Population structure and species description of aquatic *Sphingomonadaceae*

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Contribution of Hong Chen to the publications listed in this thesis

Publication 1:

Hong Chen performed the isolation and identification of all the 95 strains of *Sphingomonadaceae* used in the analysis; she chose and designed the primers for the 9 housekeeping genes, tested and established the PCR protocols. She also run all the molecular work such as PCR and gene sequencing, edited the sequence data; set up the clone library of *gyrB* gene, finished all the phenotypic characterization using the BiOLOG system. Hong Chen performed the data analysis together with Johannes Sikorski. Hong Chen together with Johannes Sikorski and Jörg Overmann created all figures and tables and participated in the writing of the publication.

Publications 2-5:

Hong Chen performed the all the physiological analysis, enzyme activity, biochemical analysis, susceptibility test (medium, pH, temperature, API NE, API ZYM, API 50CH, BiOLOG, antibiotic test). For chemotaxonomic analysis, Hong Chen prepared all the biomass for fatty acid analysis, respiratory quinone, polar lipids and polyamines analysis. For genetic analysis, Hong Chen performed the PCR, the 16S rRNA sequencing and prepared the biomass for genomic DNA G+C content determination and the DNA-DNA hybridization. Hong Chen performed all the data collection and analysis. Hong Chen created all figures and tables and together with and Jörg Overmann and the Co-authors participated in writing of the publications.

I herby confirm the above statements

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Chapter 1
Summary

Prokaryotes consist of the domains of *Bacteria* and *Archaea* and exist since approximately 3.8 billion years. Prokaryotes, despite the small size of the individual cells, are regarded to represent the 'unseen majority' among the living world as they occur numerously in all types of habitats and contribute greatly to the biogeochemical cycle. They diversified strongly throughout their long evolutionary history. Prokaryotes have usually a short generation time and relatively small amount of genetic information as compared to eukaryotes, and large census population sizes. This renders them suitable test organisms for studying their evolutionary processes. The discipline of population genetics analyses the evolutionary change of the genotypic and phenotypic variants at the level of species. Most of the recent bacterial population genetic studies have focussed on pathogens.

Little is known of the population structure of freshwater bacteria. Natural freshwater lakes harbor a considerably lower diversity of bacteria, this facilitating the study of the genetic variability of bacteria. *Sphingomonadaceae* represent typical constituents of freshwater bacterioplankton communities and therefore served as a target group for a high-resolution multilocus sequence analysis (MLSA) of nine housekeeping genes (*atpD*, *dnaK*, *fusA*, *tufA*, *gap*, *groEL*, *gyrB*, *recA*, *rpoB*) and a parallel phenotypic characterization. Among 95 strains recovered from two trophically different freshwater lakes (Starnberger See and Walchensee), only 19 different 16S rRNA gene sequences were found. Yet, each strain represented a unique MLSA haplotype and the population displayed extraordinary high levels of nucleotide diversity. A split decomposition analysis revealed eight genetically distinct subpopulations, three of which comprised a single phylotype G1A with 52 strains. The population recombination rate ρ was comparable to that of other bacteria but two to eight-fold lower than the population mutation rates θ_S. Consequently, the impact of recombination on the population structure of freshwater *Sphingomonadaceae* is markedly lower than in most other free-living aquatic bacteria investigated to date. This was supported by a linkage disequilibrium analysis on the

allele distribution. Together with the large effective population size (estimate, ~$6{\cdot}10^8$), our data suggest that the incipient sexual isolation of subpopulations is caused by natural selection rather than genetic drift or demographic effects. Since neutrality tests did not provide evidence for an effect of selective forces on the housekeeping genes and no consistent physiological differences were detected between the G1A subpopulations, alternative phenotypic traits are supposed to provide a selective advantage for individual subpopulations of *Sphingomonadaceae*. This conclusion is supported by discrete seasonal abundance patterns that were detected based on pyrosequencing of internal transcribed spacer sequences in the natural samples.

MLSA is a widely applied genotyping tool in studies of the evolution and population structure of microbial organism and also represents a novel standard in microbial molecular systematics. Population genetic analysis of *Sphingomonadaceae* by MLSA revealed a distinct population substructure among individual 16S rRNA phylotypes, providing insights into the diversity within bacterial species. A 'species' is the main taxonomic unit in the systematics of prokaryotes, but the subject of the species concept of prokaryotes has always been controversial. Until now there is no prokaryotic species concept that is accepted by all scientists. But for practical reasons, bacterial strains are affiliated to different species on the basis of DNA-DNA reassociation and diagnostic phenotypes. As DNA-DNA hybridization is difficult to be compared between laboratories and time consuming, MLSA becomes a valuable alternative to it. The population genetic structure revealed by MLSA is strongly associated with the results from DNA-DNA relatedness values. When sufficient numbers of suitable loci are selected, the concatenated sequence similarity values can in principle be used for species delineation.

To assess the population and subpopulation structure revealed by MLSA also from a taxonomic perspective, four *Sphingomonadaceae* strains belonging to four different subpopulations were chosen for new species description. Based on morphological, physiological and biochemical characterization, strain 247 from group G3B was affiliated to a species formerly named '*Caulobacter leidyi*' and which was now reclassified as '*Sphingomonas leidyi*'. Strain 382 from group G1A2 was proposed as type strain of a novel species

'*Sphingomonas limneticum*'. Strain 301 from group G2D was proposed as type strain of a novel species '*Sphingobium oligotrophica*', and a strain 469 was proposed as type strain of a novel species '*Sphingobium boeckii*', and the closely related species formerly names '*Sphingomonas suberifaciens*' was reclassified as '*Sphingobium suberifaciens*'.

Chapter 2
Introduction

Microevolution of bacteria

Evolution is the process that has produced the enormous diversity of organisms alive today. Evolution is the change over time in one or more inherited traits present in populations of individuals. Evolutionary changes are classified into two types, macroevolutionary and microevolutionary changes. Macroevolution refers to any evolutionary changes at or above the level of species, such as evolution of genera, families and phyla. Microevolution refers to any evolutionary changes below the level of species, normally refers to the changes in allele frequencies that occur over time within a population or a species (Hennig, 1966). Thus, microevolution results in splitting a species into two (cladogenesis) or change a species into another (anagenesis) (Rensch, 1959): Population genetics is the branch of biology that studies the process of microevolution. The change of microevolution is due to four different processes: mutation, selection, gene flow and genetic drift.

Bacteria reproduce by the asexual process of binary fission. So, the daughter cells get the identical chromosome from their mother cell. There is no sexual process to exchange or retrieve new genetic information within the course of replication. The genetic variation between bacteria results then from the accumulation of mutations which may occur because of replication errors or other external factors (Bertram, 2000; Aminetzach *et al.*, 2005; Burrus & Waldor, 2004). After a sufficient number of generations, the variation may be enough to delineate them into different lineages. The diversity can also arise by horizontal gene transfer (HGT), by which cells may obtain or lose genetic materials between different lineages. Gene flow refers to the horizontal gene transfer between different populations. For bacterial populations, HGT plays a major role during the evolutionary process.

The genetic variation may be neutral at the beginning. But under specific conditions like competing for the same source of nutrient, the novel genetic variation (either by mutation or HGT) may increase the fitness of one of the lineages, i.e., by increasing the survival or

reproductive capability, resulting in an increase of the abundance of this lineage in their population by periodic selection, whereas other lineages may decrease in abundance or even get extinct (Levin, 1981). As a consequence, the frequency of alleles changes in the course of generations. The lower the sequence diversity in a population, the higher the frequency of gene drifts in their offspring generation (Spratt & Maiden, 1999; Boucher *et al.*, 2003; Walsh, 2006).

Evolution at the species or population level proceeds through a differential reproductive success of individuals. Populations and species are levels of diversity in which evolutionary changes can be observed. One central issue in evolution is the mechanisms maintaining genetic and phenotypic variation in natural populations. Prokaryotes like bacteria have often short generation times but have large population sizes and are suitable to genetic-level analysis in the controlled environments in the field of 'experimental evolution', in which the complexity of the analysis is reduced allowing researchers to better understand microevolutionary dynamics (Rainey *et al.*, 2000). But prokaryotes represent one of the least understood groups with respect to evolutionary biology because of their high diversity. To more fully understand and better explain the forces which bring about changes in these groups, it has been necessary to apply our knowledge of genetics to populations.

The species concept of prokaryotes

Population genetics is concerned with the allelic variation within species over generations. Thus, the field of population genetics is ultimately linked to the field of taxonomy. The category 'species' represents the main taxonomic unit in classification. Speciation is the evolutionary process by which new biological species arise. Natural speciation take place over the course of evolution. But the nature of the process of speciation is still a subject of debate among microbiologists because of the existence of different mechanism (Baker, 2005). The disciplines of eukaryotic populations are divided by the species boundary, so the population genetics is summarized in Mayr's definition of biological species: "species are groups of interbreeding natural populations that are reproductively isolated from other groups" (Mayr, 1970; de Queiroz, 2005). For prokaryotes, the difficult

task is to set up the limits of the populations and to define a species. The problem arises because of their predominantly asexual reproduction and the apparent promiscuity of certain genetic elements.

The prokaryotes vary by responding to changes in the biotic and abiotic components of ecosystems. For eukaryotes it is well known by the fossil records that species got extinct. But for prokaryotes, there is no direct evidence of microbial extinctions because fossil records cannot be captured.

The prokaryotes, which constitute the domain of *Archaea* and *Bacteria* may possibly contain millions of different species. The diversity is the product of about 3.8 billion years of evolution, which may be the reason for the extraordinary diversity and habitat range of prokaryotes (Torsvik *et al.*, 2002). Numerous species have a worldwide distribution, and a local population may contain the full range of variation that exists worldwide (O' Rourke & Stevens, 1993). The Global Biodiversity Assessment program suspects that there are millions of species of prokaryotic organisms that exist in nature, but only around 9000 of them have been described taxonomically (Straininfo http://www.straininfo.net/stats; LPSN http://www.bacterio.cict.fr/; DSMZ http://www.dsmz.de/). The vast majority of naturally occurring prokaryotic organisms cannot yet be cultured. So the full diversity of the physiological and biochemical reactions, the biotechnological potential of prokaryotes and their ecological role are still unknown. Some complex environments like soil inhabit a very high degree of diversity of prokaryotes, genetic studies of soil estimate that 10 grams of unpolluted soil typically have millions of different species of prokaryotes (Lengeler *et al.*, 1999; Gans *et al.*, 2005).

What determining the driving force behind the speciation of prokaryotes, people found that prokaryotic organisms have evolved sophisticated ways for acquiring and losing genetic material (Margulis & Sagan, 2002). The three major mechanisms (transformation, transduction and conjugation) of genetic exchange are well understood, and have been documented to exist in nature with major ramifications for acquisition of new traits and speciation. Natural genetic exchange is the uptake of genetic materials from the environment or other cells. It is potentially relevant for speciation because the source of the obtained DNA

can be from different species. Therefore, these genetic exchanges can introduce novel genes into a microbial population ultimately leading to niche specialization and speciation (Aravind *et al.*, 1998; Cohan, 1994; Ochman *et al.*, 2000; Ogunseitan, 1995). But on the other side it is these genetic exchanges which make it difficult to delineate the species by genetic analysis, leading a debate of whether there are any naturally entities of prokaryotic species or not.

Speciation is the result of evolution and it may ultimately lead to the generation of major taxonomic groups. The investigation of speciation and diversity of prokaryotes are related to the research on the origin of planetary life, and on how the physiological characteristics of prehistoric microorganisms contributed to the emergent signature of a co-evolving life and environment. So the investigation can also shed light on biogeochemical cycles that are sensitive to industrial ecological problems such as energy crisis, disease epidemics, global warming, toxic pollutants and environmental problems (Martin & Mueller, 1998; Lake *et al.*, 1985; Brown *et al.*, 2001; Rasmussen, 2000). Cohan (2002) believes that “Bacterial species exist- on this much bacteriologists can agree. Bacteriologists widely recognize that bacterial diversity is organized into discrete phenotypic and genetic clusters, which are separated by large phenotypic and genetic gaps, and these clusters are recognized as species”. Additionally, the definition of species is necessary for the research of the bacterial populations, the study of evolutionary theories and its practical application (Wald *et al.*, 2008; Fraser *et al.*, 2009). There is little support for the non-existence of bacterial species, species taxon realism holds that recognized species can be real, they are bacteria which are variously, well-defined spatio-temporally and cohesive in their properties. Individual organisms in a species should share one or more traits that unite them to be the same species and distinguish them from other species (Diamond & Bishop 1999; Pigliucci, 2003; Riley and Lizotte-Waniewski, 2009).

There are a large number of criteria to be used to define the prokaryotic species depending on the different objectives of the researches. The understanding of prokaryotic species has traditionally relied on the criteria used to the species concept of eukaryotic organisms. For example, the term “taxospecies” refers to phenotypic clusters of bacteria and was proposed by Ravin (1960) who tried to apply the biological species concept (BSC) to

bacteria. The species was defined as a group of organisms with mutually high phenotypic similarity that forms an independent phenotypic cluster. This concept is analogous to the morphological species concept by which organisms are classified in the same species if they appear identical by morphological (anatomical) criteria (Eldredge & Cracraft, 1980), but in addition to anatomical features, it includes consideration of physiological characteristics. The "genospecies" concept was also proposed by Ravin (1963) by defining groups of bacteria that can exchange genes, but with very little correlation between groups of organisms described as taxospecies. This incongruence provoked further dissatisfaction among microbiologists holding traditional species concepts.

More recent attempts to make the biological species concept applicable to prokaryotes fall into four categorial groups: the influence of recombination, the influence of the ecology, the application of phylogeny and nominalism (Ereshefsky, 2010). Dykhuizen and Green (1991) proposed bacterial species to be "groups of strains that recombine with one another but not with strains from other such groups." The recognition of historic events of genetic recombination was built into this definition because it had become feasible to reconstruct phylogenetic relationships according to molecular sequence data, which presumably can be used to delineate groups according to the genetic exchange criterion (Fraser, 2007). However, this approach is also questionable on the basis of several observations that many bacteria are capable of exchanging and recombining genes both within and between the groups currently nominated as species, genera, or even domains (Xu, 2004; Gogarten & Townsend, 2005). The exchange and recombination gene among prokaryotes is not hindered by reproductive barriers, as it was cited by biological species concept (Doolittle & Papke, 2006).

An ecotype is defined as a bacterial population occupying the same ecological niche and its divergence is purged recurrently by natural selection. Cohan proposed a species concept as "A species in the bacterial world maybe understood as an evolutionary lineage bound by ecotype-periodic selection." Most named bacterial species may contain many bacterial ecotypes, the bacterial ecotype is equal to the eukaryotic species, and the prokaryotic species are consistent with eukaryotic genus (Cohan, 2001; 2002). Ward (1998) also proposes a "natural species concept" for prokaryotes based on the ecotype concept.

Bacterial strains showing high DNA-DNA hybridization values (approximately 70% or greater DNA-DNA relatedness, and 5 °C or less ΔT_m, both values must be considered) are regarded to belong to the same species based on the "genomic species concept" (Wayne *et al.*, 1987). A further concept is the “phylogenetic species concept”, various types of genetic data like 16S rRNA, DNA-DNA hybridization, average nucleotide identity (ANI) and the core genes or housekeeping genes are used to reconstruct phylogenies and to thereby recognize species (Rossello-Mora & Amann, 2001; Stackebrandt, 2006; Nesbo *et al.*, 2006). While different genes are used for phylogenetic analysis, the classification (clades) of the same group of organism may change, as each gene tree reflects the phylogeny only of the specific gene. To avoid this, it is recommended to use as the final result the multiplicity of phylogenetic trees (Doolittle & Bapteste, 2007; Franklin, 2007). There is another species concept called “phylophenetic species concept” which anchors the genomic species concept within an ecological and evolutionary framework (Rossello-Mora & Amann, 2001). Species are defined as “a monophyletic and genomically coherent cluster of individual organisms that shows a high degree of overall similarity in many independent characteristics, and is diagnosable by a discriminative phenotypic property.” This definition integrates character-based concepts that emphasize the presence of an apparent organism attribute with history-based concepts that emphasize the degree of relatedness of a new isolate to previously characterized organisms. The phylophenetic species concept was extended by Sicheritz-Ponten and Andersson, they developed a “phylogenomic” way to illustrate microbial evolution, and they linked the phylogenetic information with the flow of biochemical pathways within and among species (Ogunseitan, 2002; Sicheritz-Ponten & Andersson, 2001).

Stackebrandt suggested a rather nominalistic microbial species concept, by stating that “The nonexistence of species as an objective category […] has been recognized by microbiologists for over 20 years. Bacteriologists in particular follow guidelines and recommendations to provide stability, reproducibility, and coherence in taxonomy—although in the final analysis, species description is still subjective”. Nomenspecies are defined on the basis of the characters of the type specimen; typological species, this is the most widely

accepted and operational species concept (Stackebrandt *et al.*, 2006; Hanage *et al.*, 2005).

There are around 22 different concepts for defining the species at present, different species concepts highlight different properties of species, such as phenotype, reproductive isolation, phylogenetic relations, ecological role and so on (Pigliucci & Kaplan, 2006). There is no clearly and universally accepted prokaryotic species concept until today, no concept which can bear the heavy load of all the meanings and all the theoretical implications can be generated. Different scientists interested in different lineages will choose the one which is suitable to their research. But the lack of agreement on the species concept has led to some problems such as that a single species can be identified as different species by using different criteria.

For practice, a species definition is recommended by the *ad hoc* committee for the re-evaluation of the species definition in bacteriology: Prokaryotes within a species should have sequence similarities of 16S rRNA gene higher than 97.0% (or 98.7%). If the 16S rRNA sequence similarity between two different bacterial strains is higher than 97% then DNA-DNA hybridization or comparable methods (e.g. genome to genome sequence comparison and average nucleotide identity (ANI) of the genome) should be applied in order to test if they belong to the same or to two different species (Stackebrandt *et al.*, 2002, 2006, Auch *et al.*, 2010; Richter and Rossello-Mora, 2009).

Genomic similarity determined by DNA-DNA hybridization was formerly considered as 'gold standard' method to determine the relatedness between bacterial species. But this technique is very time consuming and cumbersome to perform; variation between experiments, techniques and laboratories makes it difficult. Whole genome sequence could solve all these problems but its cost are still high and also time consuming. As reported, the number of genes included in the core genome which contains the genes presented in all strains of the targeted species, appears to decrease as more genomes of strains are compared, whereas the relative size of the flexible gene pool increases (Konstantinidis & Tiedje, 2005; Goris *et al.*, 2007; Kuhnert *et al.*, 2009). So, to find the taxonomic and phylogenetic relationship of bacteria, only the comparison of the core genome appears to be suitable. The question is which genes should be used for defining the genome similarity. For this, using sequences of

multiple protein-coding genes for genotypic characterization of diverse groups of prokaryotes has been proposed (Gevers *et al.*, 2005). Multilocus sequenc typing (MLST) is a molecular tool for epidemiological purposes and aims at the identification of allelic mismatches at the loci of closely related organisms (Urwin & Maiden, 2003). The main advantage is to provide unambiguous sequence data, which is the availability and comparability from any laboratory, avoiding the problems of lack of comparability like using DNA-DNA data. To establish MLST, a set of housekeeping genes will be determined which have a higher level of sequence divergence than rRNA genes to provide the resolution to distinguish closely related bacteria lineages, but which are also conserved enough to retain genetic information. Also, these genes should be distributed across all members of the target groups and also be present as a single copy only within the given genome (Martens *et al*, 2007; Gevers *et al.*, 2005; Thompson *et al.*, 2005). Expectations are that these analyses will have a positive impact on performing taxonomic and biodiversity studies (Thompson & Swings, 2006). In MLSA, phylogenetic analysis is based on the sequence similarity. The divergence level of the sequence influenced by mutation or recombination which is ignored by MLST is taken into account in MLSA. So MLSA is most suitable for species separation, but first the cut-off values for the similarities values used to separation should be set (Gevers *et al.*, 2005). Based on the former MLSA of family *Pasteurellaceae*, they proposed that sequence comparison of the three genes *recN*, *rpoA* and *thdF* could replace DNA–DNA hybridizations (Zeigler, 2003; Kuhnert & Korczak, 2006). By using conserved primers for PCR and sequencing, they deduced the genome similarity of the family *Pasteurellaceae* based on the sequence of these three genes. They suggested that strains with the deduced genome similarity value below 40% are belonging to the different genera, and the strains with similarity above 85% linked to the same species. The MLSA analysis results are in good agreement with past results for DNA–DNA hybridizations. This strategy was shown to be a valuable taxonomic alternative to DNA–DNA hybridization (Mutters *et al.*, 1989; Christensen *et al.*, 2005 & 2007; Kuhnert *et al.*, 2007; Bisgaard *et al.*, 2007). From the MLSA analysis of genus *Haeamophilus*, four genes were used for MLSA comparison, the results also supported the DNA–DNA hybridization results (Norskov-Lauristen *et al.*, 2005). For different taxa, the selected housekeeping genes were

different, this value might be different.

