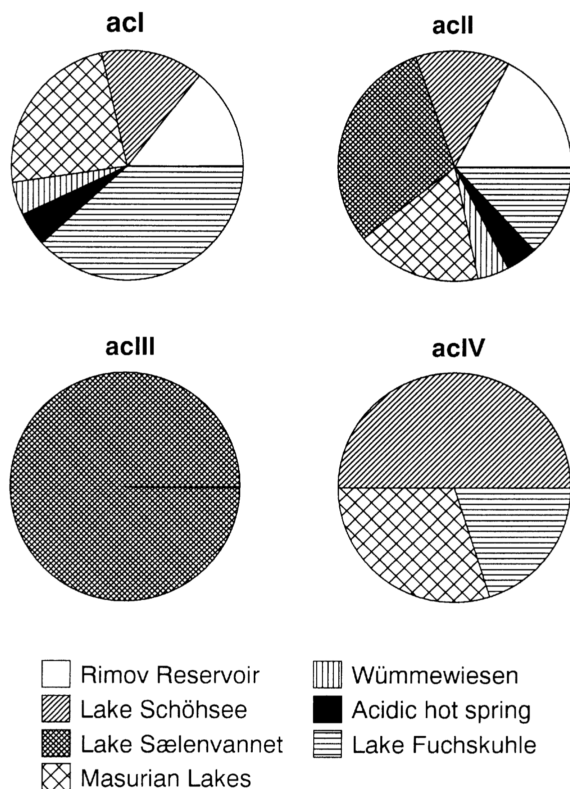

Fig. 4. Relative contribution of the studied habitats to the actinobacterial clusters acI, acII, acIII and acIV. Only three out of the 63 sequences were not affiliated with these clusters.

The actinobacterial cluster acIV contains more than 150 partial and full-length sequences in total (Fig. 7). Again, this cluster harbours no cultured representatives. AcIV is predominantly constituted of sequences from freshwaters and estuaries (78%), and a smaller fraction of sequences originates from marine (8%) and soil habitats (7%). Sequence types affiliated to cluster acIV were only retrieved from three of the study sites (Fig. 4). Owing to the limited number of complete sequences, only two separated lineages can be postulated within acIV (acIV-A and acIV-B). The lineage acIV-A is consistent with the previously described cluster CL500-29 (Zwart *et al*., 2002), whereas the proposed clusters Med0-06 and Urk0-14 (Zwart *et al*., 2002) are not confirmed by our analysis.

Discussion

PCR screening assay

With the rise of environmental metagenomics (DeLong, 2002), protocols for the detection of rare sequence motifs (in particular of specific rRNA genes) in large clone libraries are of increasing importance. We screened 18 libraries by PCR with the specific primers HGC236F and HGC664R (Glöckner *et al*., 2000) to produce a large set of 16S rRNA sequences affiliated with the Actinobacteria. This was essential because of the low frequencies of such sequence types in our libraries (mean <5%). We wanted to minimize the risk of losing potentially novel actinobacterial lineages by setting the PCR conditions to relatively low stringencies without affecting the reliability of the assay (Fig. 2). Indeed, more than half the positive actinobacterial clones contained inserts with one or two mismatches to the general actinobacterial primers/probes HGC236 and HGC664. This implies that a substantial fraction of the collected actinobacterial phylotypes would not have been detected in lakewater samples by FISH with these probes at optimal hybridization conditions (Glöckner *et al*., 2000; Sekar *et al*., 2003) or by PCR at stringent conditions. Moreover, in contrast to sequence collection by PCR with group-specific primers (Stach *et al*., 2003), our protocol recovers almost full-length sequences, which is essential for FISH probe design and reliable phylogenetic analysis. In fact, our low-stringency PCR screening assay was probably essential for the detection of the freshwater actinobacterial groups. The novel primers S-C-Act-235-a-s-20 and S-C-Act-878-a-s-19 proposed by Stach *et al*. (2003) have one mismatch each to sequences from clusters acII and acIII, and acI-A and acI-B respectively.

Reliability of phylogenetic analysis

Our phylogenetic reconstructions are partially in contradiction with previous analyses (Urbach *et al*., 2001; Zwart *et al*., 2002; Humayoun *et al*., 2003), and they also outline hitherto undetected lineages within individual actinobacterial clusters (e.g. acI-C, acII-A, acII-C) (Figs 5 and 6). These results underline the importance of using almost full-length sequences for the reconstruction of microbial

Table 3. Origin of sequences affiliated with the freshwater actinobacterial clusters.