Population genetics of prokaryotes

Population genetics is the study of evolutionary change in the genetic composition of populations, so it focused on the mechanisms of mutation, natural selection, recombination, migration and genetic drift that influence the evolutionary rate of change in the populations and also the amount and nature, distribution over space and time, and the biological significance of these changes (Whittam, 1995). The outcomes of such investigations will illustrate the population genetic structure, knowledge of the nature of allelic variation and the role of different modes of recombination in generating genotypic variation.

To understand genetic variation of populations, a wide range of phenomena must be taken into account. There are three aspects to population genetics: the description of variation within populations, an assessment of the processes that cause this variation (which may involve both field and laboratory studies), and a body of theory that connects the cause of variation with the effect of the variation. So that means we must know the processes that generate new genotypes, which are mutation, rearrangements within genomes, and the passage of genes from one individual to another; the processes that determine the fate of genotypes: selection, migration between populations, and the laws of chance (i.e., genetic drift). After we have sufficient informations on all these processes of particular populations, the pattern of genetic variation can be detected by the level of genetic polymorphism, linkage disequilibrium, spatial heterogeneity, temporal changes, and other indices from the sequence analysis. Conversely, the observed pattern of variation can inform us about the processes that generated it.

The most likely starting point for a population genetic event is a mutation. The progeny of a prokaryotic cell should in principle be genetically identical to its ancestor, but mutations and HGT tend to abolish this identity over time. Mutations increase the genetic diversity between individuals. Point mutations accumulate with a nearly constant rate at random positions in the sequences and we can analyze them by sequence comparison until a certain level of divergence (Hartl & Clark, 2007).

HGT is a process in which genetic material is transfered from one organism to the others which are not their offspring organisms. HGT is a highly significant phenomenon amongst prokaryotic cells, and it maybe the dominant form of genetic transfer among the single-celled organisms. HGT between prokaryotes can take place by transformation, transduction and conjugation and usually occurs as an asymmetrical exchange events between partners.

Transformation is the uptake of exogenous genetic material from its surrounding environment and its heritable incorporation into the genome (Lorenz and Wackenmagel, 1994). In bacteria, DNA is taken up as a double strand across the outer and as a linear single stranded across the inner membrane, also, uptake signal sequence have been found to favor uptake (Maughan *et al.*, 2010). Transduction is the process by which DNA is transferred from one bacterium to another by transducing bacteriophages. There is high variability in the ability of bacteriophages to cross-react with strains within the same species, between different species of the same genus and between different genera of the same family (Fink *et al.*, 2001). Bacterial conjugation is the transfer of genetic material between bacterial cells by direct cell-to-cell contact or by a bridge-like connection between two cells. It occurs between donor cells which have an F-plasmid (F+) and recipient cells that lack it (F-). The F+ initiate the conjugation via the F pilus, then the genetic materials can be transferred from F+ to F- cells (Griffiths *et al.*, 2000).

The prevalence of HGT among prokaryotes may be explained by their often single-celled lifestyle (Lan & Reeves 2000), as a newly transferred gene sometime can give the whole organism immediate benefit like adaptive superiority, sometimes even in the absence of sophisticated regulation. In eukaryotes, recombination refers to the results of crossing over in a symmetrical way between chromosomes during the zygotene stage of meiosis. This happens between members of the same species and even with members of the same population. The change of the genes happens frequently and normally together with reproduction. But in prokaryotes, the genetic exchange occurs less frequently than in eukaryotes as it is independent from reproduction. As such, HGT in bacteria may occur between different species, genera or even domains, hence it is not limited to the species level (Nelson *et al.*, 1999). In bacterial HGT, parts of the genome which range in size from a few kilobases in

natural transformation to several tens of kilobases in phage-mediated transduction and, potentially, hundreds of kilobases in conjugation can be transferred from one cell to another cell (Lan & Reeves, 2000).

While the level of mutation rates is rather constant in bacteria (Drake *et al*., 1998; Lynch & Conery, 2003), the frequency of homologous recombination varies strongly between clonal and panmictic populations (Cohan & Perry, 2007; Vos & Didelot, 2008). The often large effective size of bacterial populations favours a strong influence of selection over drift (Lynch & Conery, 2003) whereas obligately host-associated bacteria such as endosymbionts or some pathogens display population bottlenecks and higher levels of genetic drift (Herbeck *et al*., 2003; Kuo *et al*., 2009). Depending on the relevance of these individual forces, the resulting evolutionary patterns vary considerably between different bacteria (Cohan & Perry, 2007; Gevers *et al*., 2005).

Methods to determine the bacterial population structure

To analyses the population structure, different methods like serotyping, monoclonal antibody typing, biotyping, bacteriophage typing, cell electrophoresis and whole protein extract electrophoresis can be used. But to detect the population genetic structure, the informations on the frequencies of alleles should be obtained. The unbiased method for studying genetic variation that would be widely applied to a large number of genes in a variety of organisms is the direct study of genes and their products. The electrophoretic separation of protein, isozymes and nucleic acids are useful tools for differentiating taxa (Ibrahim *et al.*, 2003). This can be used for detecting a mutation which results in the difference of the electrophoretic patterns of the genes or the different mobility of the protein it encodes. The methods based on the electrophoresis are SDS-PAGE, MLEE, RFLP, RAPD, DGGE, REP-PCR. SDS-PAGE and MLEE analyze the differences of the gene products, i.e, the proteins. SDS-PAGE has been used extensively for identification and classification at the strain and species level. MLEE can give us a variety of insights into the genetics, ecology and taxonomy and identify clusters of closely related strains. RFLP, RAPD, REP-PCR and DGGE detect the differences between strains on the level of the DNA itself. RFLP is highly sensitive

and rapid in detecting mutations. RAPD detects the mutations randomly in the genome and REP-PCR detects the distribution of repetitive elements. DGGE provides genetic diversity and richness of the diversity in the microbial populations. The major problem of these methods is that the results obtained in different laboratories are difficult to compare or limited by targeting only at special sites (Danger *et al.*, 2010; Selander *et al.*, 1986; Pourzand & Cerutti, 1993; Williams *et al.*, 1990; de Bruijn *et al.*, 1996). MLMT is a method targeting microsatellite regions and using the sequence of them to detect the population and genetic characteristics of different species (Ochsenreither *et al.*, 2006; Meece *et al*., 2011). All the above methods are trying to achieve the high levels of discrimination and giving the maximal variation within the bacterial population, and then the population genetic structure can be found more clearly and convincible.

MLST / MLSA As the sequence technology developed very fast and became cheap and easy to apply, MLEE has been adapted by identifying alleles directly from the nucleotide sequences of the internal fragments of housekeeping genes rather than comparing the electrophoretic mobilities of the enzyme they encoded. Multilocus sequence typing (MLST) determine the sequence of a set of housekeeping genes (normally approximate 400-500 bp of their internal fragment), then different sequences of the same gene are assigned as alleles. Each strain used in the analysis will get an allele profile which is also called sequence type (ST). In MLST, the number of nucleotide differences between alleles is ignored and sequences are given different allele numbers without taking into account whether the difference between two sequences is at a single nucleotide site only or at many sites, e.g., as a result of multiple mutations or even recombination events. So, the number of nucleotide polymorphisms between alleles affected by recombination events is normally higher than by alleles affected only by point mutations. Multilocus sequence analysis (MLSA) compares the primary DNA sequences from multiple conserved protein-coding loci for assessing the diversity and relationship of different isolates across related taxa, thereby using an appropriate phylogenetic or cladistic approach to illustrate the population genetic structure (Naser *et al.*, 2005). The sequences of multiple (often seven) loci from many hundreds of isolates of a bacterial species, provide data that can be used to address the population genetic structure and

evolutionary biology of the species (Feil *et al.*, 2001; 2003). The distribution of alleles investigated by MLST can be used to predict if the population structure is clonal or non-clonal (panmictic). MLST is usually applied to strains that belong to a well-defined species while MLSA is more often used when species boundaries are not well known and MLSA data are used to improve species description (Young *et al.*, 2008). MLSA has been widely used in studies of several bacterial populations, such as *Lactobacillus* genera (Naser *et al.*, 2006), *Borrelia* spp. (Richter *et al.*, 2006), *Mycobacteria* (Mignard & Flandrois, 2008), *Pseudomonads* (Young & Park, 2007), *Burkholderia* (Gevers *et al.*, 2005), *Bradyrhizobium* (de las Rivas *et al.*, 2009), *Haemophilus* (Norskov-Lauritsen *et al.*, 2005), *Ensifer* (Martens *et al.,* 2007; Martens *et al.*, 2008) and *Vibrionaceae* (Goarant *et al.*, 2006; Urbanczyk *et al.*, 2007; Thompson *et al.*, 2008; Thompson *et al.*, 2005).

Most of the population structures have been analyzed for pathogens. From the dataset of MLST and MLSA analysis, most of the online dataset (http://www.mlst.net/databases/default.asp, http://pubmlst.org/) are pathogens (Achtman, 2008; Maiden, 2006; Kilian *et al.*, 2008; Nuebel *et al.*, 2010; Keymer & Boehm, 2011). But pathogenic bacteria represent only a minority of the total bacterial diversity. The evolution mechanism of pathogenic bacterial are governed by the particular dispersal of their hosts and immune selection pressures, which are distinctly different from those mechanism experienced by the majority of bacteria occurring in the environment (Lange & Ferguson, 2009; Grenfell *et al.*, 2004). So, more attention should be drawn to the environmental prokaryotes (soil, sediment, lake Water, marine). The often low cultivation success of the majority of environmentally dominant bacteria has impeded the analysis of population structure of natural bacterial populations. As a result, only few studies focussed on environmental and free-living prokaryotes (Doroghazi & Buckley, 2010; Sikorski & Nevo, 2007; Vergin *et al.*, 2007; Hunt *et al.*, 2008; Sikorski *et al.*, 2008a; Connor *et al.*, 2010; Melendrez *et al.*, 2011).

The planktonic environment is especially well suited to investigate the evolutionary basis of diversity in environmentally significant bacteria. The major environments are the marine and freshwater environments. An aquatic environment is a small self-sustaining ecosystem. The abundance and distribution of the plankton is dependent on the nutrients, light

and temperature. Bacterioplankton is important for the nutrient cycles (nitrogen and phosphorus). The composition of freshwater bacteria appears to be different from the marine. The natural freshwater lakes harbor a considerably lower diversity of bacteria, there is a set of "typical fresh water bacterioplankton" that were found to be globaly distributed. *Proteobacteria*, *Actinobacteria*, *Bacteroidetes*, *Cyanobacteria*, *Verrucomicrobia* and *Planctomycetes* are the predominant component of the typical freshwater bacterial community. The abundance of microbial cells in the freshwater Puma Yumco was determined as 1.59×10^5 to 3.37×10^5 cells mL^{-1} (Logue *et al.*, 2008; Liu *et al.*, 2009). Planktonic cells were expected to be subject to lower rates of lateral gene transfer than occur in denser bacterial communities such as biofilms. This facilitated the study of the genetic variability of bacterial ecotypes as it is comparatively homogeneous and has a lower bacterial diversity compared to other environments, yet still offers numerous niches for closely related strains to coexist (Jaspers and Overmann 2004; Acinas *et al.* 2004). In particular, there are only few studies on the freshwater bacterial population genetics available. The oligotrophic alpine Walchensee and mesotrophic Starnberger See are located in southern Germany, both were selected for the present study since a larger dataset on environmental parameters and several isolates are already available from our previous investigations (Bruns *et al.*, 2003; Gich *et al.*, 2005). The family of *Sphingomonadaceae* (Kosako *et al.*, 2000; Lee *et al.*, 2005) within the *Alphaproteobacteria* exist in different environments and are abundant in freshwater environments. *Sphingomonadaceae* can account for up to 22% of the cells in bacterioplankton communities (Gloeckner *et al.*, 2000; Zwart *et al*., 2002) and include physiologically diverse bacteria such as aromatic-compounds degrading strains and aerobic anoxygenic phototrophs (Kim *et al.*, 2007). Therefore, the family *Sphingomonadaceae* has been chosen as the target group of the current study. Closely related ribotypes of this typical freshwater *Alphaproteobacteria* co-occur and many can be retrieved by cultivation (Gich & Overmann, 2006; Jaspers & Overmann, 2004). The studies of *Alphaproteobacteria* by Gich *et al*. suggested that there were genetically and physiologically different strains of the same 16S rRNA phylotype existing in the same oligotrophic alpine lake (Gich *et al.*, 2005).

In the present study, the population structure of *Sphingomonadaceae* in two lakes of

different trophic state was elucidated based on a high-resolution multilocus sequence analysis of nine housekeeping genes. 95 *Sphingomonadaceae* strains were isolated, and some strains could not be distinguished by 16S rRNA gene. Amongst them, a set of 52 strains was found to have identical 16S rRNA gene sequences. However, some of these strains differed by their growth rates, cell colors and shapes, and by their 23S rRNA gene and ITS1 sequences, indicating some level of divergence despite identical 16S gene sequences. In order to study the internal diversity structure amongst these strains, including potential taxonomic implications at the level of speciation processes, MLSA was chosen as method of analysis for this purpose. The genes *atpD* (F0F1 ATP synthase, beta subunit), *dnaK* (bacterial homolog of 70kD heat-shock protein), *fusA*/*tufA* (elongation factors G/Tu), *gap* (glyceraldehyde-3-phosphate dehydrogenase), *groEL* (bacterial homolog of 60kD heat- shock protein), *gyrB* (DNA gyrase, B subunit), *recA* (recombinase A), and *rpoB* (DNA-directed RNA polymerase, beta subunit) were chosen for the analysis. These housekeeping genes have been found suitable for the analysis of other *Alphaproteobacteria* (van Berkum *et al*., 2006; Vinuesa *et al*., 2008). The MLSA results were combined with phenotypic, physiological characteristics for new species description.

Aims of the present study

The goal of the project is to elucidate the role of mutation, recombination, genetic drift and selection (such as selective sweeps) in shaping bacterial diversity. Here, bacteria from the family *Sphingomonadaceae* of the *Alphaproteobacteria* are used as model groups. Compared to soil and sediment, the planktonic environment is suitable for the investigation of the bacterial evolution and diversity, because it is comparatively homogeneous and has a lower bacterial diversity, but it still offers numerous niches for closely related strains to coexist (Jaspers & Overmann 2004; Acinas *et al.* 2004). Molecular analyses of natural population structrue and isolated strains in combination with ecophysiological tests in the laboratory were used to the elucidate factors governing bacterial evolution and diversification in the model groups.

The population structure and the significance of different evolutionary events was

analyzed based on the multilocus sequence analysis of a set of housekeeping genes using a collection of isolates of *Sphingomonadaceae*. Together with the results of a physiological characterization of the strains, this approach should allow to identify the role of adaptation and selection in the origin and divergence of closely related bacterial lineages. From the result of the analysis, the population structure concept potentially could be extended to the taxonomic field of bacterial species delineation.

Several distinct lineages represent the subpopulations revealed by MLSA were analysed; phenotypic and genotypic characteristics were identified and analysed. The combination of MLSA with phenotypic and physiological characteristics will illustrate whether MLSA is suitable and powerful for performing species taxonomic and biodiversity studies leading to new species description. Driving forces and the process of the speciation will also be under discussion.

Chapter 3
Material and Methods

Sampling site and targeted isolation of bacterial strains

The oligotrophic alpine Walchensee (802 m above sea level; maximum water depth of 190 m) is located in southern Germany and was sampled at a distance of 30 m from the western shore (47°35′N, 11°20′E). Mesotrophic Starnberger See (584 m above sea level; maximum water depth 128 m) is situated 23 km north of Walchensee and was sampled from a pier located on the eastern shore near Ammerland (47°54′11N, 11°19′54E). Water samples were collected on December 20, 2007, at a water depth of 1 m using a bilge pump connected to isoversinic tubing as previously described (Overmann *et al*., 1998). For cultivation, basic synthetic freshwater medium buffered with 10 mM HEPES (Bartscht *et al*., 1999) was supplemented with 20 amino acids, glucose, pyruvate, citrate, 2-oxoglutarate, succinate (200 µM each), Tween 80 (0.001% v/v), a mixture of formate, acetate and propionate (200 µM each), trace element solution SL 10 and 10-vitamin solution (Jaspers *et al*., 2001). Signal molecules (cAMP, *N*-butyryl homoserine lactone, *N*-oxohexanoyl-DL-homoserine lactone, ATP) were added at final concentrations of 10 µM each for growth stimulation (Bruns *et al*., 2002). Growth medium (200 µl) was dispensed into the wells of sterile 96-well round bottom microtiter plates and each well inoculated with aliquots of lake water containing 50 cells. After incubated for 6 weeks at 15°C, bacterial cell growth was monitored by turbidity. Positive cultures were screened for the presence of *Sphingomonadaceae* using a specific PCR protocol employing novel primer Sphingo866f (5'-CGCATTAAGTTATCCGCC-3') and primer Alf968r (5'-GGTAAGGTTCTGCGCGTT-3') (Neef, 1997). 36-46% of the cultures obtained from both lakes were identified as *Sphingomonadaceae* and were subcultivated on agar plates prepared with washed agar, basic synthetic freshwater medium and 1:10 diluted HD (0.05% casein peptone, 0.01% glucose, 0.025% yeast extract, w/v). Of the 95 strains recovered, 52 strains originated from Walchensee and 43 from Starnberger See.

Sequencing and phylogeny of 16S rRNA genes

16S rRNA genes were amplified with primers Alf19F (5'-CTGGCTCAGARCGAACG-3') (Manz *et al.*, 1992) and 1492R (5'-GGTTACCTTGTTACGACTT-3') (Lane, 1991). PCR products were purified with the QIAquick PCR purification kit (Qiagen) and sequenced by the dideoxynucleotid method on an ABI Prism 3730 genetic analyzer (Applied Biosystems), employing the AmpliTaq FS Big Dye terminator cycle sequencing kit. Small nucleotide differences were confirmed by repeating the PCR reactions and sequencing. Sequences were edited and assembled with the Vector NTI computer package (Invitrogen). Phylogenetic analysis of 16S rRNA gene sequences was performed with the ARB software package (Ludwig *et al.*, 2004). Sequences were automatically aligned with the integrated Fast Aligner tool, and the alignment corrected manually according to secondary-structure information. The resulting alignment spanned 1231 bp. Phylogenetic trees were constructed based on the Maximum Likelihood, Neighbor Joining and Maximum Parsimony algorithms. Bootstrap values were calculated with 1,000 bootstrap resamplings. 16S rRNA gene sequences of the closest relatives were identified by the NCBI BLAST online tool (http://blast.ncbi.nlm.nih.gov) and classification was verified by the RDP classifier.

Multilocus sequence analysis (MLSA)

Sequencing of nine housekeeping genes. *AtpD* (F0F1 ATP synthase, beta subunit), *dnaK* (bacterial homolog of 70kD heat-shock protein), *fusA*/*tufA* (elongation factors G/Tu), *gap* (glyceraldehyde-3-phosphate dehydrogenase), *groEL* (bacterial homolog of 60kD heat- shock protein), *gyrB* (DNA gyrase, B subunit), *recA* (recombinase A), and *rpoB* (DNA-directed RNA polymerase, beta subunit) were chosen for MLSA. These housekeeping genes have been found suitable for the analysis of other *Alphaproteobacteria* (van Berkum *et al.*, 2006; Vinuesa *et al.*, 2008). In the genomes of *Sphingopyxis alaskensis* RB2256^T (DSM 13593^T), *Sphingomonas wittichii* RW1^T (DSM 6014^T), *Sphingobium japonicum* UT26^T, *Novosphingobium aromaticivorans* DSM 12444^T, *Zymomonas mobilis* subsp. *mobilis* ZM4, and *Zymomonas mobilis* subsp. *mobilis* NCIMB 11163, the nine genes occur only in a single copy (with the exception of groEL in DSM 12444^T). Except *fusA*/*tufA*, most of the genes are rather evenly

Table 1. Fragments, primers sequences for amplification and sequencing, and Genbank accession numbers of the obtained partial sequences.

Locus	Primers	Fragment length (bp)*	Annealing temperature**	Genbank accession No.
atpD	F: 5'-CAGGTCATYGGHSCVGTCG-3'	882	62°C, 57°C	HQ881405-
	F: 5'-GCAYCTSGGYGARAACACCGT-3'			HQ881449
	R: 5'-GCCGACCATGTAGAAVGC-3'			
danK	F: 5'-TVGGHACVACCAACAGCTGYGT-3'	1245	62°C, 57°C	HQ881350-
	R: 5'-TTRCCBGTGCCYTTGTCCT-3'			HQ881404
fusA	F: 5'-ACCGGYAARTCCTACAAGATCG-3'	546	62°C, 57°C	HQ881313-
	R: 5'-ACCTTGAAYTCGCGCTTCAT-3'			HQ881349
	F: 5'-ATGGAYTGGATGGAGCAGGAG-3'			
	R: 5'-ATCCTGTTCGACGGCGAGTT-3'			
tuf	F: 5'-GCCGAYTAYGTGAAGAACAT-3'	702	65°C, 60°C	HQ881252-
	R: 5'-GTSGTGCGGAAGTAGAACT-3'			HQ881312
	F: 5'-GCCGAYTAYGTGAAGAACAT-3'			
	R: 5'-GTSGTGCGGAAGTAGAACT-3'			
gap	F: 5'-TGCACSACCAACTGCCT-3'	435	59°C, 54°C	HQ881229-
	R: 5'-TTSGAGAAGCCCCATT-3'			HQ881251
groEl	F: 5'-GTCGARGGCATGCAGTT-3'	789	65°C, 60°C	HQ881204-
	R: 5'-ACCTTSGYCGGRTCGAT-3'			HQ881228
	F: 5'-GCSGGCATGAACCCGAT-3'			
	R: 5'-GCRCGNAYGATGTCGAT-3'			
gyrB	F: 5'-TCGGGBCTSCACCAYAT-3'	813	60°C, 56°C	HQ881135-
	R: 5'-ACRTCVGCGTCGGTCAT-3'			HQ881203
recA	F: 5'-CGCTBGAYCCBGTCTATG-3'	468	59°C, 54°C	HQ881102-
	R: 5'-CGAAYTCRACCTGCTTG-3'			HQ881134
	F: 5'-ATCGTSTCGCAGCCCG-3'			
	R: 5'-YTTSGCATTYTCRCGBCCCTGVCCG-3'			
rpoB	F: 5'-GCCVAAYGAYCTGATCAACG-3'	1080	63°C, 58°C	HQ881021-
	R: 5'-CTTYTCTTCCGGCGTCATCG-3'			HQ881101

* Fragment lengths are based on the genome of *Sphingopyxis alaskensis* RB2256.

** Using step-down PCR, 10 cycles at the first annealing temperature followed by 20 cycles at the second temperature.

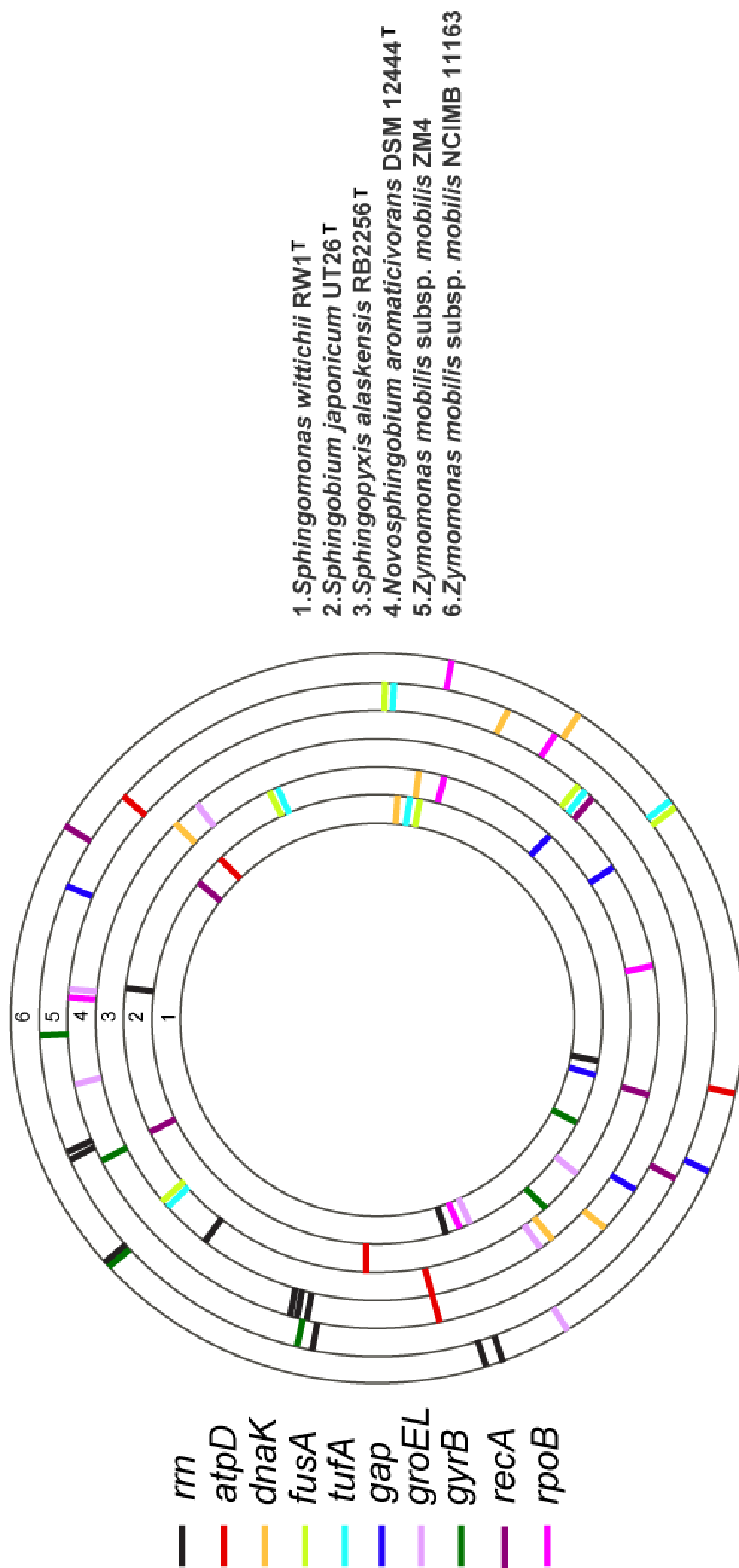


Fig. 1. Relative location of the 9 housekeeping genes in genomes of 6 different *Sphingomonadaceae*.

distributed across the genomes (at a mean distance of 11.1 % of the genome size; Fig. 1).

Specific primers (Table 1) were newly designed for the *Sphingomonadaceae* based on the available genomes listed above. Suitable annealing temperatures were determined using genomic DNA of strains *Sphingomonas abaci* DSM15867^T, *S. aerolata* DSM14746^T, *S. aurantiaca* DSM14748^T, *S. faeni* DSM14747^T, *S. fennica* DSM13665^T, *S. haloaromaticamans* DSM13477^T, *S. panni* DSM15761^T, *S. pseudosanguinis* DSM19512^T, *Sphingopyxis witflariensis* DSM14551^T, *Novosphingobium hassiacum* DSM14552^T, *Sphingomonas kaistensis* DSM16846^T, and *Sandarakinorhabdus limnophila* DSM17366^T to cover the broad phylogenetic diversity of the group. PCR products were purified with the ExoSAP-IT clean-up kit (GE Healthcare). 6 µl of PCR product and 0.5 µl ExoSAP-IT were mixed and incubated in a thermal cycler at 80°C for 15 min and then at 37°C for 45 min to remove the remaining deoxynucleotides and primers. Purified products were directly sequenced in the forward and reverse direction employing suitable sequencing primers (Table 1).

For the determination of environmental *gyrB* sequences, cells from 250 to 500 ml of lake water were collected onto Isopore polycarbonate membrane filters (pore size 0.1 µm pore, diameter 47 mm; Millipore GmbH, Schwalbach, Germany) and DNA was extracted using the protocol of Fuhrman *et al.* (1988) as modified by Marschall *et al.* (2010). DNA concentrations were determined by fluorescent dye binding with PicoGreen (Invitrogen, Karlsruhe, Germany) employing a microtiter plate reader (Tecan Infinite M200, Männedorf, Switzerland). A library of 59 clones was generated from the *gyrB* amplicons and sequenced.

Phylogenetic analysis. Maximum Likelihood, Neighbor Joining and Maximum Parsimony trees were constructed from alignments of the concatenated sequences of housekeeping genes using ARB. The output was used to construct a majority rule consensus tree with the CONSENSE program of Phylip 3.69 (Felsenstein 1993). Split decomposition analysis was conducted based on the concatenated sequences using SplitsTree 4.11.3 (Huson and Bryant, 2006). Nucleotide sequence accession numbers are given in Table 1.

Population genetics

Recombination analyses. Initial evidence for recombination was obtained by the phi test

(Bruen *et al.*, 2006) as implemented in Splitstree (Huson and Bryant, 2006). Maximum Likelihood trees were reconstructed from both the concatenated and the individual gene sequences using PhyML (version 3.0) (Guindon and Gascuel, 2003) with the general time reversible (GTR) model of nucleotide substitution, a BioNJ tree as initial tree, and a discrete gamma model with 4 categories and the gamma shape parameter estimated by PhyML. The approximate likelihood-ratio test (aLRT) was used to estimate the reliability of branching patterns in PhyML (Anisimova and Gascuel, 2006). The resulting trees were compared to identify individual recombination events for each gene. Recombination breakpoints were detected using seven non-parametric methods RDP, GENECONV, Bootscan, Maxchi, Chimaera, SiScan, and 3Seq of the RDP 3.0 software package under default parameters, a highest p value of 0.05, and Bonferroni correction for multiple comparisons (Martin *et al.*, 2010). The order of genes in the concatenated sequence was *atpD* (position 1-882), *dnaK* (886-2118), *fusA* (2122-2667), *tufA* (2671-3372), *gap* (3376-3810), *groEL* (3814-4602), *gyrB* (4606-5418), *recA* (5422-5889), and *rpoB*(5893-6972), with the genes separated from each other by three N. To compare levels of recombination between the different housekeeping genes, canonical correlation were calculated from their distance matrices using the Mantel-test implemented in the R! package ade4 and 1,000 Monte-Carlo permutation (Chessel *et al.*, 2004).

The average number of nucleotide differences per site between two sequences π and the proportion of segregating (polymorphic) sites S among all sites were calculated with DnaSP (Librado and Rozas, 2009). The significance of recombination relative to mutation was assessed using ClonalFrame (Didelot and Falush, 2007) based (i) on the ratio r/m of the probability r for an individual nucleotide to be altered through recombination and the probability m of point mutation, and (ii) on the ratio of the absolute population-scaled number of events (ρ/θ_π). In addition to ρ/θ_π, the parameter r/m takes into account the average number of nucleotide differences per site π and the average tract length of recombining sequences δ (Jolley *et al.*, 2005). 100,000 Monte Carlo Markov Chain steps were performed, discarding the first half as burn-in. Another estimate of the ratio of absolute numbers of recombinations to mutations, ρ/θ_S, was derived from the population-scaled recombination rate ρ and Watterson´s

estimator for the population mutation rate θ_S that were both estimated using the program *Pairwise* of the LDhat package (McVean *et al*., 2002).

Linkage equilibrium was estimated on the level of alleles using LIAN 3.0 with a nonparametric Monte Carlo procedure and 100,000 resamplings (Haubold and Hudson, 2000).

Neutrality tests. π and S were used to determine two different estimators of the population mutation rate, θ_π and θ_S that in turn served to determine Tajima´s D (Tajima, 1989a,b) value with DnaSP. In addition, the McDonald-Kreitman (MK) test (McDonald and Kreitman, 1991) was performed as implemented in DnaSP (Librado and Rozas, 2009).

Effective population size. The effective population size N_e is the number of individuals in an ideal population that exhibits the same evolutionary and population genetic properties as the real population under observation (Whitlock, 2006) and was calculated following Lynch and Conery (2003). The average number of substitutions between silent sites (*H*) was determined for the concatenated set of genes using DnaSP. The estimator η for the prokaryotic population mutation rate $N_e\mu$ was calculated according to

$$\eta = \frac{1.5 \cdot H}{(3 - 4 \cdot H)}$$

and then used to estimate N_e assuming a mutation rate μ of ~ $2.3 \cdot 10^{-10}$ per base and per generation (Drake *et al*., 1998; Lynch and Conery, 2003).

Ecotype simulation. The number of putative ecotypes (PE) was estimated using Ecotype Simulation (ES) (Koeppel *et al*., 2008) that tests combinations of different values for mutation, selection and drift in a coalescence simulation and using complete linkage clustering (Jain *et al*., 1999; Martin, 2002). Ecotype demarcations were performed automatically using the single-gene PhyML trees as input trees.

Phenotypic characterization

Strains were streaked on agar plates containing 1:10 diluted HD and incubated for 2-7 days to yield sufficient cell mass. The Gen III microplates (BiOLOG, Hayward, CA, USA) were inoculated with cells resuspended in the inoculation fluid IF-A according to the recommendations of the manufacturer, incubated in the dark at 28°C and read after 3-6 days

using the Omnilog-PM reader in the Single Read ID mode. After subtraction of the value of negative control, raw data were analysed using the base installation of R! (www.r-project.org). The distribution of the mean values from two repetitions for 94 phenotypic reactions from 48 strains (Fig. 8) suggested a reading of 100 to be an appropriate threshold value to distinguish positive from negative (coded as 1 and 0, respectively) reactions. The resulting matrix was used to generate a UPGMA tree with MEGA5.0 (Tamura *et al*., 2011) and evolutionary distances were computed using the p-distance method. Variances of the readings for all strains and each substrate were plotted as jittered values and as boxplots using ggplot2 (Wickham, 2009).

Physiological and chemotaxonomic characterization of new species

Cultivation and growth media For a systematic phenotypic comparison of the environmental isolates with validly named reference strains of taxonomic value, the type strains of species in the genera of *Sphingomonas* and *Sphingobium*, *Caulobacter leidyi* DSM 4733^T, *Sphingomonas paucimobilis* DSM 1098^T, *Sphingomonas wittichii* DSM 6014^T, *Sphingomonas panni* DSM 15761^T, *Sphingomonas echinoides* DSM 1805^T, *Sphingomonas alaskensis* DSM 13593^T, *Sphingomonas aquatilis* DSM 15581^T, *Sphingobium yanoikuyae* DSM 7462^T, *Sphingobium amiense* DSM 16289^T, *Sphingobium scionense* DSM 19371^T, *Sphingobium vermicomposti* DSM 21299^T, *Sphingobium japonicum* DSM 16413^T were chosen from the DSMZ collection. Strains 247, 301^T, 382^T were isolated from mesotrophic prealpine Lake Starnberger See, strain 469^T was isolated from oligotrophic alpine Walchensee. All strains mentioned above were routinely cultivated on DSM 830 medium (R2A agar) (containing 0.05% w/v yeast extract, 0.05% peptone, 0.05% Casamino acids, 0.05% glucose, 0.05% starch, 0.03% sodium pyruvate, 0.03% K_2HPO_4, 0.005% $MgSO_4$, 1.5% agar; pH=7.2) (Reasoner & Geldreich, 1985) at 28 °C.

Physiological analysis Growth was tested between pH values of 5 to 10 (at intervals of 1 pH). Temperature dependence of growth was assessed between 8 and 45°C (at intervals of 3°C) using temperature gradient incubator (Model TN-3, Sangyo). Cell morphology was determined by phase-contrast microscopy using agar-coated slides (Pfennig & Wagener, 1986)

and transmission electron microscopy. Bacteria were fixed with 2% glutaraldehyde and 5% formaldehyde in cacodylate buffer, treated with 1% aqueous osmium and then dehydrated with a graded series of acetone on formvar-coated grids. For contrasting, 2% uranyl acetate in 70% acetone was used. Samples were examined in a TEM910 transmission electron microscope (Carl Zeiss, Oberkochen) at an acceleration voltage of 80 kV. Cell motility was evaluated using light microscopy and soft agar (0.1% w/v yeast extract, 0.01% K_2HPO_4, 0.2% Agar).

The Gram-type was determined using the Bactident Amino peptidase kit and confirmed by the KOH test. Catalase activity was determined by using 10% (v/v) H_2O_2 and oxidase activity using N, N, N′, N′-tetramethyl-ρ-phenylenediamine. Physiological and biochemical characteristics, and enzyme activities were determined by employing API 20NE, API ZYM, API 50CH test strips (bioMérieux) and Biolog Gen III microplates (BiOLOG, Hayward, CA, USA) according to the instructions of the manufacturer. Susceptibility to antibiotics was determined on R2A agar plates using the discs diffusion method (NCCLS, 2007) (in µg per disc): penicillin G (10), oxacillin (5), ampicillin (10), ticarcillin (75), cefalotin (30), mezlocillin (30), cefazolin (30), cefotaxim (30), aztreonam (30), chloramphenicol (30), tetracyclin (30), imipenem (10), gentamycin (10), amikacin (30), vancomycin (30), erythromycin (15), lincomycin (15), ofloxacin (5), colistin (10), norfloxacin (10), pipemidic (20), bacitracin (10), polymyxin B (300), nitrofurantoin (100), neomycin (30), kanamycin (30), doxycycin (30), clindamycin (10), ceftriaxone (30), fosfomycin (50), nystatin (100), linezolid (10), moxifloxacin (5), quinupristin/dalfopristin (15), piperacillin/tazobactam (40), and teicoplanin (30).

Biomarker analysis Fatty acids, respiratory quinones, polar lipids, and polyamines were extracted from cells grown on R2A medium. For fatty acid analysis, 40 mg wet weight of fresh cells were harvested from the agar plates, and extracted according to the standard protocol of the Microbial Identification system (Sasser, 1990). Respiratory quinones were extracted from 200 mg freeze-dried cell material and analysed according to the method described by Tindall and Collins (Tindall 1990a, b; Tindall, 2005; Collins, 1994). Respiratory quinones were first separated by their structural classes (such as menaquinones, ubiquinones) using thin later chromatography (TLC). The resulting bands were eluted and further separated

and identified by HPLC, using a RP18 column (Tindall, 1996). Polar lipids were extracted from 100 mg freeze-dried cell material, separated by two-dimensional chromatography and identified by their R_F values in combination with their reaction with specific staining reagents (Tindall 1990a, b). Polyamines were extracted from 40 mg of freeze-dried cells and analyzed using the method of Busse & Auling (1988). The detection was carried out on a Hypersil ODS RP 18 column (25 by 4.6 cm, 5 µ particles) using a Beckman gradient liquid chromatograph (model 332) equipped with a Waters model 420 EC fluorescence detector.

Genetic and phylogenetic analysis Genomic DNA extraction and 16S rRNA gene sequencing were performed as described above. The primer used here were 8F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3') (Lane, 1991). Additional 16S rRNA gene sequences of the type strains of all species of *Caulobacter*, of all type species of relevant genera of the *Sphingomonas, Sphingobium*, and of the type strains of all other dimorphic prosthecate alphaproteobacterial species (in the genera *Brevundimonas*, *Phenylobacterium*, *Asticcacaulis*, *Maricaulis*) were retrieved from the GenBank database (Altschul *et al.*, 1997) and imported into the ARB program package (Ludwig *et al.*, 2004). Automated alignments of the Fast Aligner tool were corrected manually according to secondary-structure information to yield an alignment of approx. 1500 bp. Phylogenetic trees were constructed with the FastdnaML maximum likelihood algorithm as implemented in the ARB software package. Sequence accession numbers are provided in Fig. 14,16. The mol% G+C content of DNA was determined as described by Mesbah *et al.* (1989). For DNA-DNA hybridization, cells were disrupted by using a French pressure cell (Thermo Spectronic) and the DNA in the crude lysate was purified by chromatography on hydroxyapatite as described by Cashion *et al*. (1977). DNA-DNA hybridization (DDH) was carried out as described by De Ley *et al*. (1970) under consideration of the modifications described by Huss *et al*. (1983) using a model Cary 100 Bio UV/VIS-spectrophotometer equipped with a Peltier-thermostatted 6*6 multicell changer and a temperature controller with in-situ temperature probe (Varian).