	Origin of sequences in clusters (%)									
Cluster	Lake	River	Estuary	Hypersaline soda lake	Hot spring	Flooded pasture	Soil	Marine	Activated sludge	Unknown
acI	65	17	13	0	1	1	0	1	0	2
acII	75	2	6	0	11	2	0	2	0	2
acIII	0	0	46	54	0	0	0	0	0	0
acIV	39	26	13	3	1	1	7	8	1	1

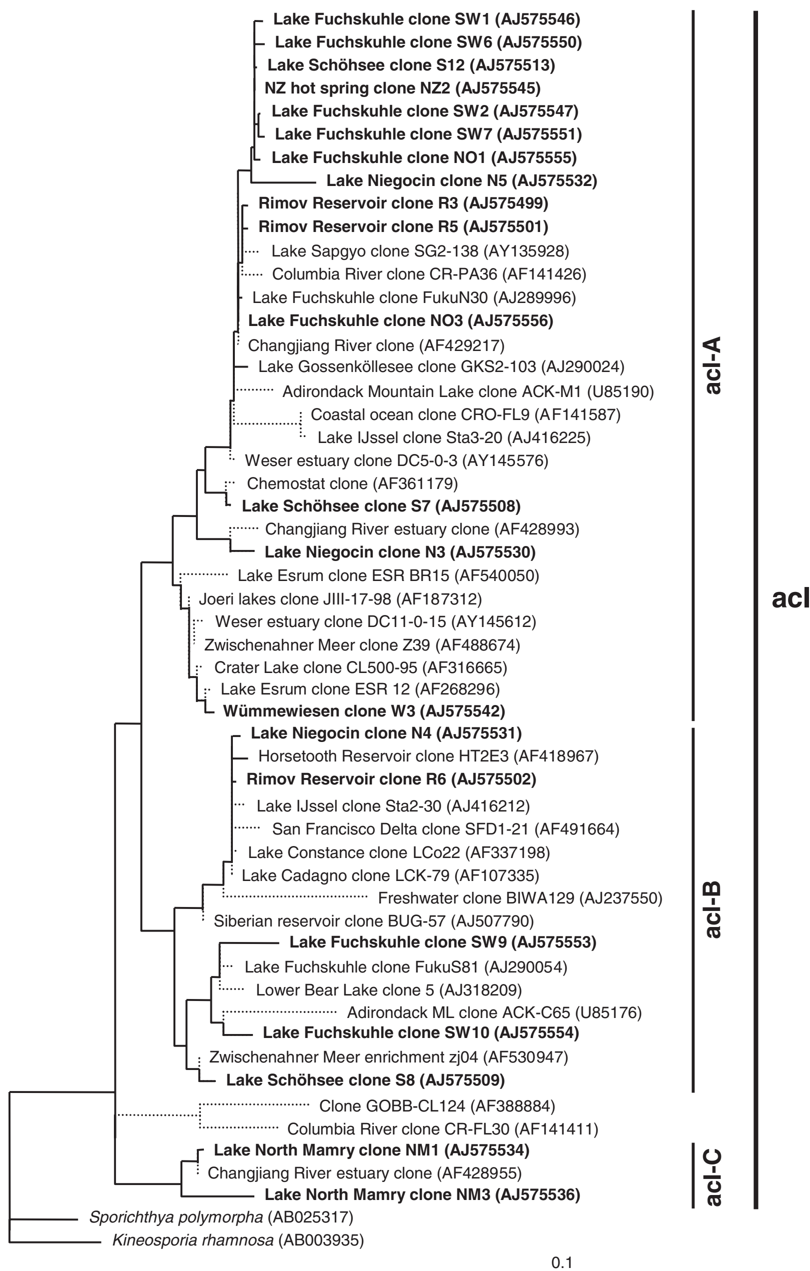

Fig. 5. Detailed view of phylogenetic relationships within the freshwater actinobacterial cluster acI, formerly termed hgcI (Glöckner *et al.*, 2000). Solid lines indicate sequences that were included in the primary analyses (i.e. sequences longer than 1400 nucleotides), whereas dotted lines indicate partial sequences. Clone sequences produced during this study are shown in bold, and GenBank accession numbers are given in parentheses. For clarity, only selected partial sequences are included in this tree. The scale bar indicates 10% estimated sequence divergence.

phylogenies (Ludwig and Klenk, 2001). Altogether, our investigation contributes >75% of almost complete sequences to the proposed freshwater actinobacterial clades (Table 2). Owing to the sequencing of different 16S rRNA regions, previous comparative studies have typically reduced the number of alignment positions to the overlapping region of all partial and full-length sequences of interest (Zwart *et al.*, 2002). Hence, previous phylogenetic tree reconstructions have been performed considering only about 200–400 bp, which is less than one-third of the information used for our analyses.

The major techniques for the reconstruction of 16S rRNA sequence relatedness are based on different hypothetical models of evolution, neither of which can be proven or dismissed (Ludwig and Klenk, 2001). Therefore, bootstrapping statistics of one particular treeing method (Crump *et al.*, 1999; Hahn *et al.*, 2003; Humayoun *et al.*, 2003) may even conceal branching uncertainties that originate either from the biases of the chosen evolutionary model or from the undersampling of diversity within a particular microbial lineage (Hughes *et al.*, 2001). We therefore compared different treeing approaches during our phylogenetic analysis, and we limited our precision to the minimal phylogenetic information that could be reliably reconstructed by our sequence data set. Consequently, multifurcational topologies were introduced if contradic-