Chapter 4

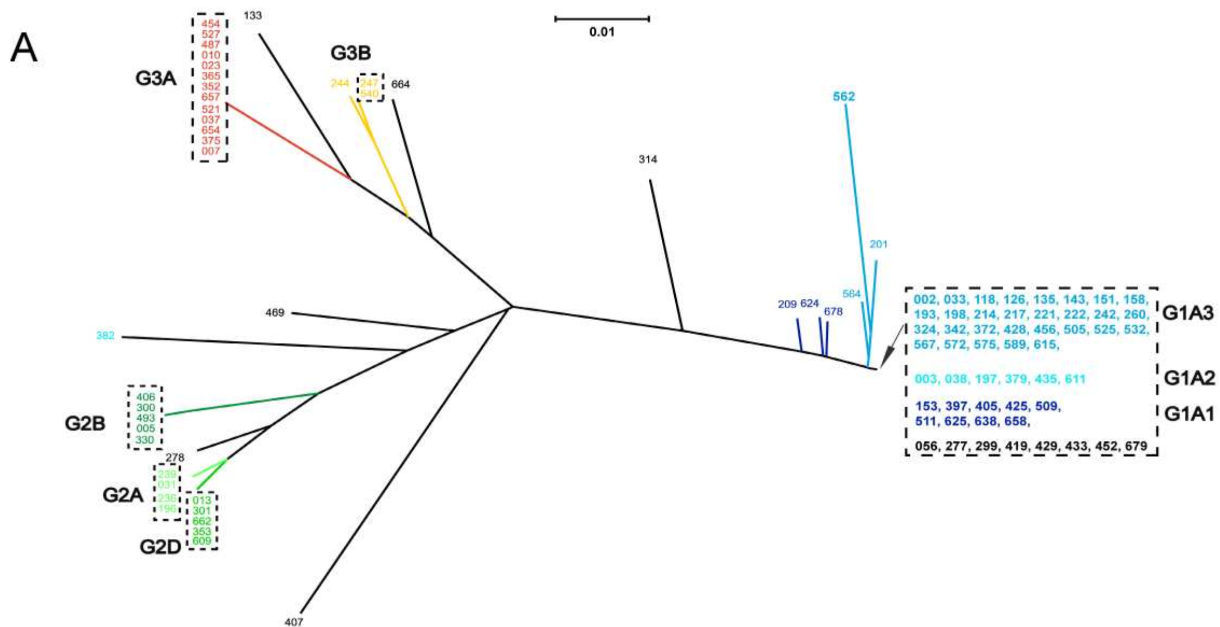
Results

Population structure of freshwater Sphingomonadaceae in the two lakes

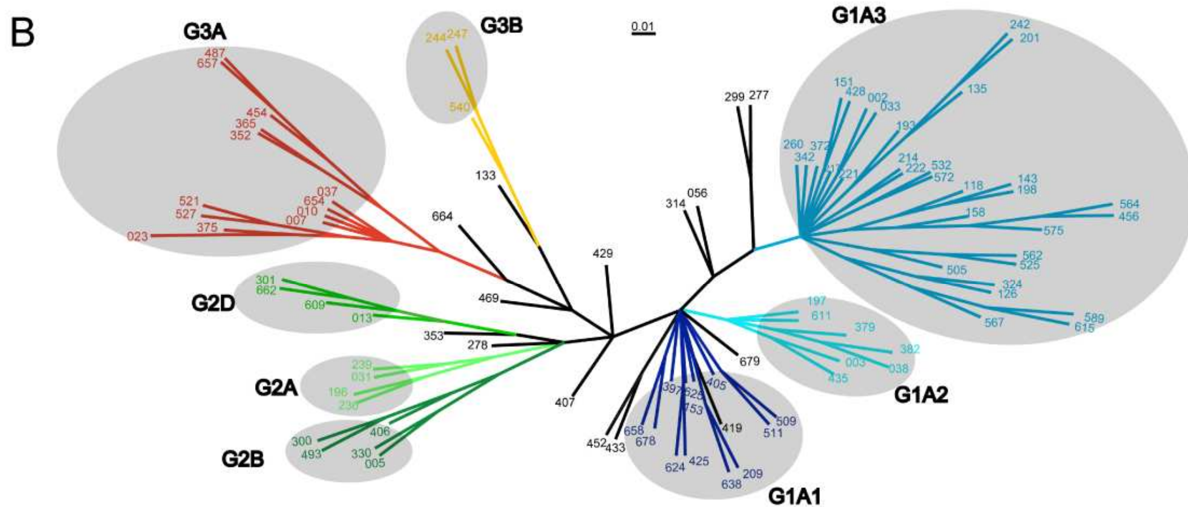
Of the 569 positive primary liquid cultures generated from Walchensee and Starnberger See samples, 46 and 36%, respectively, were identified as *Sphingomonadaceae* using the specific PCR screening protocol. From the *Sphingomonadaceae* enrichments, a total of 95 strains were subsequently isolated in pure culture. Phylogenetic analysis revealed that 6 distinct phylotypes (G1A, G2A, G2B, G2D, G3A, G3B) had been isolated multiple times (Fig. 2A, dashed boxes). For each phylotype, multiple isolates were available. One phylotype (designated G1A) strongly dominated the culture collection and was represented by 52 isolates. G1A also be the dominant phylotype in the nature population by *in situ* analysis.

To assess the DNA polymorphisms, the population structure of *Sphingomonadaceae* was elucidated based on the sequences of nine housekeeping genes. Compared to most previous studies that typically employed ≤ 7 housekeeping genes (compare the PubMLST website at http://pubmlst.org/ listing 74 MLST projects), we increased the number of loci in order to improve the robustness of our analysis. Each of the 95 strains was found to represent a unique haplotype, i.e., a unique combination of allelic states of genetic markers (Fig. 2B). Similarly, all isolates were unique on the level of the deduced amino acid sequences. We determined high levels of nucleotide diversity π of >0.1 and high proportions of polymorphic sequence sites of up to 79% for the individual housekeeping genes (Table 2). Watterson´s estimator for the population mutation rate θ_S ranged from 0.073 to 0.145 bp^{-1}generation^{-1}. Of the genes analyzed, *gyrB* exhibited the by far highest sequence diversity whereas comparatively low nucleotide diversity was detected for *dnaK* and *groEL* within the G1A cluster (Table 2). The pairwise divergence values of concatenated sequences of the 52 identical phylotypes within the G1A cluster were 0.4 to 19.6 %. The genetic diversity values detected for G1A strain which sharing the same 16S gene sequences were range from 0.016 (*groEL*) to 0.252 (*gyrB*). They are unusually high values comparing to other studies.

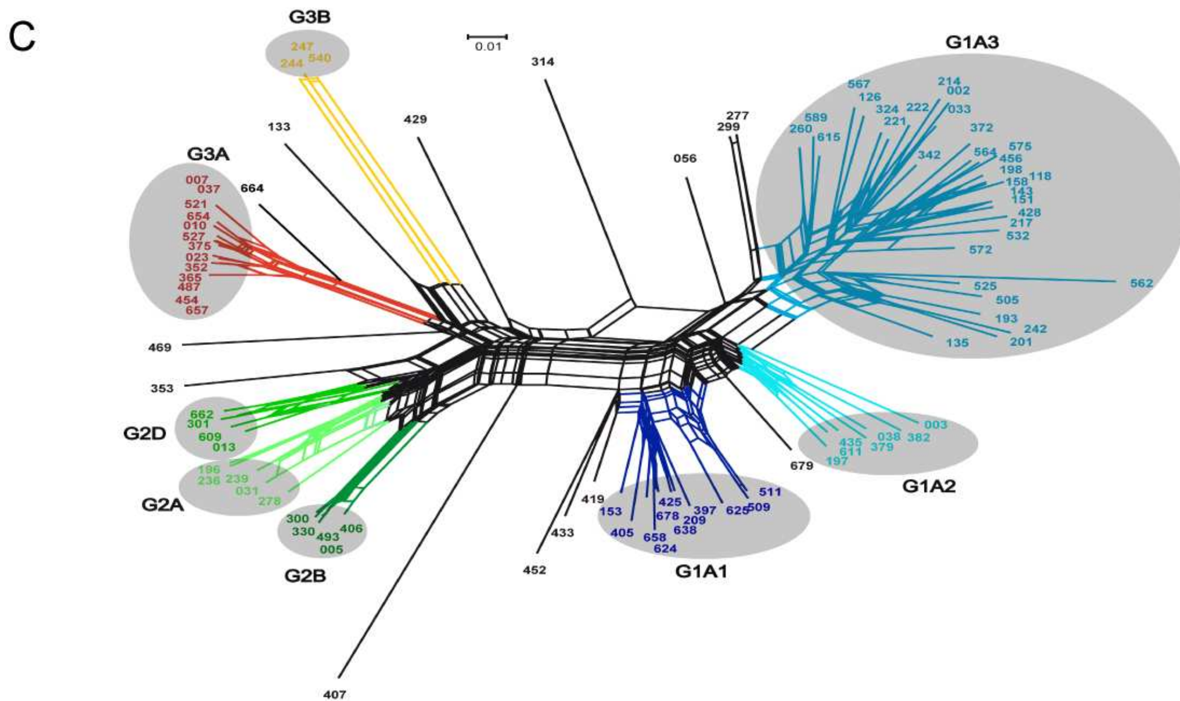
The majority rule consensus tree for the concatenated housekeeping genes followed the overall phylogenetic structure of the 16S rRNA gene tree, confirming the presence of



A
0.01
G3A
454
527
487
010
023
365
352
657
521
037
654
375
007
133
G3B
244
247
540
664
562
314
201
564
209 624
678
469
382
002, 033, 118, 126, 135, 143, 151, 158,
193, 198, 214, 217, 221, 222, 242, 260,
324, 342, 372, 428, 456, 505, 525, 532,
567, 572, 575, 589, 615,
G1A3
003, 038, 197, 379, 435, 611
G1A2
153, 397, 405, 425, 509,
511, 625, 638, 658,
G1A1
056, 277, 299, 419, 429, 433, 452, 679
G2B
406
300
493
005
330
278
G2A
239
031
236
196
013
301
662
353
609
G2D
407



B
G3A
G3B
0.01
G1A3
G2D
G2A
G2B
G1A1
G1A2



C
G3B
0.01
G1A3
G3A
G2D
G2A
G2B
G1A1
G1A2

Fig. 2. Relatedness of 95 strains of *Sphingomonadaceae* from Walchensee and Starnberger See based on 16S rRNA gene sequences and concatenated sequences of nine housekeeping genes **A.** Unrooted Maximum Likelihood reconstruction of the 16S rRNA gene sequence phylogeny. Strains with identical 16S rRNA gene sequences are placed within dashed boxes. **B.** Majority rule consensus tree calculated from Maximum Likelihood, Maximum Parsimony and Neighbor Joining phylogenetic trees of the concatenated sequences of 9 housekeeping genes. **C.** Phylogenetic network generated by SplitsTree based on the concatenated set of nine housekeeping genes. Strains within the same grey area are inferred to be panmictic (see text). Bars denote 0.01 fixed point mutations per nucleotide. Different colours code for different subclusters according to the SplitsTree analysis (see C.).

phylogenetically distinct groups (Fig. 2B). There are numerous polytomies present in the consensus tree, and the initial phi test for recombination which implemented in Splitstree revealed statistically significant evidence for recombination for the entire dataset. A phylogenetic network provides a better representation of the relationships between the 95 different *Sphingomonadaceae* haplotypes (Fig. 2C). The split decomposition analysis revealed the presence of eight distinct subclusters among the 95 strains. Most notably, three of the subclusters fell into the same 16S rRNA phylotype G1A.

To address the potential influence of a selection bias during cultivation on the population structure of *Sphingomonadaceae*, we tested for the presence and phylogenetic affiliation of additional sequence types of housekeeping genes in the two lakes. The *gyrB* gene was chosen for this analysis, as it displayed the by far largest sequence diversity π among all 9 housekeeping genes (Table 2) which renders this gene most suitable to detect additional diversity. 59 unambiguous cloned sequences, representing 32 different sequence types were obtained of which six (marked by dashed boxes in Fig. 3) corresponded to types already present in the culture collection. Analysis of the phylogenetic position of the cloned sequences was conducted using SplitsTree for a better comparability with the results of the MLSA. Whereas 22 of the environmental sequences formed two additional and previously undetected sequence clusters within the radiation of *gyrB*, the remaining environmental sequences clustered with sequences of the cultivated strains (Fig. 3). There was no distinct population substructure of the freshwater *Sphingomonadaceae* in Walchensee and Starnberger See.

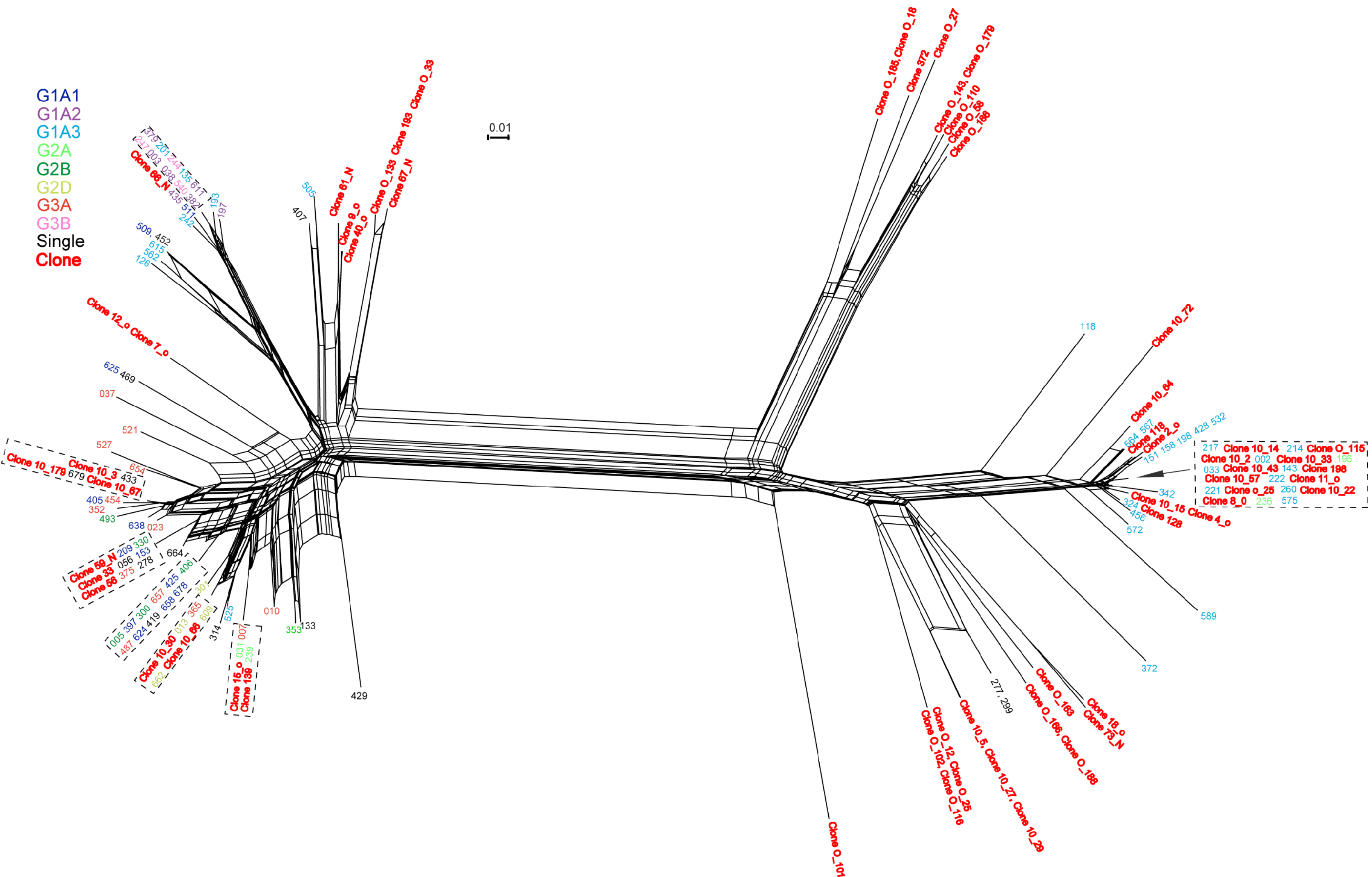
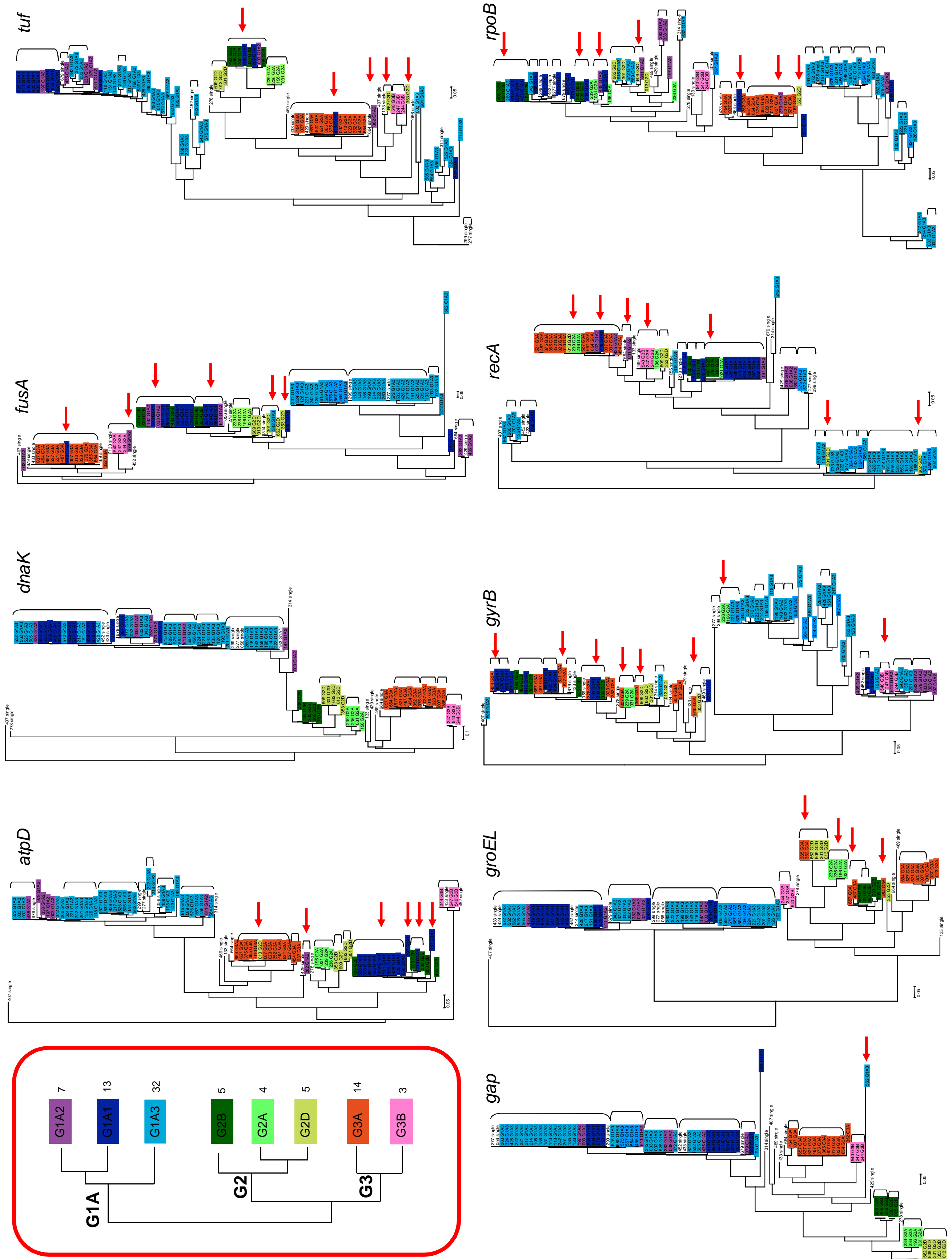


Fig. 3. Phylogenetic network generated by SplitsTree based on a sequence comparison of the *gyrB* gene sequences of *Sphingomonadaceae* isolates and environmental clones from Walchensee and Starnberger See. Isolates are color-coded based on MLSA subclusters (compare Fig. 2C). Environmental clones are depicted in bold red. Dashed boxes denote the six sequence types found among the cultured *Sphingomonadaceae* as well as the clones. Bar denotes 0.01 fixed point mutations per nucleotide.



tuf
rpoB
fusA
recA
dnaK
gyrB
atpD
groEL
gap
G1A2
7
G1A1
13
G1A3
32
G2B
5
G2A
4
G2D
5
G3A
14
G3B
3
G1A
G2
G3

Fig. 4. Comparison of single-gene phylogenies of the nine housekeeping genes. The number of strains within each MLSA subgroup (compare Fig. 2C) and its color-code are given in the upper left. The upper left tree surrounded with red line is the condensed consensus tree of the nine housekeeping genes. For the other nine trees, every single tree corresponds to a housekeeping gene. The name of the gene is marked in the upper right of every tree. Red errors denote recombination events that were identified by comparison of the branching patterns. Bars denote 0.01 fixed point mutations per nucleotide.

Analysis of the evolutionary forces underlying the population structure

A comparative analysis of the single-gene phylogenies of the nine housekeeping genes revealed candidates for numerous recombination events across the groups G1A, G2, and G3 (Fig. 4). 150 recombination breakpoints were detected using seven non-parametric recombination detection methods as implemented in the RDP package. All recombination breakpoints were detected with at least two methods, 86% with at least three methods, 73% of the breakpoints were detected with four methods, and approximately one quarter of the breakpoints (24.7%) was detected with all seven methods. This number of recombination breakpoints is somewhat higher than that reported for a comparable number of *Vibrio cholerae* strains and for 7 genes (Keymer and Boehm, 2011). We then analyzed the congruence of distance matrices of the single genes by applying a Mantel test (Chessel *et al.*, 2004). For most genes, a significant positive correlation of the distance matrices could be shown (Fig. 5). Of the nine housekeeping genes, *dnaK*, *groEL* and *gap* displayed the highest correlation among each other and with the concatenated tree. In contrast, a clear lack of congruence was observed between *rpoB* and *gyrB* as compared to *dnaK*, *groEL,* and *gap*. Notably, *gyrB* of *Pseudomonas syringae* has also been documented to yield phylogenetic trees that are incongruent to those of other housekeeping genes (Sakar and Guttman, 2004). Based on this initial evidence, the impact of recombination in shaping the diversity of freshwater *Sphingomonadaceae* was assessed further.

The population recombination rates ρ calculated for the individual housekeeping genes ranged between 0.005 and 0.033 $bp^{-1}generation^{-1}$ for the entire set of strains and between 0 and 0.031 bp^{-1} $generation^{-1}$ for the G1A cluster (Table 2). These rates are comparable with the gene-specific values of other species and indicate that the presence of extraordinarily effective recombination barriers between the different *Sphingomonadaceae* genomes is rather unlikely. In contrast to ρ, Watterson´s estimator for the population mutation rate θ_S (Table 2) surpassed the values reported in the literature (see above) and resulted in a ratio of ρ/θ_S that is lower than in many other bacterial species. Similarly, the second estimate for the ratio of recombination to mutation, ρ/θ_π, was low.

Our results indicate that the mutation rate for all housekeeping genes investigated was between 2 and 8-fold larger than the per locus recombination rate. Taking into account the average tract length of recombination events (δ) yields the ratio of probabilities that an individual nucleotide will be altered through recombination and point mutation, *r*/*m* (Jolley *et al.*, 2005). Our corresponding values for individual housekeeping genes reached 7.88 for all strains and 4.42 for G1A (Table 2). Calculations based on the concatenated sequences yielded *r*/*m* values of 5.83 (confidence interval, 5.30-6.38) for all 95 strains of freshwater *Sphingomonadaceae*, 4.04 (C.I., 2.81-5.61) for G1A but only 0.06 to 1.73 for the remaining seven subpopulations. These results place our *Sphingomonadaceae* isolates well in the middle in a ranking list of homologous recombination rates of approximately 48 species, and the relative impact of recombination on population structure of *Sphingomonadaceae* is markedly lower than most of other free-living aquatic bacteria investigated (Vos and Didelot, 2009).

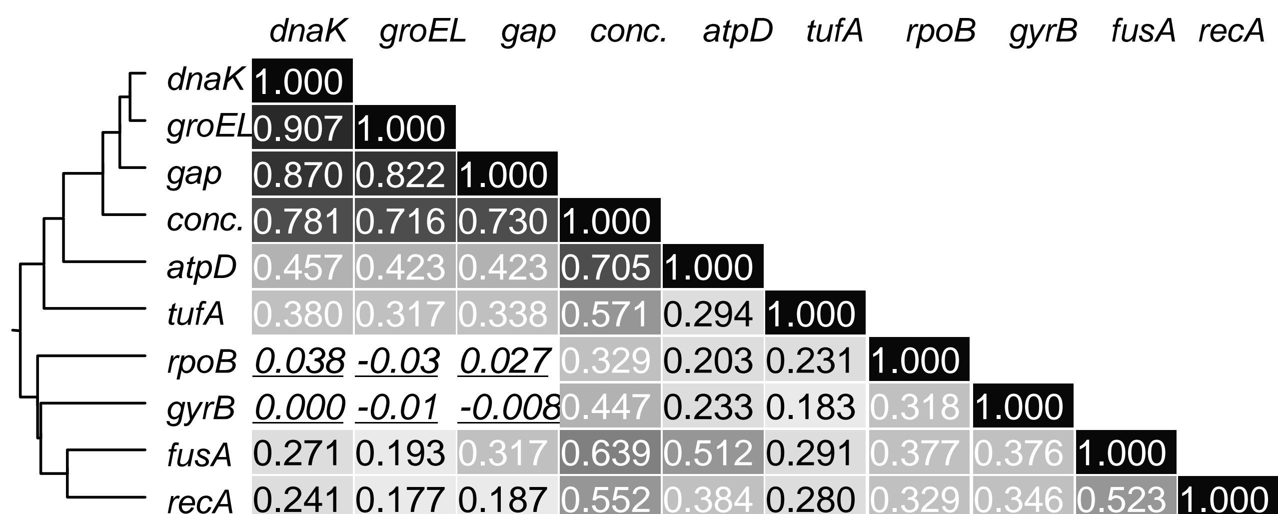


Fig. 5. Congruence of distance matrices for 9 housekeeping genes of 95 *Sphingomonadaceae* strains from Walchensee and Starnberger See. Canonical correlation values as determined with the Mantel-test are given. Values in italics indicate a lack of correlation ($p > 0.17$). All other correlation values were highly significant ($p < 0.001$). The grey shades indicate varying levels of correlation. The canonical correlation values were used as input for the cluster analysis by UPGMA depicted on the left.

Based on simulation studies, the ρ/θ_S values of all strains range from 0.064 to 0.283, for G1A strains range from 0 to 0.261, only the value from gene *tuf* was above 0.25. Though our estimates, the θ_S (Table 2) was larger than those used in these simulations.

Table 2. Population genetic parameters for 95 isolates of *Sphingomonadaceae* isolated from Walchensee and Starnberger See.

		π [1]		S [%][2]		ρ[3] [per bp/kb]		θ_S[4]		ρ/θ_S[5]		$\rho/\theta\pi$[6]		δ[bp][7]		r/m[8]		D[9]	
Gene	length [bp]	all	G1A	all	G1A	all	G1A	all	G1A	all	G1A	all	G1A	all	G1A	all	G1A	all	G1A
atpD	**882**	0.161	0.122	54.8	48.4	0.007	0.005	0.1068	0.1071	0.064	0.042	0.452	0.365	43	110	2.85	4.42	0.019	-0.418
dnaK	**1233**	0.122	0.023	43.9	14.8	0.005	0.000	0.0865	0.0515	0.056	0.000	0.311	0.098	157	321	3.64	2.32	-0.057	-1.156
fusA	**546**	0.165	0.151	49.9	48.2	0.009	0.005	0.0983	0.1049	0.093	0.052	0.521	0.489	50	70	3.53	4.2	0.395	0.129
tufA	**702**	0.181	0.161	59.2	55.3	0.033	0.031	0.1156	0.1202	0.283	0.261	0.609	0.452	22	25	2.14	1.80	-0.071	-0.428
gap	**435**	0.169	0.044	70.4	54.9	0.016	0.002	0.1368	0.1252	0.118	0.018	0.188	0.0016	12	69.708	0.84	0.15	-0.695	-2.433**
groEL	**789**	0.110	0.016	37.3	5.6	0.005	0.003	0.0727	0.0120	0.070	0.211	0.749	0.988	219	1.322	7.88	3.55	0.370	1.015
gyrB	**813**	0.236	0.252	79.0	76.6	0.017	0.015	0.1445	0.1546	0.119	0.095	0.381	0.313	29	35	2.65	2.92	-0.397	-0.288
recA	**468**	0.153	0.144	59.4	50.2	0.015	0.019	0.1159	0.1085	0.129	0.177	0.265	0.755	11	46	1.07	4.13	-0.304	-0.158
rpoB	**1080**	0.151	0.162	57.6	55.5	0.024	0.017	0.1116	0.1196	0.216	0.142	0.667	0.552	48	51	3.79	3.42	-0.450	-0.328

all = all 95 strains

[1] average number of nucleotide differences per site between two sequences, determined using DnaSP.

[2] the proportion of segregating (polymorphic) sites among all sites, determined using DnaSP.

[3] the population recombination rate values were obtained by dividing the recombination rate per region estimate (LDhat, not shown) by the length [bp] of the gene.

[4] Watterson´s estimator for the population mutation rate θ_S as based on the number of segregating sites, determined using LDhat.

[5] the ratio of rates at which recombination occurs relative to point mutation, determined from the LDhat values of ρ and θ_S.

[6] the ratio of rates at which recombination occurs relative to point mutation, as determined using ClonalFrame. Note that ClonalFrame applies θ_π instead of θ_S (LDhat), and that ClonalFrame determines ρ also differently than in LDhat.

[7] the mean estimated average tract length of recombining sequences, as determined using ClonalFrame.

[8] the ratio of probabilities that a nucleotide is changed as the result of recombination relative to point mutation, as determined by ClonalFrame. The determination of r/m takes into account the average number of nucleotide differences (π), the average tract length of recombining sequences (δ), and ρ/θ_π (Jolley *et al.*, 2005).

[9] Tajima´s D value, determined using DnaSP. All values were non-significant, except **, where $p < 0.01$.

To further assess whether the recombination rate is sufficient to maintain a panmictic population structure of the *Sphingomanadaceae*, we finally tested for the linkage equilibrium of the 9 housekeeping genes among and between all strains and its subpopulations. Low values for the standardized index of association I_A^S between 0 and 0.117 were found to be not significant ($p \geq$ 0.075) for alleles within the eight individual subpopulations G1A1, G1A2, G1A3, G2A, G2B, G2D, G3A, and G3B (colored rectangles in Fig. 6). Hence, the null hypothesis of linkage equilibrium within the eight subpopulations was not rejected. In contrast, the I_A^S for pairwise combinations of the eight subpopulations and for the three subpopulations of G1A or G2 were significant ($p \leq$ 0.0041) and reached values of I_A^S of up to 0.380. Consequently, the null hypothesis of free combination between the eight different subpopulations has to be rejected. Taken together, our multipronged recombination analysis indicated incipient sexual isolation (denoted as distinct grey area in Fig. 2C).

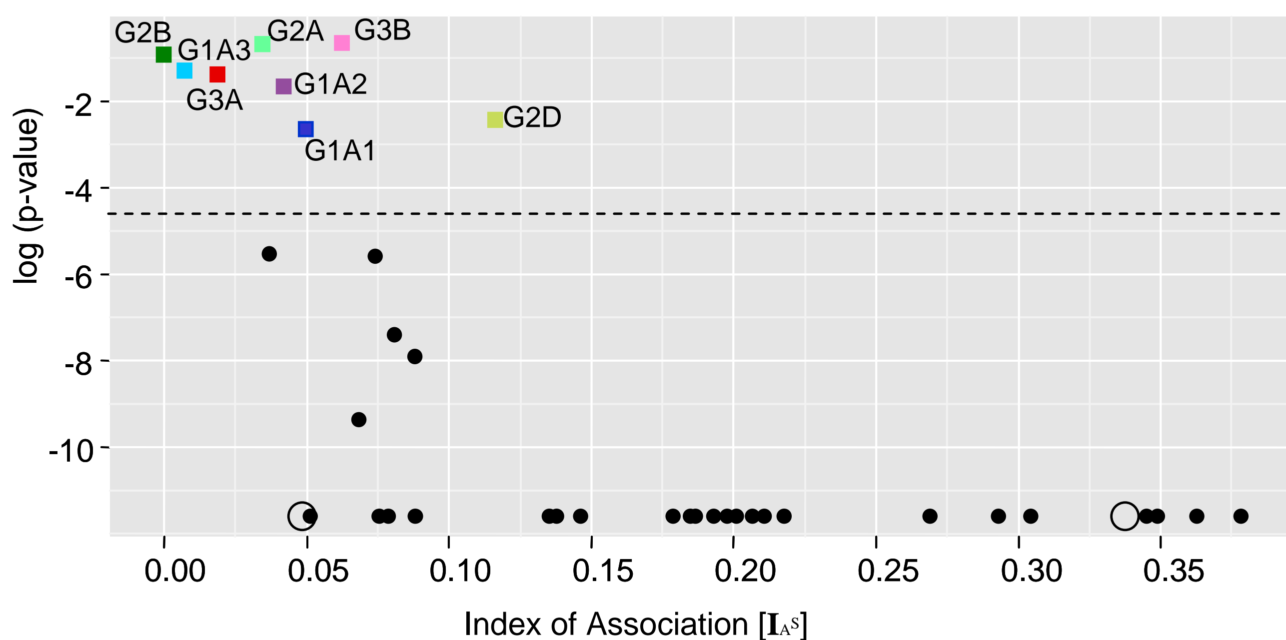


Fig. 6. Analysis of linkage disequilibrium within and between the 8 individual subpopulations G1A1, G1A2, G1A3, G2A, G2B, G2D, G3A, and G3B based on the alleles of 9 housekeeping genes. The standardized index of association (I_A^S) and its significance level as determined with LIAN 3.0 are given. Results for analysis within individual subpopulations are color-coded as in Fig. 2C. Results for pairwise combinations of subpopulations are shown as black dots, and those for multiple combinations (i.e., all subpopulations of G1A or G2) are shown as open circles. The dotted line denotes a significance level of $p = 0.01$.

Table 3. Summary of the results of the McDonald-Kreitman (MK) test

	G1A1	G1A2	G1A3	G2A	G2B	G2D	G3A
G1A2	0						
G1A3	0	0					
G2A	*dnaK* (0.012) *groEL* (0.013)	*dnaK* (0.037) *gap* (0.021) *groEL* (0.0072)	*dnaK* (0.00014) *tuf* (0.0088) *groEL* (0.00013)				
G2B	*groEL* (0.034)	*Gap* (0.005) *groEL* (0.015) *gyrB* (0.017)	*dnaK* (0.0078) *tuf* (0.024) *groEL* (0.0023)	0			
G2D	*dnaK* (0.027)	*gyrB* (0.0098)	*dnaK* (0.0017)	0	0		
G3A	*dnaK* (0.0011) *tuf* (0.0008)	*dnaK* (0.0018) *tuf* (0.0045) *gyrB* (0.00038)	*rpoB* (0.041)	*atpD* (0.00022) *dnaK* (0.0013) *gap* (0.0013)	*atpD* (0.0076) *dnaK* (0.017) *tuf* (0.0041) *gap* (0.00024)	*dnaK* (0.011) *gap* (0.011)	
G3B	*atpD* (0.0042) *dnaK* (0.014) *tuf* (0.0008)	*atpD* (<0.0000) *dnaK* (0.014) *tuf* (0.0016) *gap* (0.018)	*dnaK* (0.0001) *tuf* (0.00017) *groEL* (0.015) *rpoB* (0.029)	0	*atpD* (0.023) *tuf* (0.026) *rpoB* (0.047)	*atpD* (<0.0000) *fusA* (0.041)	0

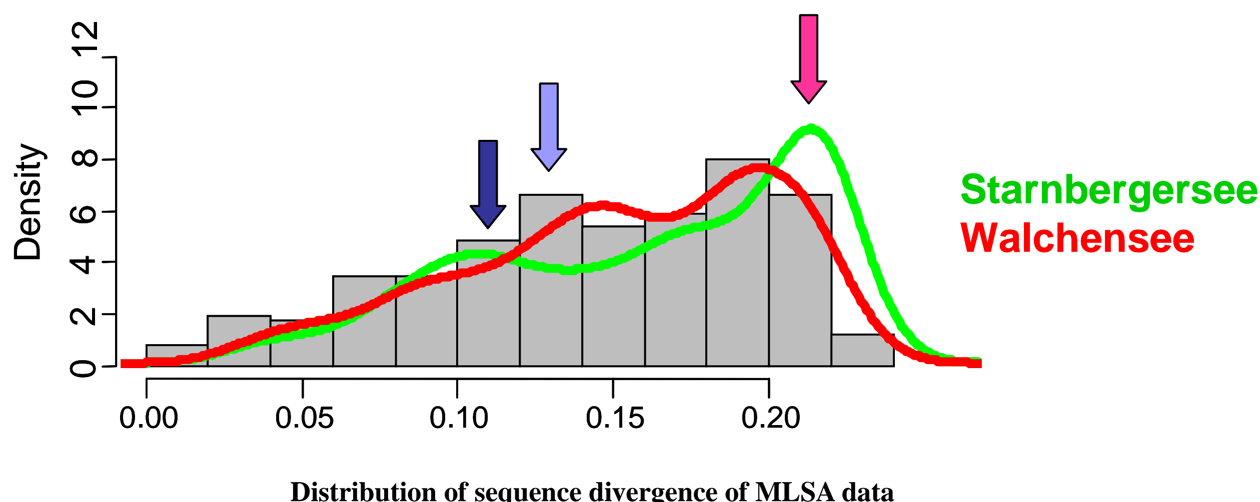
* Only genes are listed for which the Neutrality Index (NI) in the MK test was significantly smaller than 1 ($p < 0.05$, G-test as implemented in DnaSP).

The values in brackets indicate the *p*-values (G-test). A zero indicates that none of the genes showed a NI value significantly smaller than 1.

In order to test for selective neutrality of the investigated genes, we used the Tajima´s D and MacDonald-Kreitman (MK) tests. None of the genes in the entire population and in G1A yielded a significant deviation from 0 for Tajima´s D (Table 3). The same holds true for the subgroups G2 and G3. The only exception was the *gap* gene that exhibited an extremely low population recombination rate and unrealistic long average tract length in G1A (Table 2). For the MK test of the observed eight individual subpopulations and big clusters (Table 3), the neutrality indices between the subgroups within each of the larger MLSA clusters G1A, G2, and G3, none of the genes showed a NI value significantly smaller than 1. But there are deviations of the neutrality indices between subpopulations which from different larger clusters G1A, G2 and G3. For every comparison, not all genes showed significant deviations, the genes which shown deviations were displayed in Table 3.

In order to address the potential power of natural selection versus genetic drift, we determined the effective population size N_e, following Lynch and Conery (2003). The effective population sizes N_e for G1A, G1A1, G1A2, G1A3, as determined from the concatenated set of genes, were 9.3, 3.6, 6.6, and 5.6 ($\times 10^8$), respectively. The N_e value detected by the freshwater *Sphingomonadaceae* is in the range of the largest values comparing to the other studies. Additionally, the Tajima´s D values were generally close to zero.

As a final aspect of the population genetics of the *Sphingomonadaceae*, the potential effect of geographic isolation by the two different lakes (Walchensee and Starnberger See) was investigated. A visual inspection of the distribution of isolates across the phylogenetic trees depicted in Fig. 4 according to their geographical origin revealed no obvious clade specific for either lake, suggesting a rather low, if at all, influence of geographic separation on the *Sphingomonadaceae* populations. It was supported by an analysis of the distribution of sequence divergence values (percent sequence dissimilarity) across all pairwise comparisons of sequences per lake (Fig. 7). Generally, the distribution of sequence divergence values was very similar in both lakes, with the majority of sequence divergence values at approximately 15-22% sequence diversity across all isolates per lake. Only at three data fractions (columns) of the histogram a significant difference between both lakes was found (Fig. 7). These findings may be supportive of the above finding of a rather low, if at all, influence of the geographic separation. However, it should be noted that the comparison of distribution of sequence divergence values is just an indirect test for geographical isolation.



Distribution of sequence divergence of MLSA data

Fig. 7. The distribution and density of the sequence divergence values from all pairwise comparisons of the concatenated sequences of nine housekeeping genes. The histogram depicts the distribution of sequence divergence values across all pairwise comparisons of sequences from Starnbergersee. The green and red lines represent the density distribution of the sequence divergence values from Starnbergersee and Walchensee, respectively. The data were obtained from the cumulated sequence divergence values by using the *hist*() and *density()* functions from the base installation of the R! software. The lightblue (p = 3.04e-05), blue (p = 0.045), and pinkish (p = 7.42e-09) arrows indicate those fractions of sequence divergence values where Starnbergersee and Walchensee differ significantly (using the *binom.test()* function in the *stats* package in R! This test performs an exact test of a simple null hypothesis about the hypothesized probability of success [here: 50%] in a Bernoulli experiment).

Metabolic diversification and its role in niche invasion

To obtain a first insight into the presence of ecologically distinct subpopulations within the freshwater *Sphingomonadaceae*, we analyzed the dataset using Ecotype Simulation (ES). This method aims to estimate the number of ecologically distinct populations within a given clade based on a simulation of evolutionary dynamics (Koeppel *et al*., 2008). Because of the considerable recombination deterected among the *Sphingomonadaceae*, ES was performed separately for the all housekeeping genes. For every gene, the number of putative ecotype of all strains was always bigger than number of the subpopulations identified by the phylogentic analysis (Fig. 2C). Within G1A, the following numbers of putative ecotypes were hypothesized: 18 (*atpD*), 11 (*dnaK*), 14 (*fusA*), 38 (*tufA*), 8 (*gap*), 6 (*groEL*), 37 (*gyrB*), 22 (*recA*), and 33 (*rpoB*). The subgroups recognized by phylogenetic analysis within G1A cluster were G1A1, G1A2 and G1A3, obviously the number of putative ecotypes within G1A hypothesized by simulation were much larger than 3.

The *Sphingomondaceae* isolates displayed different colony colors, different temperature preferences (between 15°C to 28°C) and also different growth rates on agar plates. In order to assess phenotypic differences at different levels of population substructure, we tested for the utilization of 71 sole carbon sources and the effect of 23 inhibitory substances. The distribution of frequency of the raw value of the BiOLOG phenotypic analysis showed two peaks at approximately 0 and 200 with a minimum at approximately 100 (Fig. 8). Therefore, all values below were regarded as a negative reaction (i.e., no cellular respiration) whereas values above 100 were regarded as a positive physiological reaction resulting in cellular respiration.