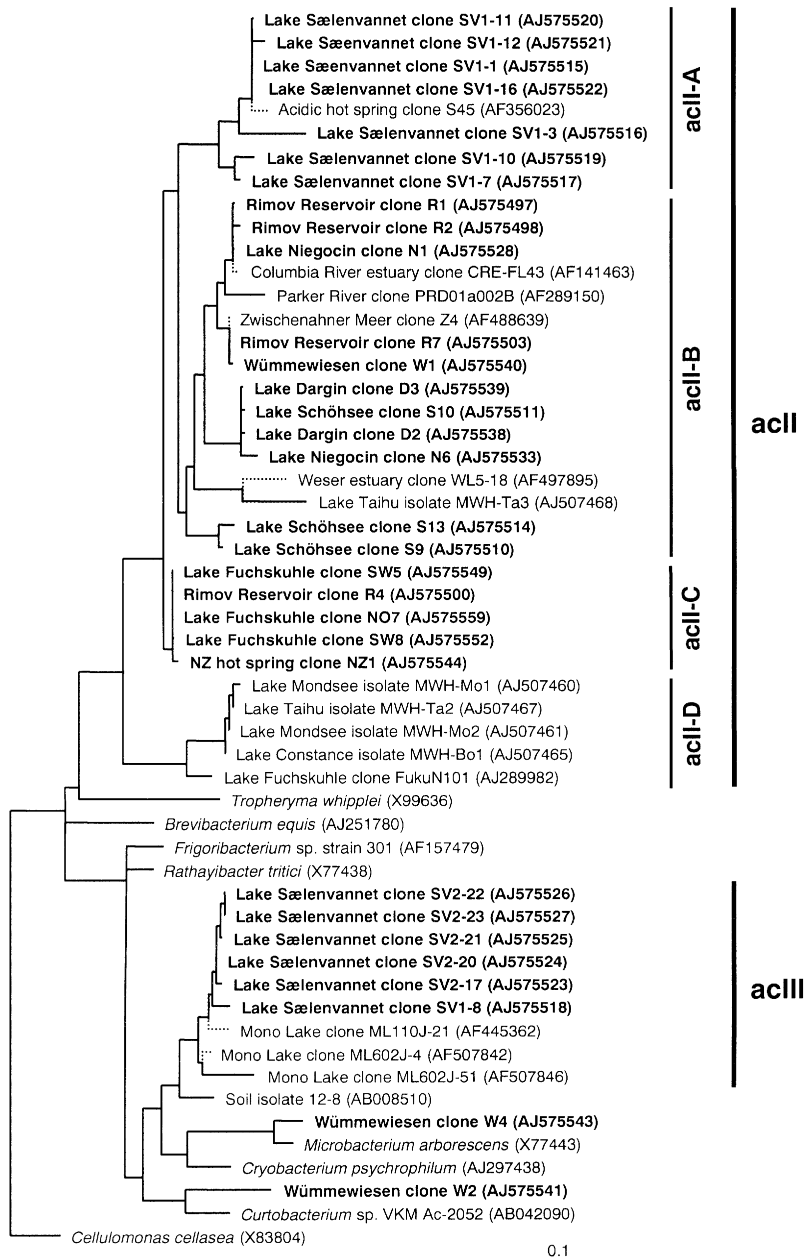

Fig. 6. Detailed view of the phylogenetic relationships within the freshwater actinobacterial lineages acII and acIII. For a further description, see the legend to Figs 3 and 5.

tions between the calculation methods could not be resolved unambiguously (e.g. Fig. 3).

Autochthonous pelagic Actinobacteria?

In evolutionary timescales, the freshwater pelagic zone might represent an independent stage for the evolution of an autochthonous planktonic microbial community. Alternatively, it might only be regarded as a transient enrichment system for bacterial lineages from other habitats, e.g. the catchment. A comprehensive phylogenetic analysis of actinobacterial 16S rRNA sequence types from freshwaters and other environments might help to decide between such hypotheses. For example, if the latter scenario was correct (i.e. distinct evolutionary lineages of limnic bacteria do not exist), then the actinobacterial 16S rRNA sequences from the different freshwaters should fall into numerous, deeply branching clades. All these lineages should moreover contain a considerable fraction of sequence types obtained from other environments.

Such a simple pattern is not supported by our data. The large majority of the sequences in the acI and acII clades originate exclusively from freshwater habitats (Table 3). Although the closest relative of acI could not be defined unambiguously, the two highly diversified lineages were nevertheless of monophyletic origin in all performed anal-