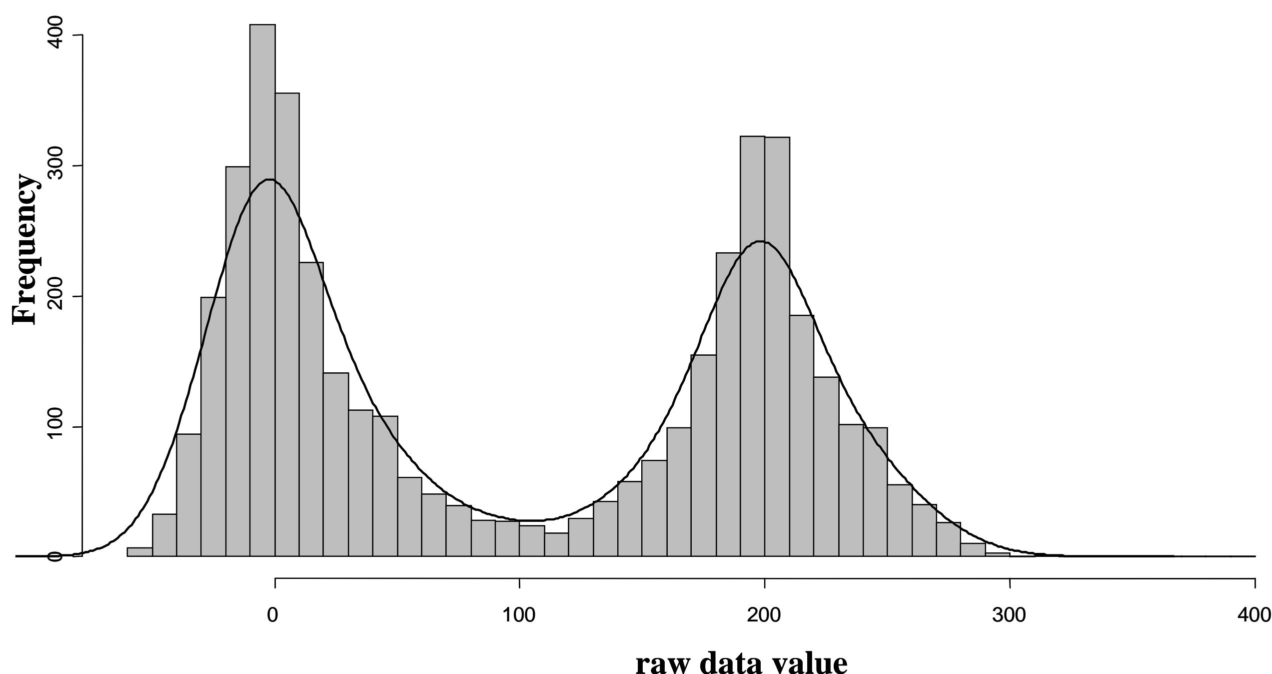


Fig. 8. Histogram and density curve of the BiOLOG raw data for 94 phenotypic tests on 48 strains using the *hist ()* and *density()* functions in the R! base installation. The maximum intensity value of the color reading that can be attained is 400. Negative processed BiOLOG values may result where the negative control raw value exceeds the raw value obtained for individual substrates.

G1A strains did not utilize several di- or polysaccharic sugars, but their monomeric consitutents (Fig. 9). This may suggest a lack or inactivity of glycosidic enzymes or of respective disaccharide transporters. A further major difference to G2 and G3 is the ability of G1A strains to utilize several additional monomeric sugars (mannose, fructose), sugar alcohols and sugar acids. G2 and G3 strains were found to be resistant to a diverse set of inhibitory substances whereas G1A strains are mainly sensitive. The G1A strains are sensitive to the inhibitory substance rifamycin SV, but G2 and G3 strain are resistant to it (Fig. 9; 10). For another inhibitory substance, vancomycin, the resistance pattern distribution is opposite to the results with rifamycin SV. When combining

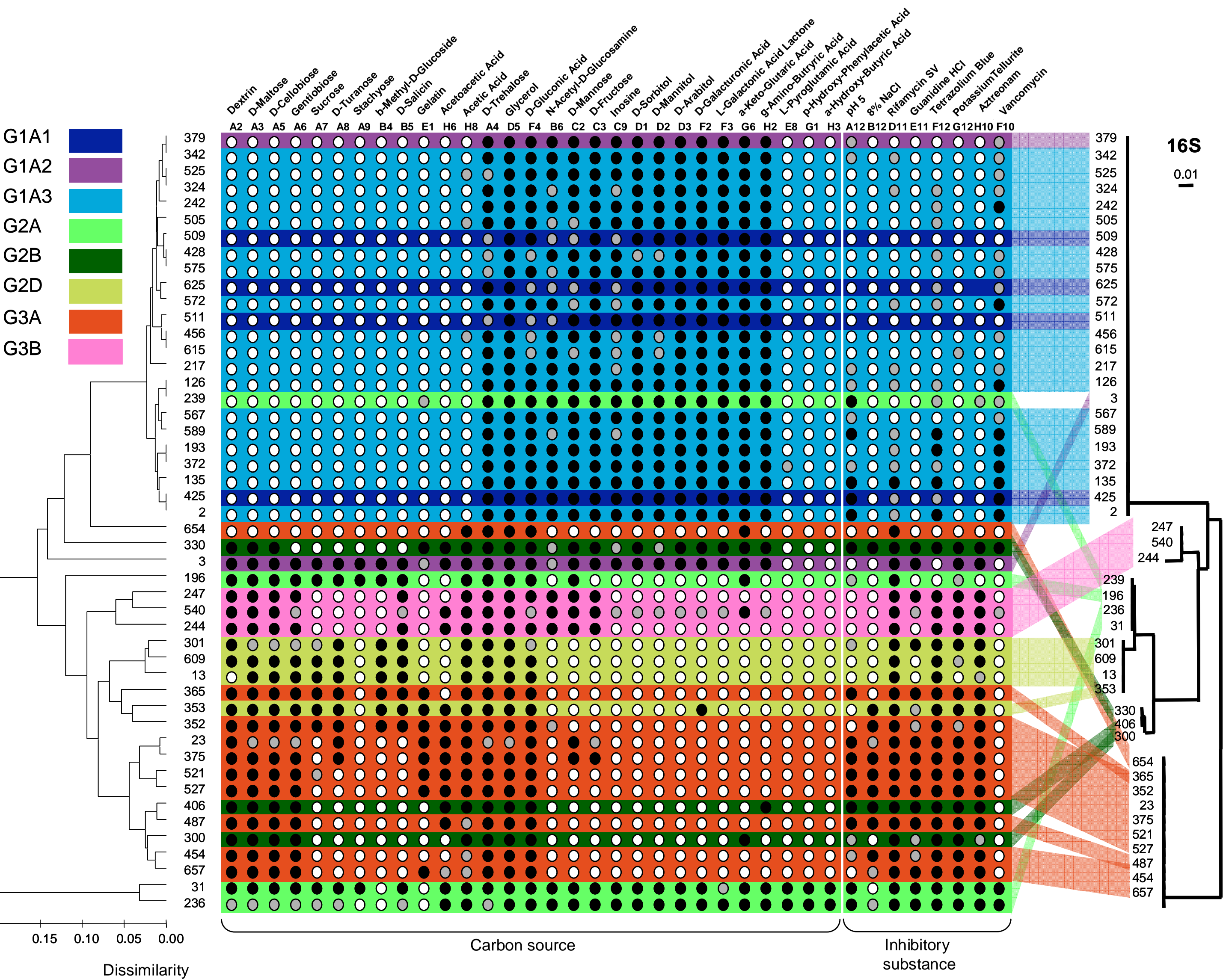


Fig. 9. Phenotypic similarity of the *Sphingomonadaceae* isolates. Phenotypic similary of the tested isolates is depicted as a UPGMA tree based on the metabolic respiratory reaction (positive versus negative) towards 94 substances on BiOLOG Gen III microplates. The black and white circles indicate discriminative positive or negative phenotypic reactions, respectively. Grey circles indicate as variable phenotypic reactions. The underlying colors code for the individual subpopulations shown in Figure 1. On the right, a maximum-likelihood phylogenetic reconstruction of the 16S sequences of the strains included in the UPGMA tree is shown. Bar denotes 0.01 fixed point mutations per nucleotide.

subpopulations of G1A group together, the phenotypic diversity did not increase when members of the two subpopulations G1A1 and G1A3 were treated together but only when strains from all groups were combined (Fig. 10).

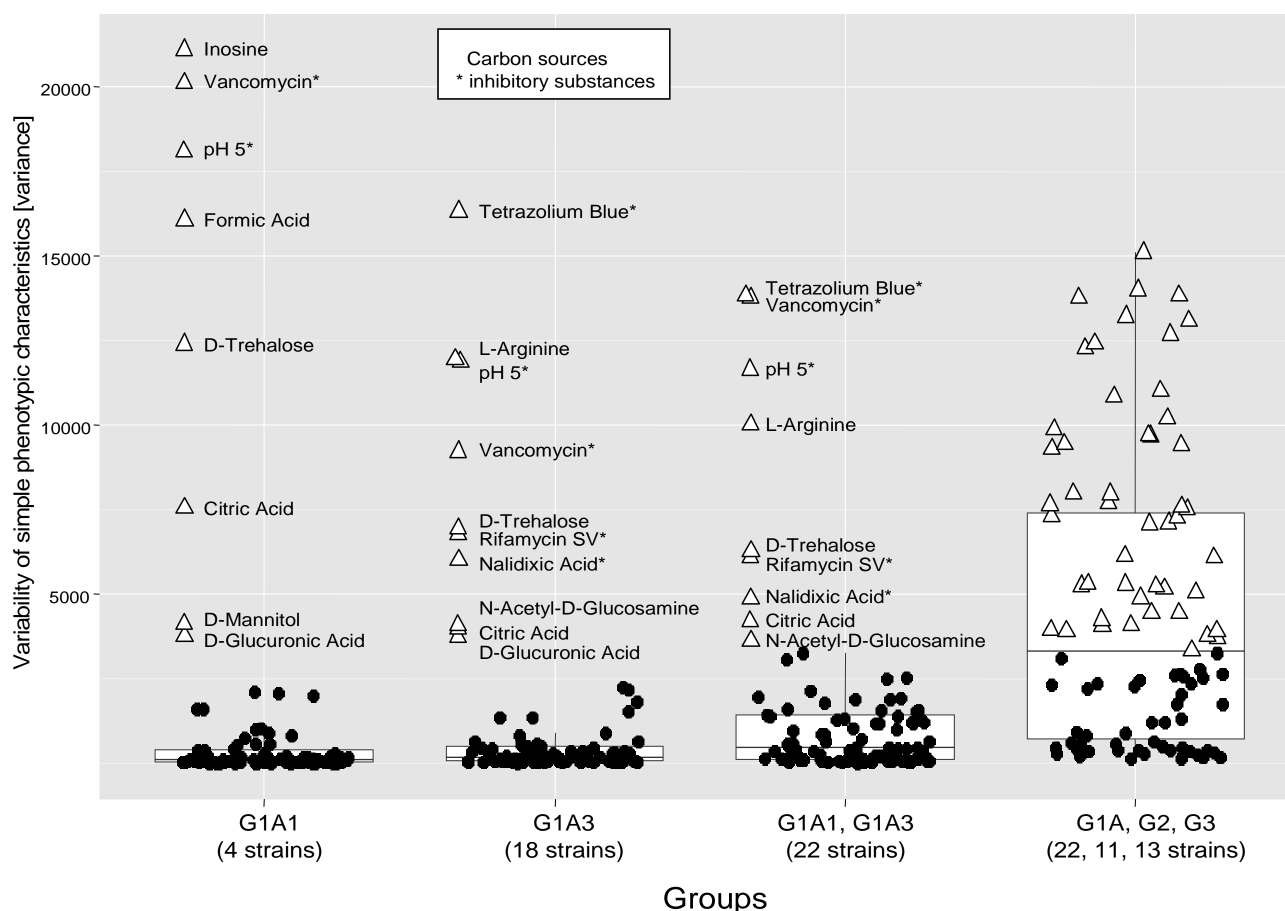


Fig. 10. Variance distribution of simple phenotypic characteristics within groups of different phylogenetic range. The mean of the processed values of two independent microplates was used for analysis. For each group of interest, the variance values from the 94 substances as jittered values and their summarizing boxplots using the R! package ggplot2 were plotted. Boxplots indicate the first and third quartile, the central horizontal line in the box the median value and the whiskers extend to a maximum of 1.5fold of the box size. Open triangles indicate all variance values equal or above the median for the combined set of G1A, G2, and G3 strains.

However, the phenotypic similarity in general only reflected the split into the major groups as identified by the 16S rRNA phylogeny (see Fig. 2A). The G1A strains differ substantially from G2 and G3 (Fig. 9), but are quite homogeneous, and do not split phenotypically into the observed MLSA subgroups (see Fig. 2C).

Polyphasic analysis of the subpopulations revealed by MLSA

We further assessed the observed subpopulation structure of the *Sphingomonadaceae* isolates (Fig. 2), from a taxonomic level, here we present a polyphasic study focussing on four strains (Fig. 11) from four different subpopulations (Fig. 2). Three of theses were proposed to belong to novel species, a fourth strain was found to be a member of a species which was reclassified.

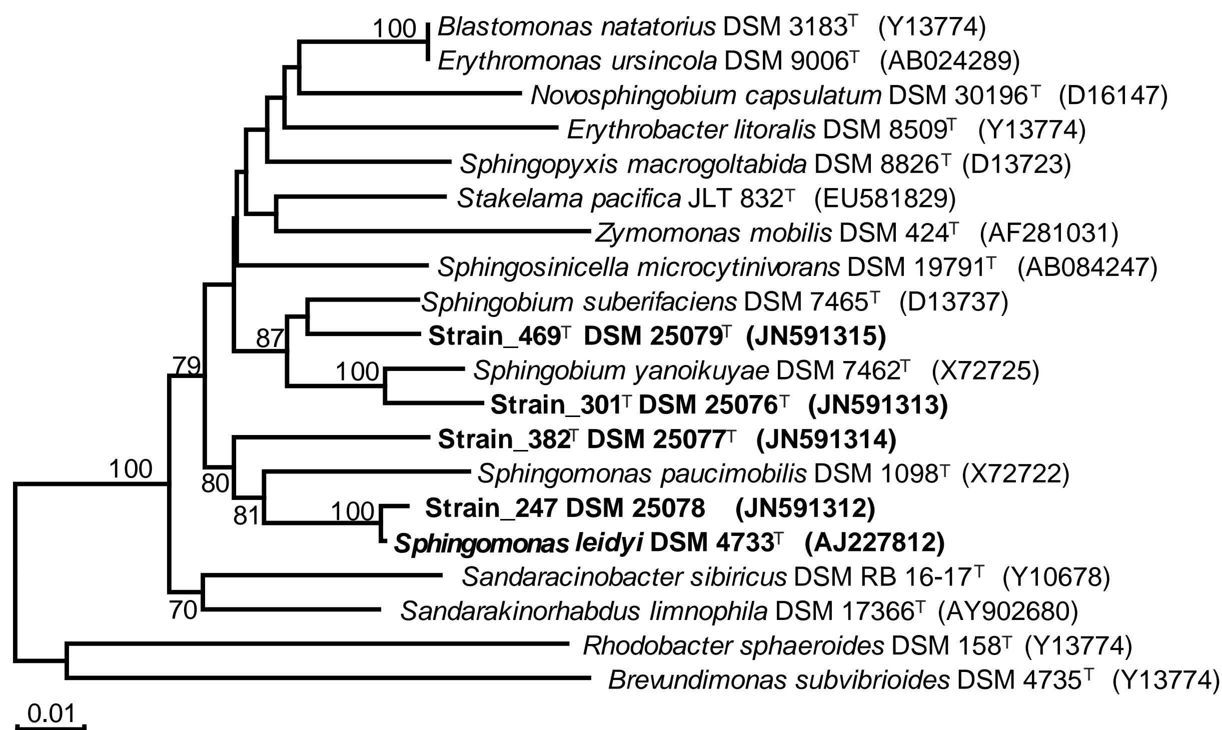


Fig. 11. Rooted neighbour-joining phylogenetic tree, based on 16 rRNA gene sequences showing the relationgships among strain 247, 301^T, 382^T, 469^T and other type species of genera in *Sphingomonadaceae*. The tree was constructed using ARB software package (Ludwig *et al.*, 2004). Numbers at nodes indicated the level (%) of bootstrap support based on 1000 re-samples dataset. (Only values > 70 are showing). *Rhodobacter sphaeroides* DSM 158^T and *Brevundimonas subvibrioides* DSM 4735^T were used as the outgroup.

Sphingomonas oligotrophica 382^T

Strain 382^T formed yellow, circular, domed, convex and smooth colonies on R2A agar, PYE agar and 1/10 HD agar on 28°C after 24 hours. Cells were Gram-negative, asporogenous, rod-shaped (1-1.8 μ m long and 0.4-0.5 μ m wide), motile by peritrichous flagella (Fig. 12), aerobic, catalase- and oxidase- positive. The isolate could grow at a pH range of 5-10 (optimum

pH 7) and a temperature range of 10- 40°C (optimum approx. 28°C). Other characteristics of strain 382^T are given in species description and Table 4, 5.

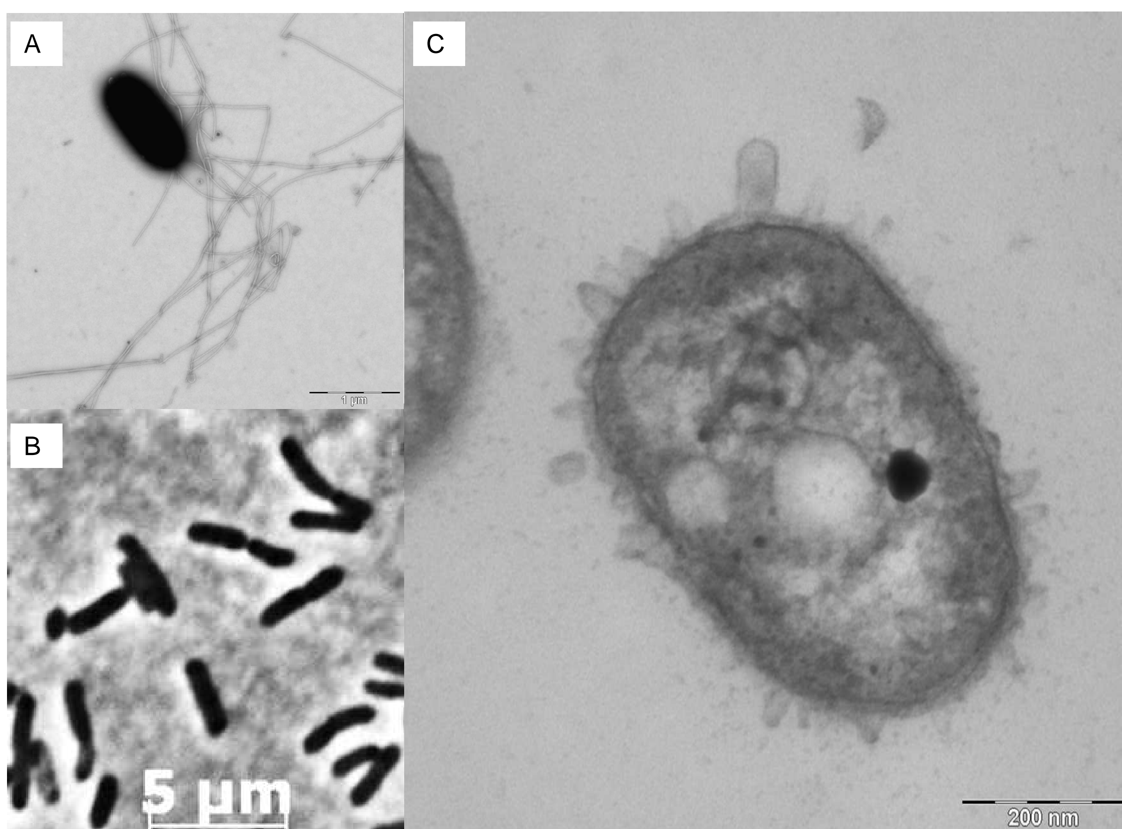


Fig. 12. Phase-contrast micrograph and transmission electron micrographs of strain 382^T. (**A**), TEM-image of negative staining cells, bar, 1 µm; (**B**), phase-contrast image, bar, 5 µm; (**C**), TEM image of thin sectioned cells, bar, 200nm.

Table 4. Different physiological and phenotypic characteristics of strain 247, 382^T, *Sphingomonas leidyi* DSM 4733^T and related type strains of the genus *Sphingomonas*.

Strains: 1, strain 247; 2, *Sphingomonas leidyi* DSM 4733^T; 3, 382^T; 4, *Sphingomonas aquatilis* DSM 15581^T, 5, *Sphingomonas paucimobilis* DSM 1098^T; 6, *Sphingomonas wittichii* DSM 6014^T; 7, *Sphingomonas echinoides* DSM 1805^T; 8, *Sphingomonas alaskensis* DSM 13593^T. +, positive; -, negative; V, weak reaction; ?, questionable reaction; Y: yellow; GW, greyish-white; CL, colorless; ND, not detected.

All strains tested negative for: arginine dihydrolase, α-fucosidase, α-galactosidase, β-glucuronidase, lipase (C14), α-mannosidase, protease, urease, Gram-staining, fermentation of glucose, indole production, nitrate reduction to nitrite, nitrate reduction to N_2, adipic acid, adonitol, D-arabinose, D-arabitol, L-arabitol, dulcitol, erythritol, L-fucose, β-gentiobiose, gluconate, glycerol, glycogen, inositol, inulin, 2-keto-gluconate, 5-keto-gluconate, mannitol, D-mannitol, β-methyl-D-xyloside, phenylacetic acid, potassium gluconate, ribose, salicin, sorbitol, L-sorbose, starch, D-tagatose,

xylitol, L-xylose.
All strains tested positive for: acid phosphatase, alkaline phosphatase, catalase, α-glucosidase, β-glucosidase, leucine arylamidase, aerobic growth, L-arabinose, cellobiose, D-fucose, D-glucose, maltose and D-xylose.