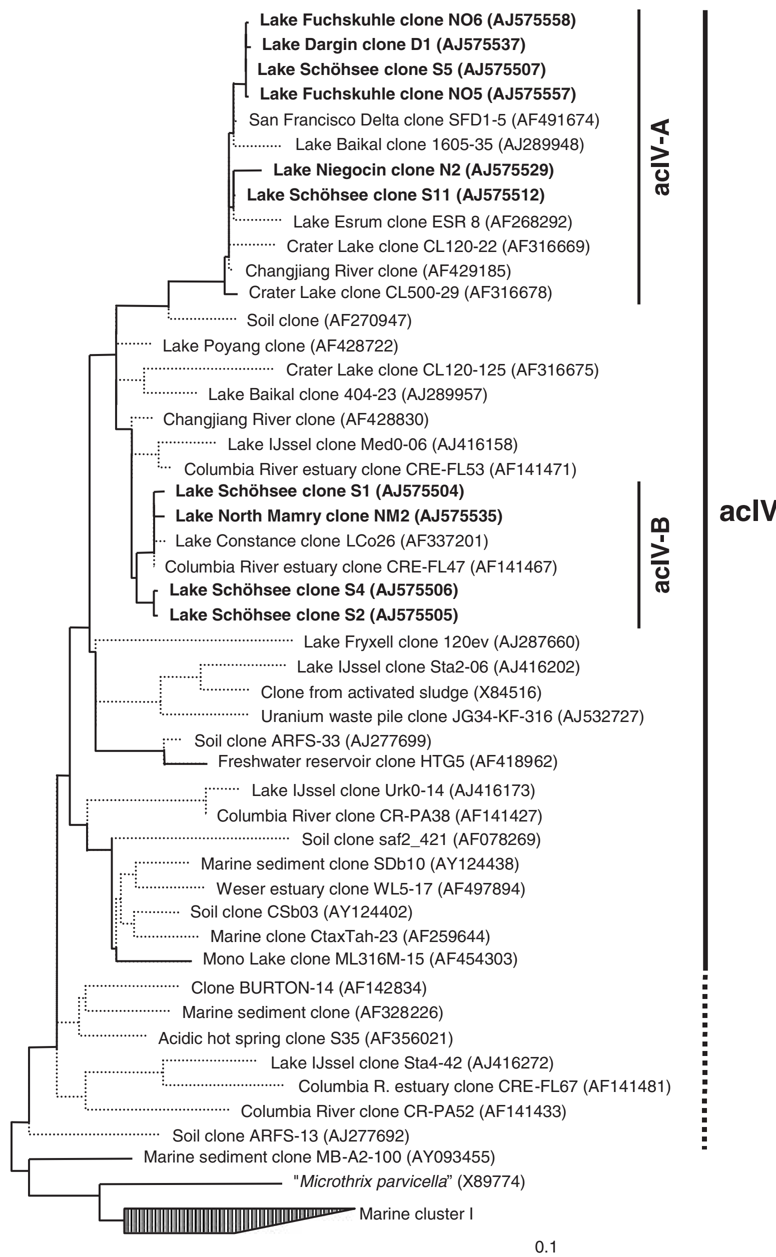

Fig. 7. Detailed view of the phylogenetic relationships within the freshwater actinobacterial lineage acIV. For a further description, see the legend to Figs 3 and 5. Note that the lower reaches of this cluster are supported by only two almost complete sequences.

yses (Fig. 3). Phylogenetic evidence therefore suggests a single ancestral acI and acII 16S rRNA sequence type and a subsequent adaptive radiation within the freshwater pelagic zone (Figs 5 and 6). In contrast, cluster acIV also harbours a considerable fraction of sequence types from soils, from marine waters and sediments and from brackish water habitats (Fig. 7, Table 3). Moreover, this lineage is closely related to other clades of uncultured Actinobacteria from the marine plankton, sediments and from soils. Members of acIV have apparently radiated across several fundamentally different habitat types.

The conspicuous accumulation of sequences from freshwater systems in acI and acII is a rather counterintuitive result, considering the close physico-chemical connection between terrestrial and aquatic systems via influx from the catchment area. One might argue that a parallel molecular survey of the watershed soil from which the samples were taken would be essential to confirm or reject the freshwater origin of the proposed actinobacterial clusters. However, although the presence of sequences related to our clusters in such libraries from the catchment areas might have been evidence that these clusters do

not represent freshwater lineages, their absence would not have proved the opposite. Interestingly, two of the three actinobacterial sequence types that did not affiliate with any of the proposed freshwater clades originated from the Wümmewiesen flood plains (Fig. 3). This again supports the notion of autochthonous aquatic lineages that are not passively inoculated from the catchment. In clone libraries from the Columbia river, Crump *et al.* (1999) found sequences related to the acI clade in the particle-attached fraction (Fig. 7). However, this is no evidence for a terrestrial origin, because particles in large rivers may well be autochthonously generated (Grossart and Ploug, 2000). In view of the several hundred 16S rRNA gene sequences that have so far been retrieved from various terrestrial habitats, it is furthermore unlikely that another clone library would have substantially revised the current picture of actinobacterial diversity in soils (Rheims *et al.*, 1999). Instead, our investigation was designed to compensate for this unbalance and analyse comparably sized data sets from the two habitat types.

Limitations of 16S rRNA diversity analysis

The present study should, however, not be confused with a truly biogeographic investigation (Papke *et al.*, 2003). The analysis of 16S rRNA genes is an essential first step in unveiling the diversity of freshwater Actinobacteria, and provides new ideas about the restriction of the proposed actinobacterial clades to particular habitat types. For example, no sequence types from these groups were detected in the library from the anoxic layer of Lake Sælenvannet. Similarly, Humayoun *et al.* (2003) recovered Actinobacteria-related sequences from the oxic surface water and the chemocline of meromictic Mono Lake, but never from the anoxic zone.

Yet there might be considerable genotypic and physiological diversity beneath the resolution of the 16S rRNA gene. Recent biogeographic studies on microbes have therefore relied upon more rapidly evolving molecular clocks (Crosbie *et al.*, 2003; Papke *et al.*, 2003; Whitaker *et al.*, 2003). Presently, it is still discussed whether biogeographic discontinuities as, e.g. reported by Whitaker *et al.* (2003), are actually rather exceptional for free-living microbes (Fenchel, 2003). Our data nevertheless hint at ecologically relevant diversity within the studied Actinobacteria lineages that could not be resolved by our approach. Groups of nearly identical phylotypes from up to three completely different habitats were obtained in three of the four freshwater clusters (Figs 5–7). For example, within cluster acI, identical sequence types were found in the humic Lake Fuchskuhle, the oligomesotrophic Schöhsee and the acidic hot spring in New Zealand (Fig. 5). This agrees with recent observations by Hahn *et al.* (2003) who isolated actinobacterial strains with identical 16S rRNA sequences but contrasting physiological traits from very different freshwaters.

Secondly, far-reaching deductions about sequence types based on the habitat they are obtained from may be misleading, because all information about their relative abundances is lost during PCR amplification (Cottrell and Kirchman, 2000). Thus, it cannot be decided by this type of study whether a specific phylotype is common or rare in the environment. FISH or equivalent techniques (MacGregor *et al.*, 1997; Pernthaler *et al.*, 1998; Papke *et al.*, 2003) would be required to quantify the frequencies of members of the individual lineages in the different habitats. There is nevertheless ample evidence that some of these actinobacteria must be among the most common bacteria in freshwaters (Glöckner *et al.*, 2000; Sekar *et al.*, 2003). In order to prove that a particular actinobacterial group is indeed indigenous to a specific environment, it would furthermore be important to show that it is also capable of growth, e.g. by immunocytochemical or autoradiographic techniques (Pernthaler *et al.*, 2002; Cottrell and Kirchman, 2003). In this context, it is important to note that so far the only successful enrichment of members from the acI cluster was observed in experimental conditions designed to mimic a typical freshwater scenario (high grazing mortality) (Posch *et al.*, 1999; Pernthaler *et al.*, 2001).

Particular sequence types in environmental 16S rRNA gene clone libraries might sometimes be the result of contaminating DNA (Tanner *et al.*, 1998). It has been suggested that the main sources of this contamination are the chemicals and enzymes used for the preparation of genomic DNA (Tanner *et al.*, 1998). All the actinobacterial 16S rRNA sequences in this study were amplified directly by PCR from cells on membrane filters (Kirchman *et al.*, 2001), thus omitting this critical step. Furthermore, several of our clone libraries did not contain any of the identical sequence types (e.g. Lake Sælenvannet clone library from the anoxic zone), which should have been the case if these sequences had originated from contamination.

In summary, the water column of various oligo- to hypereutrophic freshwaters appears to harbour actinobacterial 16S rRNA sequence types that are predominantly affiliated with only four major phylogenetic lineages. Particular identical actinobacterial sequence types were obtained from very different environments. This suggests that these bacteria might be physiologically highly diversified (Hahn *et al.*, 2003), and might have undergone adaptive radiation into different habitats in evolutionary rather recent times. However, without information about *in situ* abundances and activity, the role of these phylotypes in the studied habitats remains unknown. Our collection of almost complete 16S rRNA gene sequences nevertheless provides a comprehensive data set for the design of specific FISH probes, and thus forms a base for future eco-

logical studies about these actinobacterial lineages in freshwater habitats.

Experimental procedures

Study sites and sampling

Water samples were collected from a variety of freshwater habitats in Europe (Fig. 1) and from one site in New Zealand (Table 1). These habitats differ substantially in size, trophic state, stratification stability, food webs, water chemistry and other parameters. We put a focus on meso- to eutrophic freshwaters, as oligotrophic systems are better covered by recent studies (Hiorns *et al*., 1997; Methé *et al*., 1998; Glöckner *et al*., 2000; Urbach *et al*., 2001). The meso-eutrophic, dimictic Římov Reservoir is located in southern Bohemia, Czech Republic (Šimek and Straskrabova, 1992). Lake Schöhsee is an oligomesotrophic shallow lake in northern Germany (Jürgens *et al*., 1994). Lake Sælenvannet is a meromictic lake in western Norway. The oxic and anoxic strata are separated by a steep salinity gradient (Tuomi *et al*., 1997). Samples were obtained from oxic waters (1 m), from the chemocline (2 m) and from the anoxic zone (6 m). The lakes Niegocin, Dargin and North Mamry are three interconnected eu- to hypereutrophic lakes in the Great Masurian Lake District, Poland (Kufel and Kufel, 1999). The Wümmewiesen are Germany's largest flood plains, an agricultural pasture land near the city of Bremen that is flooded during the winter months by the river Wümme. Sulphur Point is an acidic hot spring in the Rotorua thermal area, New Zealand (geographic location not shown on Fig. 1) with an average temperature of 53°C and a pH of 2.5. This habitat was included because recently 16S rRNA sequences related to freshwater Actinobacteria have been reported from similar systems (Donachie *et al*., 2002). Lake Fuchskuhle is a small meso- to acidotrophic and dystrophic forest lake in the Brandenburg–Mecklenburg lake district, Germany (Babenzien and Babenzien, 1990). In 1990, the lake was artificially divided into four basins with different catchment areas, two of which were sampled for this study. Two enrichments of 0.8 μm filtrates from these basins were also included in the screening.