Substrate or test	1	2	3	4	5	6	7	8
Cell width cell length (µm)	0.4-0.5 0.7-1.2	0.4-0.5 1.2	0.4-0.5 1-1.8	ND	0.7 1.4	0.5-0.7 1.2-2.0	0.8 1.9	0.2-0.5 0.5-3
Motility	+	+	+	+	+	-	+	+
G+C content (mol%)	67.6	67	65.5	63	65	ND	65.8	65
Pigmentation of colonies	CL	CL	Y	Y	Y	Y	Y	Y
Growth in presence of 4% NaCl	-	+	+	-	+	+	+	-
N-acetyl-β-glucosaminidase	-	V	-	V	+	+	-	-
α-chymotrypsine	-	-	+	-	+	-	-	-
Cystine arylamidase	+	V	-	V	-	-	-	-
Esterase (C4)	V	V	-	-	+	-	V	V
Esterase Lipase(C8)	V	+	-	-	-	+	V	V
β-galactosidase	-	-	+	+	+	+	?	-
β-glucosidase	+	+	-	+	+	+	+	-
Naphtol phosphohydrolase	V	+	+	-	-	V	V	V
Oxidase	+	+	+	+	+	-	+	-
Trypsine	+	V	-	-	V	-	-	+
Valine arylamidase	+	+	+	+	+	+	+	-
L-arabinose	-	-	+	+	+	+	+	-
D-fructose	-	-	-	+	+	+	-	-
Galactose	-	-	+	+	+	+	+	-
Lactose	-	-	?	V	+	+	V	-
D-lyxose	V	V	-	V	V	+	+	V
D-maltose	+	+	+	+	+	-	+	+
D-mannose	-	+	-	+	+	+	-	-
Melezitose	-	-	-	V	+	-	-	-
Melibiose	V	-	-	-	+	-	-	-
D-raffinose	-	-	-	-	+	-	-	-
Rhamnose	-	+	+	+	-	+	-	-
Sucrose	-	-	-	+	+	+	+	-
Trehalose	+	+	V	+	+	+	+	-
D-turanose	-	-	-	-	+	+	+	-
Amygdalin	-	-	-	-	+	+	-	-
Arbutin	-	-	-	V	V	+	-	-
α-methyl-D-glucoside	-	-	-	-	+	-	-	-
α-methyl-D-mannopyranoside	-	-	-	-	+	-	-	-
N-acetyl-glucosamine	+	+	-	+	+	+	+	-
Capric acid	-	-	-	-	+	-	-	-
Malic acid	+	+	-	+	+	+	-	+
Trisodium citrate	-	-	-	-	-	+	-	-

Table 5. Susceptibilities of strain 247, 382^T and *Sphingomonas leidyi* DSM 4377^T against 36 antibiotic agents.

Strains: 1, strain 247; 2, *Sphingomonas leidyi* DSM 4733^T; 3, strain 382^T;4, *Sphingomonas aquatilis* DSM 15581^T; 5*, *Sphingomonas histidinilytica* UM2^T (Nigam *et al.*, 2010); 6, *Sphingomonas paucimobilis* DSM 1098^T; 7, *Sphingomonas wittichii* DSM 6014^T; 8, *Sphingomonas panni* DSM 15761^T; 9, *Sphingomonas echinoides* DSM 1805^T; 10, *Sphingomonas alaskensis* DSM 13593^T. R, resistant; IM, intermediate; S, susceptible; ND, not detected. All data except the strain 5* were generated in this study.

antibiotic	1	2	3	4	5*	6	7	8	9	10
Penicillin G	R	R	IM	R	S	R	IM	IM	IM	S
Oxacillin	R	R	IM	R	ND	R	R	R	R	S
Ampicillin	R	R	IM	R	S	IM	S	IM	S	S
Ticarcillin	R	R	S	R	ND	IM	S	IM	IM	S
Cefalotin	R	R	IM	R	ND	IM	R	R	R	S
Mezlocillin	R	R	IM	R	ND	R	IM	IM	R	S
Cefazolin	R	R	IM	R	ND	IM	R	R	R	S
Cefotaxim	R	R	S	R	ND	S	IM	IM	S	S
Aztreonam	R	R	IM	R	ND	R	R	IM	R	S
Chloramphenicol	R	R	S	S	S	S	S	R	S	S
Tetracyclin	S	S	S	S	ND	S	S	S	S	S
Imipenem	IM	IM	S	S	ND	S	S	IM	S	S
Gentamycin	IM	IM	IM	IM	S	IM	IM	IM	IM	IM
Amikacin	S	S	S	IM	S	IM	S	IM	S	S
Vancomycin	S	S	S	S	S	S	S	S	S	S
Erythromycin	IM	IM	S	S	ND	S	IM	S	IM	S
Lincomycin	R	R	R	R	ND	R	R	R	R	R
Ofloxacin	IM	IM	S	S	ND	S	IM	R	S	S
Colistin	R	IM	IM	R	ND	IM	IM	IM	IM	IM
Norfloxacin	IM	IM	S	IM	ND	IM	IM	R	S	S
Pipemidic	R	R	S	IM	ND	R	R	R	R	IM
Bacitracin	R	R	IM	R	ND	IM	R	IM	R	IM
Polymyxin B	IM	IM	IM	IM	S	IM	IM	IM	IM	IM
Nitrofurantoin	R	R	IM	IM	ND	R	R	IM	IM	IM
Neomycin	IM	IM	IM	IM	R	IM	S	IM	S	IM
Kanamycin	IM	S	S	S	S	IM	S	IM	S	S
Doxycycin	S	S	S	S	ND	S	S	S	S	S
Clindamycin	R	R	IM	R	ND	IM	R	R	R	IM
Ceftriaxone	R	R	IM	R	ND	IM	IM	IM	IM	S
Fosfomycin	S	IM	IM	S	ND	IM	S	IM	S	R
Nystatin	R	R	R	R	ND	R	R	R	R	R
Linezolid	R	R	IM	IM	ND	IM	R	R	IM	IM
Moxifloxacin	S	S	S	S	ND	S	IM	R	IM	S
Quinupristin/Dalfopristin	R	R	S	IM	ND	IM	R	S	IM	S
Piperacillin/tazobactam	R	R	IM	R	ND	IM	IM	IM	IM	S
Teicoplanin	IM	IM	S	IM	ND	IM	IM	IM	IM	S

Table 6. Cellular fatty acids profiles of strain 247, *Sphingomonas leidyi* DSM 4733^T, strain 382^T and the related species of the genus *Sphingomonas*

Strains: 1, strain 247; 2, *Sphingomonas leidyi* DSM 4733^T; 3, strain 382^T;4, *Sphingomonas aquatilis* DSM 15581^T; 5*, *Sphingomonas histidinilytica* UM2^T (Nigam *et al.*, 2010); 6, *Sphingomonas paucimobilis* DSM 1098^T; 7, *Sphingomonas wittichii* DSM 6014^T; 8, *Sphingomonas panni* DSM 15761^T; 9, *Sphingomonas echinoides* DSM 1805^T; 10, *Sphingomonas alaskensis* DSM 13593^T. All data except the (5*) were generated in this study. Values shown are percentages of the total fatty acids. -, not detected; tr, trace (<1%).

Fatty acids	1	2	3	4	5*	6	7	8	9	10
$C_{14:0}$	tr	-	-	tr	1.4	tr	1.71	tr	tr	-
$C_{14:0}$ 2OH	6.72	6.63	9.71	13.57	7.6	6.9	8.35	4.24	8.14	1.33
$C_{15:0}$	-	-		-	-	-	-	3.07	-	2.77
$C_{15:0}$ 2OH	-	-	tr	-	-	-	-	1.28	-	8.82
Summed feature 3	tr	1.52	7.81	7.09	8.1	4.71	11.38	13.15	2.37	6.54
$C_{16:1}\omega5c$	tr	tr	1.07	1.26	4.4	tr	2.04	1.84	tr	tr
$C_{16:0}$	10.73	14.61	7.13	10.95	12.1	8.75	15.9	8.69	12.31	4.94
$C_{16:0}$ 2OH	tr	tr	tr	-	-	-	-	-	-	1.14
$C_{16:1}$ 2OH	-	-	-	-	-	-	-	-	tr	-
$C_{17:1}\omega8c$	-	-	-	-	tr	-	-	1.37	-	8.52
$C_{17:1}\omega6c$	tr	1.12	2.31	-	9.1	tr	-	12.01	tr	38.8
$C_{17:0}$	-	-	-	-	1.2	-	-	1.52	tr	3.66
$C_{18:1}\omega7c$	67.15	70.47	66.43	61.31	32.9	75.29	53.75	51.62	64.04	19.7
$C_{18:1}\omega5c$	1.6	tr	1.05	tr	2.1	1.77	-	tr	1.48	tr
$C_{18:0}$	tr	-	-	tr	tr	tr	-	-	tr	tr
11-Methyl $C_{18:1}\omega7c$	8.39	2.73	3.15	3.26	4.4	-	3.13	-	8.71	1.59
$C_{19:0}$ cyclo $\omega8c$	1.93	tr	tr	-	15.5	-	3.73	-	tr	-
Summed feature 7	-	-	-	-	tr	-	-	-	-	tr

Summed features represent groups of two or more fatty acids that could not be separated by gas chromatography with the MIDI system. Summed feature 3 contains C16:1ω7c a-/or C15:0 ISO 2-OH. Summed feature 7 contains C19 Cyclo ω10c a-/or C19 ω6c.

The chemotaxonomic results of strain 382^T also supported its assignment to the genus *Sphingomonas*. The predominant respiratory lipiquinone around 98.22% was ubiquinone-10 (Q-10) and 1.78% was detected as Q-9. The major fatty acids were $C_{18:1}\omega7c$ (66.43%), Summed feature 3 (contains $C_{16:1}\omega7c$ and/or C_{15} ISO2-OH) (7.81%), $C_{16:0}$ (7.13%), and $C_{14:0}$ 2OH (9.71%) was the major 2-hydroxy fatty acids. In addition, 11-methyl $C_{18:1}\omega7c$ (3.15%), $C_{17:1}\omega6c$ (2.31%), $C_{16:1}\omega5c$ (1.07%), $C_{18:1}\omega5c$ (1.05%) were identified as minor peaks (Table 6). The difference of the composition of fatty acid indicated that strain 382^T was a new species of *Sphingomonas*. The major polar lipids were diphosphatidylglycerol, phosphatidylglycerol, phosphatidyldimethylethanolamine, phosphatidylethanolamine, phosphatidylcholine,

phosphatidylmonomethylethanolamine and sphingoglycolipids. The presence of the sphingoglycolipid was the characteristic components reported for member of *Sphingomonas* (Fig. 13). The major polyamine was sym-homospermidine, other polyamines were detected only in trace amounts. This also a characteristic feature of the genus *Sphingomonas*.

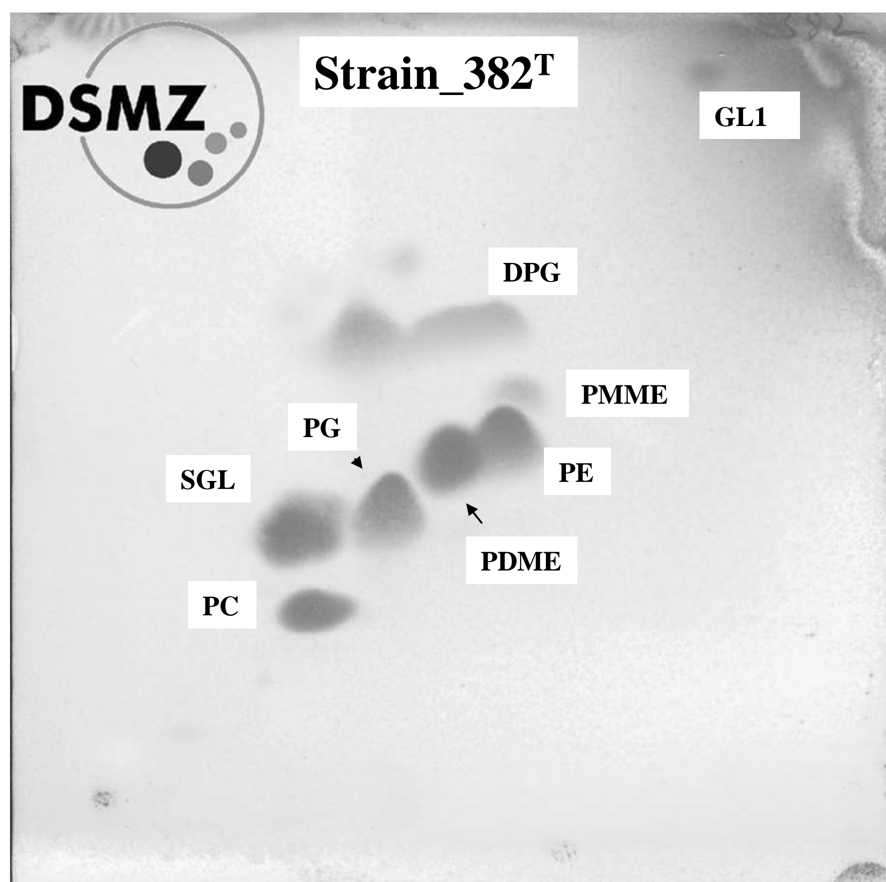


Fig. 13. Polar lipid pattern of strain 382^T after separation by two-dimensional thin-layer chromatogram (TLC), detected with anis aldehyde (for all lipids), ninhydrin (for amino groups), cis-aconitinacid-anhydride (for phosphorus-containing lipids), meta-periodate/schiff (for vicinal hydroxy groups), molybdenum blue (for phosphate groups).

DPG, diphosphatidylglycerol; PG, phosphatidylglycerol; PE, phosphatidylethanolamine; PC, phosphatidylcholine; PMME, phosphatidylmonomethylethanolamine; PDME, phosphatidyldimethylethanolamine; GL1, glycolipids; SGL：sphingoglycolipids.

The 16S rRNA gene sequence of strain 382^T were 1482 bp with the GenBank accession number JN591314. Based on the comprehensive phylogenetic analysis of 16S rRNA gene sequences which included sequences from the type strains of species in the genera *Sphingomonas* and *Sphingobium*, strain 382^T was clustered within the genus *Sphingomonas.*

Sphingomonas histidinilytica UM 2^T, *Sphingomonas wittichii* DSM 6014^T, *Sphingomonas haloaromaticamans* DSM 13477^T and *Sphingmonas fennica* DSM 13665^T were the closest neighbors (97.1%, 96.6%, 96.0% and 95.8% similarity, respectively). The 16S rRNA gene sequence similarity of strain 382^T to *Sphingomonas paucimobilis* DSM 1098^T (type species of this genus) was 95.3%. A phylogenetic tree showing the relationship between strain 382^T and type strains of species in genus *Sphingomonas* is shown in Fig. 14, Fig. S1, S2.

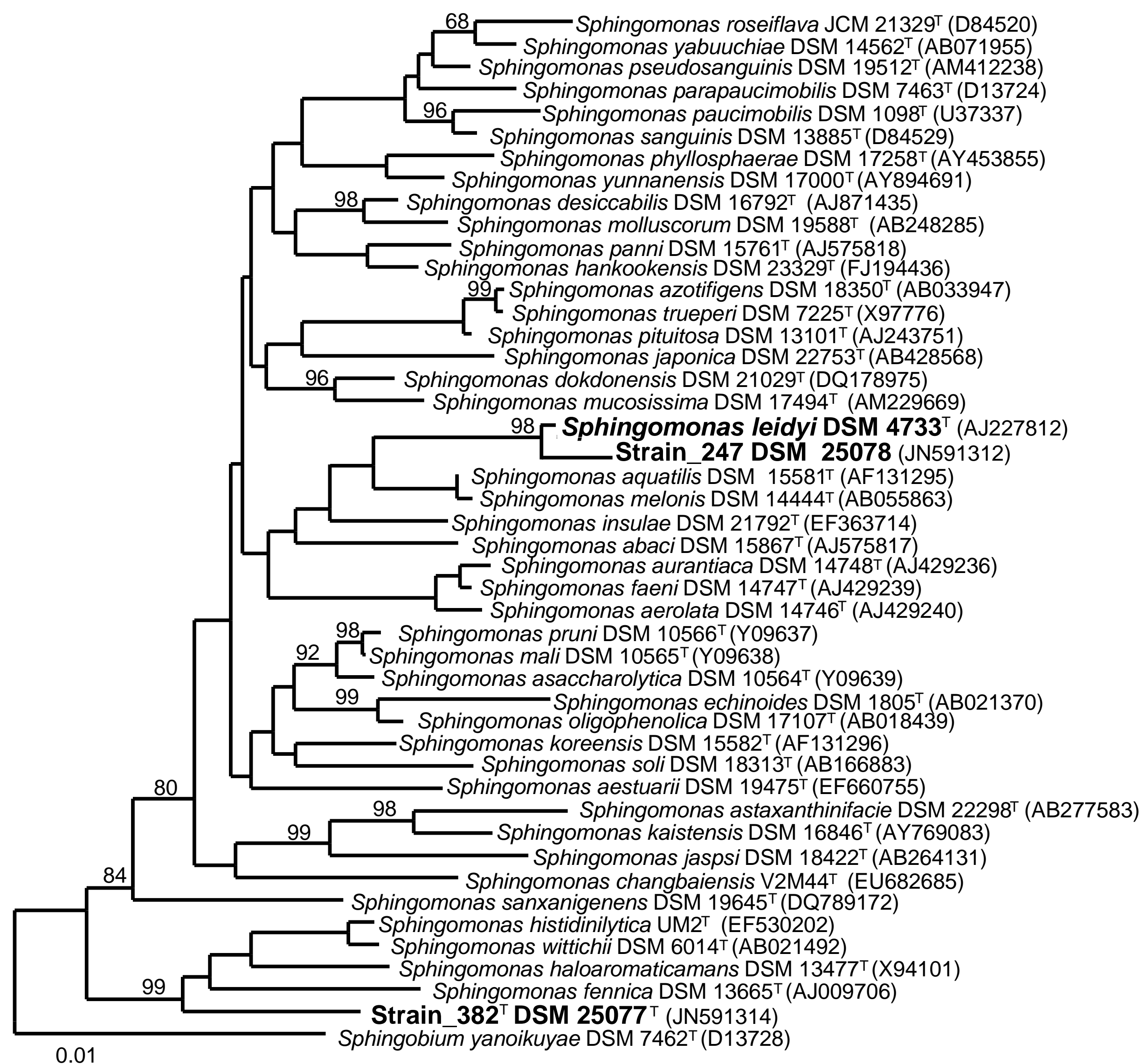


Fig. 14. Rooted neighbor-joining phylogenetic tree, based on 16 rRNA gene sequences showing the relationships among strain 247, 382^T, *Spingomonas leidyi* DSM 4733^T and other type species of *Sphingomonas*. The tree was constructed using ARB software package (Ludwig *et al.*, 2004). Numbers at nodes indicated the level (%) of bootstrap support based on 1000 re-samples dataset. *Sphingobium yanokuyae* DSM 7462^T was used as the outgroup.

For the comparison of strain 382^T with closely related type strains, the type strain UM2^T of species *Sphingomonas histidinilytica* (=MTCC 9473^T = CCM 7545^T) could not be included. During the course of the study, it turned out that despite the literature information (Nigam *et al.*, 2010), strain UM2^T is not existing in the MTCC collection. Also, the characteristics of strain UM2^T obtained from the CCM collection do not match the characteristics published (Nigam *et al.*, 2010). Hence, the strain at CCM is probably a wrong deposit. Therefore, in this study, the information of this strain was taken from the species description by Nigam *et al.* (2010). Even the 16S rRNA gene sequence of strain 382^T was 97.1% similar to *S. histidinilytica* UM2^T, but there were lots of differences between these two strains, the phenotypic differences like color of the cells, oxidase reaction, resistance to different antibiotics, the fatty acids composition, polar lipid composition suggested that strain 382^T does not belong to the species *S. histidinilytica* but should be proposed as a novel species. The genomic G+C content of strain 382^T was 65.6 mol%.

Description of *Sphingomonas oligotrophica* sp. nov. *Sphingomonas oligotrophica* (oligo. tro. phi. ca Gr. adj. *oligos*, few; Gr. adj. *trophikos*, nursing, tending or feeding; N.L. fem. adj. *oligotrophica*, eating little, utilizing only a few growth substrates).

Colonies are yellow, circular, domed, convex on R2A agar, 1:10 diluted HD agar after 24 hours incubation. Cells are Gram-negative, asporogenous, rod-shaped,1-1.8 μ m long and 0.4-0.5 μ m wide, motile by peritrichous flagella, aerobic, catalase- and oxidase- positive. The isolate can grow at pH values between 5 and 10 (optimum pH 7) and temperatures between 10 and 40°C (optimum approx. 28°C). Nitrate is not reduced; produces acid phosphatase, alkaline phosphatase, α-chymotrypsine, esculin, β-galactosidase, α-glucosidase, β- glucosidase, leucine arylamidase, valine arylamidase are hydrolysed. Naphthol-AS-BI-phosphohydrolase, D-glucose, and arabinose, L-arabinose, cellobiose, esculin, D-fucose, galactose, maltose, rhamnose, trehalose, D-xylose are utilized and lactose, starch are weakly utilized. Cells are sensitive (inhibition zones >30 mm) to ticarcillin (75 µg per disk), cefotaxim (30), chloramphenicol (30), tetracyclin (30), imipenem (10), amikacin (30), vancomycin (30), erythromycin (15), ofloxacin (5), norfloxacin (10), pipemidic (20), kanamycin (30), doxycycin (30), moxifloxacin (5), quinupristin/dalfopristin (15), teicoplanin (30); resistant to lincomycin (15) and nystatin (100).