Samples (10–50 ml) were fixed with formaldehyde (2% final concentration) for 2–24 h and filtered onto membrane filters (type GTTP; pore size 0.2 μm; diameter 47 mm; Millipore). Filters were stored at −20°C until further processing.

Environmental 16S rRNA gene clone libraries

Small subunit rRNA genes were amplified using the primers GM3F and GM4R, specific for the domain Bacteria (Muyzer *et al*., 1995). Small pieces of the membrane filters were added directly to PCR tubes as inocula, and PCRs were performed as described previously (Kirchman *et al*., 2001). The resulting PCR product was purified using the QIAquick PCR purification kit (Qiagen) and ligated into the pCR4-TOPO vector using the TOPO TA cloning kit (Invitrogen) according to the manufacturer's instructions. The transformed cells were plated on LB agar plates containing 50 μg ml^{-1} ampicillin and incubated overnight at 37°C. Clones were picked and transferred into microtitre plates (MTP) containing 100 μl of LB medium amended with 50 μg ml^{-1} ampicillin and cultivated overnight at 37°C. Glycerol (50% final concentration) was added to each well, and the MTPs were stored at −80°C until further processing. Subsequently, these MTPs served as master plates for the inoculation of the PCR assays.

Screening of clone libraries

For the detection of actinobacterial phylotypes, the environmental clone libraries were screened by PCR with the primers HGC236F (5′-GCGGCCTATCAGCTTGTT-3′, *Escherichia coli* position 236–253) (Brosius *et al*., 1981) and HGC664R (5′-AGGAATTCCAGTCTCCCC-3′, position 664–681). These primers were derived from FISH probes that specifically target Actinobacteria (Glöckner *et al*., 2000). The accumulation of the ≈430 bp amplicons was detected with an ABI SDS 7700 instrument ('Taqman', Applied Biosystems) using the double-stranded DNA-binding dye SYBR Green I®. One microlitre of the respective clone culture was added to 11 μl of PCR mixture [1× ABI SYBR Green Mastermix (Applied Biosystems), 5 pmol of each primer] in the wells of ABgene PCR-MTPs. Reactions were performed in the following conditions: initial denaturation at 50°C for 2 min and at 95°C for 10 min, followed by 30 cycles consisting of denaturation (15 s at 95°C), annealing (30 s at 52°C) and extension (1 min at 72°C). Each PCR-MTP featured one well with a positive control (previously sequenced actinobacterial clone) and one well with a no-template control. The relative fluorescence values after 30 PCR cycles were used to detect clones with actinobacterial 16S rRNA inserts.

The reliability of the specific PCR assay was tested by assembling 48 actinobacterial clones and 44 negative control clones in one MTP. These negative controls represented sequenced clones of phylogenetic lineages commonly found in freshwater 16S rRNA gene clone libraries, e.g. alpha-, beta-, gamma-, delta-Proteobacteria, Cytophaga/Flavobacterium/Bacteroidetes, Acidobacteria, Gram-positive bacteria with low genomic G+C content and Verrucomicrobia. Four replicate PCR-MTPs with this assemblage were subsequently subjected to independent PCR runs.

Sequence analysis and phylogenetic reconstruction

Plasmids were isolated from clones with the QIAprep Spin Miniprep kit (Qiagen). Sequencing reactions were performed using the ABI BigDye® chemistry and an ABI 3100 genetic analyser (Applied Biosystems) according to the manufacturer's instructions. For sequencing, the following primers were used: GM1F (Muyzer *et al*., 1993), M13F (5′-GTAAAACGACGGCCAG-3′) and M13R (5′-CAGGAAA CAGCTATGAC-3′). Partial sequencing files were assembled and corrected manually using the software SEQUENCHER (Gene Codes). Sequencing reactions were repeated if the obtained partial sequences contained ambiguities, so that, on average, more than five sequencing reactions per assembled sequence were performed. The mean number of ambiguous bases per almost complete sequence was <1, and double or triple coverage was given for >500 bases. The assembled sequences were tested for chimeric origin with the program CHIMERA_CHECK (http://rdp.cme.msu.edu/).

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Phylogenetic analyses were performed using the ARB software package (http://www.arb-home.de). The ARB database (release June 2002) was completed with actinobacterial sequences deposited in GenBank. All sequences were automatically prealigned using the ARB tool FAST_ALIGNER, and subsequently checked and corrected manually considering the secondary structure of the rRNA molecule. The complete data set contained 4831 partial and full-length small subunit rRNA sequences affiliated with the class Actinobacteria. Actinobacterial sequences used for the phylogenetic analyses are available in aligned ARB format from the authors.

For the reconstruction of phylogenetic trees, only nearly complete 16S rRNA sequences (i.e. longer than 1400 nucleotides; 2533 sequences) were considered. A 50% base frequency filter was calculated on these sequences to exclude highly variable positions. The respective ARB tools were used to perform maximum parsimony (MP), neighbour-joining (NJ) and maximum likelihood (ML) analyses. All 2533 sequences were considered for the first two algorithms and up to 130 selected sequences for the ML analyses. The calculation methods were combined with different filters, correction models and outgroups. The ML analyses were repeated with different subsets of the sequences to evaluate the stability of the obtained tree topology. The resulting phylogenetic trees were compared manually. The final consensus tree shows bifurcations only if branchings appeared to be stable and well separated from neighbouring branchings in the great majority of analyses. Multifurcations were introduced if tree topologies could not be resolved unambiguously. Partial sequences (shorter than 1400 nucleotides) were added to this consensus tree with the respective ARB tool according to maximum parsimony criteria, without allowing changes in the overall tree topology and applying a 50% base frequency filter for Actinobacteria.

Nucleotide sequence accession numbers

The 16S rRNA gene sequences of the actinobacterial clones obtained during this study were deposited in GenBank with the following accession numbers: AJ575497 to AJ575559 (see also Figs 3, 5, 6 and 7).

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References

Babenzien, D., and Babenzien, C. (1990) Microbial activities in a naturally acidotrophic lake. *Arch Hydrobiol Beih* **34:** 175–181.

Brosius, J., Dull, T.J., Sleeter, D.D., and Noller, H.F. (1981) Gene organization and primary structure of a ribosomal RNA operon from *Escherichia coli*. *J Mol Biol* **148:** 107–127.

Cottrell, M.T., and Kirchman, D.L. (2000) Community composition of marine bacterioplankton determined by 16S rRNA gene clone libraries and fluorescence *in situ* hybridization. *Appl Environ Microbiol* **66:** 5116–5122.

Cottrell, M.T., and Kirchman, D.L. (2003) Contribution of major bacterial groups to bacterial biomass production (thymidine and leucine incorporation) in the Delaware estuary. *Limnol Oceanogr* **48:** 168–178.

Crosbie, N.D., Pockl, M., and Weisse, T. (2003) Dispersal and phylogenetic diversity of nonmarine picocyanobacteria, inferred from 16S rRNA gene and cpcBA-intergenic spacer sequence analyses. *Appl Environ Microbiol* **69:** 5716–5721.

Crump, B.C., Armbrust, E.V., and Baross, J.A. (1999) Phylogenetic analysis of particle-attached and free-living bacterial communities in the Columbia River, its estuary, and the adjacent coastal ocean. *Appl Environ Microbiol* **65:** 3192–3204.

DeLong, E.F. (2002) Microbial population genomics and ecology. *Curr Opin Microbiol* **5:** 520–524.

Donachie, S.P., Christenson, B.W., Kunkel, D.D., Malahoff, A., and Alam, M. (2002) Microbial community in acidic hydrothermal waters of volcanically active White Island, New Zealand. *Extremophiles* **6:** 419–425.

Fenchel, T. (2003) Biogeography for bacteria. *Science* **301:** 925–926.

Glöckner, F.O., Fuchs, B.M., and Amann, R. (1999) Bacterioplankton compositions of lakes and oceans: a first comparison based on fluorescence *in situ* hybridization. *Appl Environ Microbiol* **65:** 3721–3726.

Glöckner, F.-O., Zaichikov, E., Belkova, N., Denissova, L., Pernthaler, J., Pernthaler, A., and Amann, R. (2000) Comparative 16S rRNA analysis of lake bacterioplankton reveals globally distributed phylogenetic clusters including an abundant group of Actinobacteria. *Appl Environ Microbiol* **66:** 5053–5065.

Goodfellow, M., and Williams, S.T. (1983) Ecology of Actinomycetes. *Annu Rev Microbiol* **37:** 189–216.

Grossart, H.P., and Ploug, H. (2000) Bacterial production and growth efficiencies: direct measurements on riverine aggregates. *Limnol Oceanogr* **45:** 436–445.

Hahn, M.W., Lünsdorf, H., Wu, Q., Schauer, M., Höfle, M.G., Boenigk, J., and Stadler, P. (2003) Isolation of novel ultramicrobacteria classified as Actinobacteria from five freshwater habitats in Europe and Asia. *Appl Environ Microbiol* **69:** 1442–1451.

Hiorns, W.D., Methe, B.A., Nierzwicki-Bauer, S.A., and Zehr, J.P. (1997) Bacterial diversity in Adirondack mountain lakes as revealed by 16S rRNA gene sequences. *Appl Environ Microbiol* **63:** 2957–2960.

Hughes, J.B., Hellmann, J.J., Ricketts, T.H., and Bohannan, B.J.M. (2001) Counting the uncountable: statistical approaches to estimating microbial diversity. *Appl Environ Microbiol* **67:** 4399–4406.

Humayoun, S.B., Bano, N., and Hollibaugh, J.T. (2003) Depth distribution of microbial diversity in Mono Lake, a meromictic soda lake in California. *Appl Environ Microbiol* **69:** 1030–1042.

Iizuka, T., Yamanaka, S., Nishiyama, T., and Hiraishi, A. (1998) Isolation and phylogenetic analysis of aerobic copiotrophic ultramicrobacteria from urban soil. *J Gen Appl Microbiol* **44:** 75–84.

Jürgens, K., Arndt, H., and Rothhaupt, K.O. (1994) Zooplankton-mediated change of bacterial community structure. *Microb Ecol* **27:** 27–42.

Kirchman, D.L., Fuchs, L.Y., Yu, B.M., and Amann, R. (2001) Structure of bacterial communities in aquatic systems as revealed by filter PCR. *Aquat Microb Ecol* **26:** 13–22.

Kufel, I., and Kufel, L. (1999) Spatial variability and long-term changes of the trophic parameters in Great Masurian Lakes (Poland). *Polish J Ecol* **47:** 323–333.

Ludwig, W., and Klenk (2001) Overview: a phylogenetic backbone and taxonomic framework for procaryotic systematics. In Bergey's Manual of Systematic Bacteriology, 2nd edn, Vol. 1. Garrety, G.M. (ed.). New York: Williams & Wilkins, pp. 49–65.

MacGregor, B.J., Moser, D.P., Alm, E.W., Nealson, K.H., and Stahl, D.A. (1997) *Crenarchaeota* in Lake Michigan sediment. *Appl Environ Microbiol* **63:** 1178–1181.

Methé, B.A., Hiorns, W.D., and Zehr, J.P. (1998) Contrasts between marine and freshwater bacterial community composition: analyses of communities in Lake George and six other Adirondack lakes. *Limnol Oceanogr* **43:** 368–374.

Muyzer, G., de Waal, E.C., and Uitterlinden, A.G. (1993) Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. *Appl Environ Microbiol* **59:** 695–700.

Muyzer, G., Teske, A., Wirsen, C.O., and Jannasch, H.W. (1995) Phylogenetic relationship of *Thiomicrospira* species and their identification in deep-sea hydrothermal vent samples by denaturing gradient gel electrophoresis of 16S rDNA fragments. *Arch Microbiol* **164:** 165–172.

Papke, R.T., Ramsing, N.B., Bateson, M.M., and Ward, D.M. (2003) Geographical isolation in hot spring cyanobacteria. *Environ Microbiol* **5:** 650–659.

Pernthaler, J., Glöckner, F.O., Unterholzner, S., Alfreider, A., Psenner, R., and Amann, R. (1998) Seasonal community and population dynamics of pelagic *Bacteria* and *Archaea* in a high mountain lake. *Appl Environ Microbiol* **64:** 4299–4306.

Pernthaler, J., Posch, T., Šimek, K., Vrba, J., Pernthaler, A., Glöckner, F.O., *et al.* (2001) Predator-specific enrichment of actinobacteria from a cosmopolitan freshwater clade in mixed continuous culture. *Appl Environ Microbiol* **67:** 2145–2155.

Pernthaler, A., Pernthaler, J., Schattenhofer, M., and Amann, R. (2002) Identification of DNA-synthesizing bacterial cells in coastal North Sea plankton. *Appl Environ Microbiol* **68:** 5728–5736.

Posch, T., Simek, K., Vrba, J., Pernthaler, S., Nedoma, J., Sattler, B., *et al.* (1999) Predator-induced changes of bacterial size-structure and productivity studied on an experimental microbial community. *Aquat Microb Ecol* **18:** 235–246.

Rappe, M.S., Gordon, D.A., Vergin, K.L., and Giovannoni, S.J. (1999) Phylogeny of actinobacteria small subunit (SSU) rRNA gene clones recovered from marine bacterioplankton. *Syst Appl Microbiol* **22:** 106–112.

Rheims, H., Felske, A., Seufert, S., and Stackebrandt, E. (1999) Molecular monitoring of an uncultured group of the class Actinobacteria in two terrestrial environments. *J Microbiol Methods* **36:** 65–75.

Sekar, R., Pernthaler, A., Pernthaler, J., Warnecke, F., Posch, T., and Amann, R. (2003) An improved protocol for the quantification of freshwater actinobacteria by fluorescence *in situ* hybridization. *Appl Environ Microbiol* **69:** 2928–2935.

Šimek, K., and Straskrabova, V. (1992) Bacterioplankton production and protozoan bacterivory in a mesotrophic reservoir. *J Plankton Res* **14:** 773–787.

Stach, J.E.M., Maldondando, L.A., Ward, A.C., Goodfellow, M., and Bull, A.T. (2003) New primers for the class *Actinobacteria*: application to marine and terrestrial environments. *Environ Microbiol* **5:** 828–841.

Tanner, M.A., Goebel, B.M., Dojka, M.A., and Pace, N.R. (1998) Specific ribosomal DNA sequences from diverse environmental settings correlate with experimental contaminants. *Appl Environ Microbiol* **64:** 3110–3113.

Tuomi, P., Torsvik, T., Heldal, M., and Bratbak, G. (1997) Bacterial population dynamics in a meromictic lake. *Appl Environ Microbiol* **63:** 2181–2188.

Urbach, E., Vergin, K.L., Young, L., Morse, A., Larson, G.L., and Giovannoni, S.J. (2001) Unusual bacterioplankton community structure in ultra-oligotrophic Crater Lake. *Limnol Oceanogr* **46:** 557–572.

Whitaker, R.J., Grogan, D.W., and Taylor, J.W. (2003) Geographic barriers isolate endemic populations of hyperthermophilic archaea. *Science* **301:** 976–978.

Zwart, G., Crump, B.C., Agterveld, M., Hagen, F., and Han, S.K. (2002) Typical freshwater bacteria: an analysis of available 16S rRNA gene sequences from plankton of lakes and rivers. *Aquat Microb Ecol* **28:** 141–155.