The Q-10 (98.22%) is the major respiratory quinone. The major fatty acids are $C_{18:1}\omega 7c$ (66.43%), summed feature 3 (contains $C_{16:1}\omega 7c$ and/or C_{15} ISO2-OH) (7.81%), $C_{16:0}$ (7.13%), and C14:0 2OH (9.71%) is the major 2-hydroxy fatty acids. The major polar lipids are diphosphatidylglycerol, phosphatidylglycerol, phosphatidyldimethylethanolamine,

phosphatidylethanolamine, phosphatidylcholine, phosphatidylmonomethylethanolamine and sphingoglycolipids. The major polyamine is sym-homospermidine.

The genomic DNA G+C content of strain 382^{T} is 65.5mol%. Based on these results, it is proposed that strain 382^{T} represents a new species of the genus *Sphingomonas*, which named as *Sphingomonas oligotrophica*. The type strain is 382^{T} (=DSM 25077^{T}=LMG xxxxx^{T}) isolated from fresh lake water of Starnberger See, Germany.

Reclassification of **Caulobacter leidyi** *as* **Sphingomonas leidyi** *comb. nov.*

Strains DSM 4733^{T} and 247 formed transparent, colorless, circular and convex colonies on R2A agar, PYE agar and 1:10 diluted HD agar and after 24 hours incubation. Individual cells of strain 247 were Gram-negative, asporogenous, rod-shaped, 0.7-1.2 μ m long and 0.4-0.5 μ m wide (Table 4) and typically formed rosettes (Fig. 15A). Electron microscopic investigations revealed the presence of cells bearing either a polar flagellum or a stalk (Fig. 15B, C) similar to strain DSM 4733^{T}.

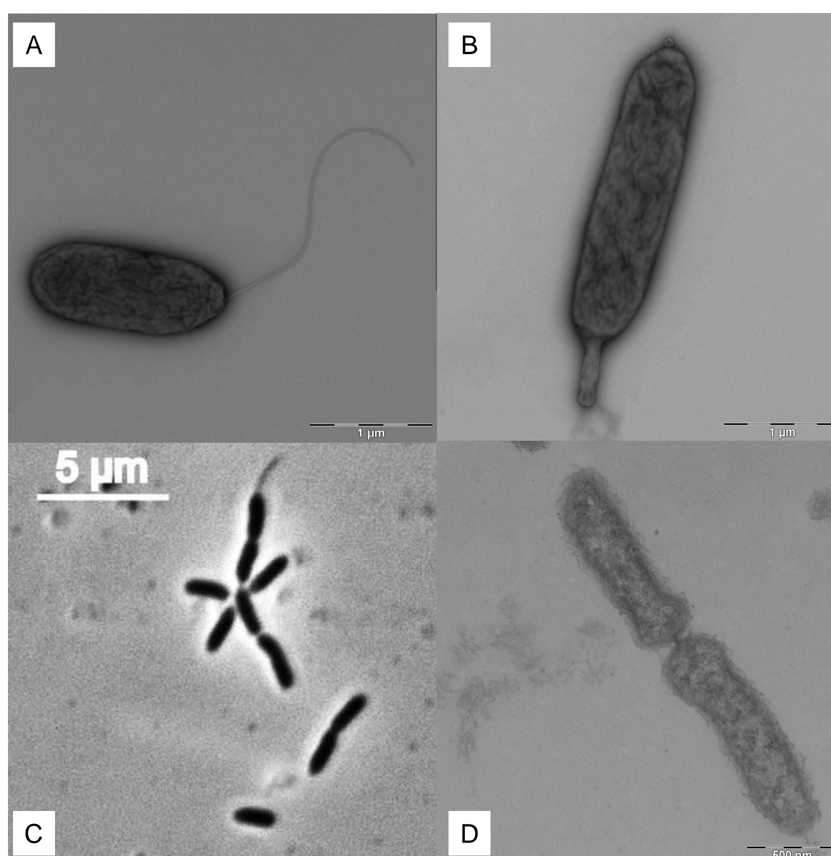


Fig. 15. Phase-contrast micrograph and transmission electron micrographs of strain 247. (**A, B**), TEM image of negative staining cells, bar, 1μm; (**C**), phase-contrast image, bar, 5 μm; (**D**), TEM image of thin sectioned cells, bar, 500nm.

Based on the nearly complete (1493 bp) 16S rRNA gene sequence of strain 247 and the available (1443 nt long) 16S rRNA gene sequence of strain DSM 4733^T, our phylogenetic analysis of all dimorphic prosthecate *Alphaproteobacteria* and the type strains of *Sphingomonadaceae* type species placed strains DSM 4733^T and 247 unambigously within the family *Sphingomonadaceae* (Yabuuchi *et al.*, 1990) (Fig. 16); results of maximum likelihood and maximimum parsimony analyses were very similar and hence are not shown). The sequence similarity of the 16S rRNA genes of strains DSM 4733^T and 247 was 99.6%. Within the *Sphingomonadaceae*, *Sphingomonas aquatilis* DSM 15581^T was identified as the phylogenetically closest relative with a sequence similarity of 98.1 % to strain DSM 4733^T and 97.7 % to strain 247. Sequence similarity with *Sphingomonas paucimobilis* DSM 1098^T (type strain of type species of genus *Sphingomonas*) was 94.5%. The 16S rRNA gene sequences were distantly related to that of the species of genus *Caulobacter*, the largest nucleotide similarity was 87.9% (Fig. 16). Both 16S rRNA gene sequences also include all 7 signature sequences of *Sphingomonadaceae*. Cells were aerobic, catalase- and oxidase-positive and motile by the single polar flagellum. The novel isolate strain 247 grew at pH values between 5 and 10 (optimum, pH 7) and temperatures between 10 and 40°C (optimum, 28°C) (Table 4). Both strains did not reduce nitrate but were capable of using esculin, glucose, N-acetyl-glucosamine, maltose, L-arabinose, D-xylose, D-glucose, N-acetyl-glucosamine, cellobiose, trehalose and malic acid. Melibiose, β-gentiobiose and D-lyxose were weakly utilized. They produced alkaline phosphatase, leucine arylamidase, valine arylamidase, cystine arylamidase, trypsin, acid phosphatase, naphthol-AS-BI-phosphohydrolase, α-glucosidase, β-glucosidase. Cells of strains DSM 4733^T and 247 were sensitive (inhibition zones >30 mm) to tetracyclin, amikacin, vancomycin, doxycycin, fosfomycin, moxifloxacin and resistant to penicillin G, oxacillin, ampicillin, ticarcillin, cefalotin, mezlocillin, cefazolin, cefotaxim, sztreonam, chloramphenicol, lincomycin, colistin, pipemidic, bacitracin, nitrofurantoin, clindamycin, ceftriaxone, nystatin, linezolid, quinupristin/dalfopristin, and piperacillin/tazobactam. Additional characteristics are listed in the species description, Table 4 and Table 5.

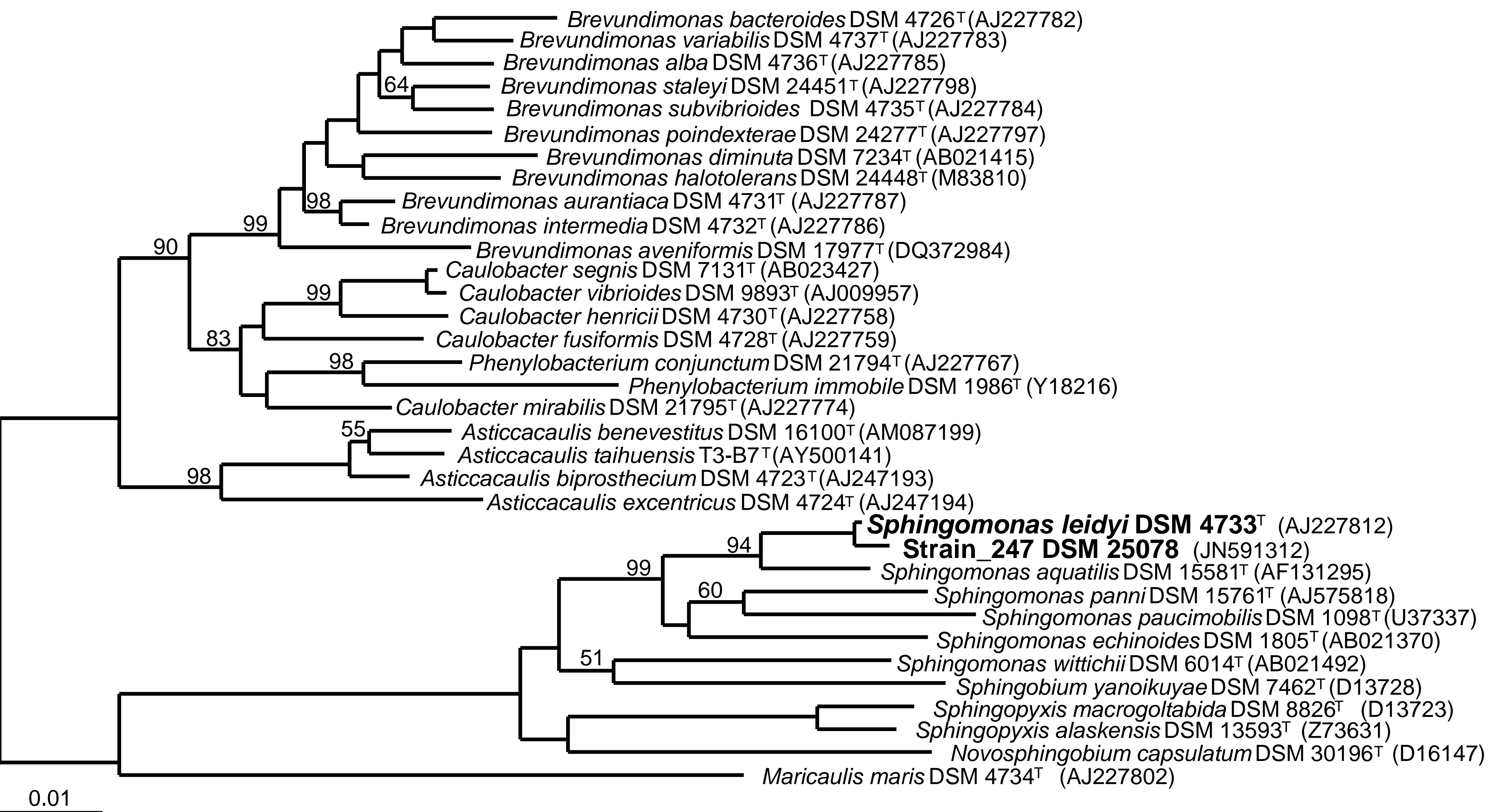


Fig. 16. Rooted neighbor-joining phylogenetic tree, based on 16 rRNA gene sequences showing the relationships between *Sphingomonas leidyi* DSM 4733^T, strain 247 and other dimorphic prosthecate *Alphaproteobacteria*. The type species of the respective genera were included in the analysis. Numbers at nodes indicated the level (in %) of bootstrap support based on 1000 resampled dataset. *Maricaulis maris* DSM4734^T was used as the out group. Bar indicates 1% nucleotide divergence.

The major respiratory quinone for strains DSM 4733^T and 247 was Q-10 (81.47% for strain 247). The major fatty acids for strain 247 were $C_{18:1}\omega7c$ (67.15%), $C_{16:0}$ (10.73%), 11-methyl $C_{18:1}\omega7c$ (8.39%). $C_{14:0}$ 2OH (6.72%) was the major 2-hydroxy fatty acids. For strain DSM 4733^T, the major fatty acids were $C_{18:1}\omega7c$ (70.47%), $C_{16:0}$ (14.61%), 11-methyl $C_{18:1}\omega7c$ (2.73%), and the major hydroxy fatty acids was also $C_{14:0}$ 2OH (6.63%) (Table 6). For both strains the major polar lipids were diphosphatidylglycerol, phosphatidyldimethylethanolamine, phosphatidylglycerol, phosphatidylmonomethylethanolamine, phosphatidylethanolamine, phosphatidylcholine, glycolipids, phosphoaminolipids and two sphingoglycolipids (Fig. 17). Dominant polyamines of strains DSM 4733^T and strain 247 were *sym*-homospermidine, which was accompanied by smaller amounts of putrescine and spermidine and traces of diaminopropane, cadaverine and spermine.

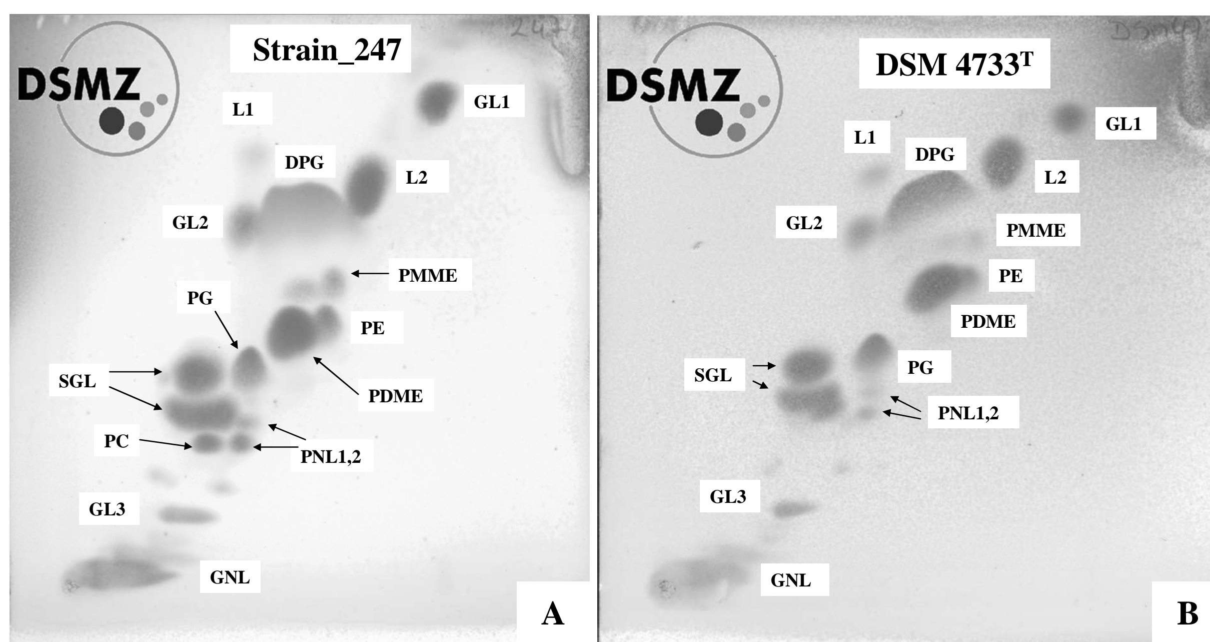


Fig. 17. Polar lipid pattern of strain 247 (**A**) and DSM 4733^T (**B**) after separation by two-dimensional thin-layer chromatogram (TLC), detected with anis aldehyde (for all lipids), ninhydrin (for amino groups), cis-aconitinacid-anhydride (for phosphate-containing lipids), meta-periodate/schiff (for vicinal hydroxy groups), molybdenum blue (for phosphate groups).

DPG, diphosphatidylglycerol; PG, phosphatidylglycerol; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PMME, phosphatidylmonomethylethanolamine; PDME, phosphatidyldimethylethanolamine; GL1, GL2, GL3, glycolipids; SGL: sphingoglycolipids; L1, L2, unidentified lipid; PNL1, PNL2, phosphoaminolipids.

Corroborating the close phylogenetic relationship to *Sphingomonas aquatilis*, the 16S rRNA gene sequences of DSM 4733^T and strain 247 include all seven signature sequences previously determined for the genus *Sphingomonas* and contain *sym*-homospermidine as the dominant polyamine similar to other species of this genus (Takeuchi *et al.*, 2001). The affiliation with the *Sphingomonadaceae* is also commensurate with the fatty acid patterns and polar lipid composition of the two strains. Characteristics that distinguish strains DSM 4733^T and strain 247 from most other *Sphingomonadaceae* include the presence of cystine arylamidase (variable in DSMZ 4733^T), absence of β-galactosidase, and the lack of L-arabinose and sucrose utilization (Table 4; Takeuchi *et al.*, 2011). So far, the dimorphic life cycle that involves a prosthecate and a flagellated stage is unique among the *Sphingomonadaceae*.

Emended description of the genus *Sphingomonas* (Yabuuchi *et al.* 1990) emend. Yabuuchi *et al.* 1999 Cells are Gram-negative, non-sporulating rods measuring 0.3-0.8 x 0.7-1.9 μ m. Motile or non-motile, some species exhibit a dimorphic life cycle that involves a prosthecate nonmotile and a flagellated motile cell. Colonies are yellow, off-white or colorless. Strictly aerobic and chemo-organotrophic. Catalase-positive. Respiratory quinone is predominantly Q-10. Major fatty acids are 18:1, saturated 16:0 and/or 17:1. Major 2-hydroxy fatty acids are 2-OH 14:0 or 2-OH 15:0. GSLs are present. Homospermidine is the major polyamine component. The DNA G + C content is 62 - 68 mol%. Phylogenetic position is in the *a*-4 subclass of *Proteobacteria*. Characteristic 16S rRNA signatures are found at positions 52:359 (C:G), 134 (G), 593 (G), 987:1218 (G:C) and 990:1215 (U:G). Habitat: soil, clinical specimens, insect guts and freshwater lakes. Currently, 67 species are assigned to the emended genus *Sphingomonas*. Type species: *Sphingomonas paucimobilis* Yabuuchi *et al*. 1990.

Description of *Sphingomonas leidyi* comb. nov. *Sphingomonas leidyi* (lei.dyi, N. L. gen. masc. n. leidyi, of Leidy, named for J. Leidy, who observed tufts of (bacterial) growth of fungi in insect guts in 1853)

The description of *Sphingomonas leidyi* is the same as that given for *Caulobacter leidyi* by Henrici and Abraham (Henrici & Johnson, 1935, emended by Poindexter, 1964; Abraham *et al.*, 1999), with the following additional characteristics. Cells are predominantly short, uncurved, slightly tapered, and sometimes nearly ovoid, with short stalks. Colonies are colorless. Isolates known from freshwater, millipede hindgut and soil. Nitrate is not reduced; esculin, glucose, N-acetyl-glucosamine, maltose and malic acid are hydrolysed. Cells are divided by binary division.

Produces alkaline phosphatase, leucine arylamidase, valine arylamidase, cystine arylamidase, trypsin, acid phosphatase, naphthol-AS-BI-phosphohydrolase, α-glucosidase, β-glucosidase. L-arabinose, D-xylose, D-glucose, N-acetyl-glucosamine, cellobiose, maltose, and trehalose are utilized. Melibiose, β-gentiobiose and D-lyxose are weakly utilized. Cells are sensitive to tetracyclin, amikacin, vancomycin, doxycycin, fosfomycin, moxifloxacin. Resistant to penicillin G, oxacillin, ampicillin, ticarcillin, cefalotin, mezlocillin, cefazolin, Cefotaxim, aztreonam, chloramphenicol, lincomycin, colistin, pipemidic, bacitracin, nitrofurantoin, clindamycin, ceftriaxone, nystatin, linezolid, quinupristin/dalfopristin, piperacillin/tazobactam.

Q-10 is the major respiratory quinone, the major fatty acids are $C_{18:1}\omega7c$, $C_{16:0}$, 11-Methyl $C_{18:1}\omega7c$, and $C_{14:0}$ 2OH is the major 2-hydroxy fatty acids. The major polar lipids are diphosphatidylglycerol, phosphatidyldimethylethanolamine, phosphatidylglycerol, phosphatidylmonomethylethanolamine, phosphatidylethanolamine, phosphatidylcholine, glycolipids, phosphoaminolipids and two sphingoglycolipids. The major polyamine is *sym*-homospermidine.The genomic DNA G+C content of strain DSM 4733^T is 67 mol%. The type strain is DSM 4733^T (=ATCC 15260^T=CIP 106443^T=VKM B-1368^T) was isolated from Millipede hind-gut. Another strain in this species is 247 (DSM 25078 = LMG 26658) with the genomic DNA G+C content 67.6 mol%, isolated from a prealpine freshwater lake (Starnberger See, Germany).

***Sphingobium limneticum* 301^T**

Strain 301^T formed yellow, circular and smooth colonies on R2A agar, PYE agar and 1/10 HD agar after 24 hours incubation. Cells were Gram-negative, asporogenous, rod-shaped, 1.3-2.2 μ m long and 0.4-0.5 μ m wide (Table 7), motile by peritrichous flagella (Fig. 18).

Based on the nearly complete (1496 bp) 16S rDNA gene sequence of isolate 301^T a comprehensive phylogenetic analysis of 16S rRNA gene sequences which included sequences from the type strains of species in the genus *Sphingobium* and strain 301^T is shown in Fig. 19, Fig. S3, S4. Strain 301^T was clustered within the genus *Sphingobium*. *Sphingobium amiense* DSM 16289^T, *Sphingobium vermicomposti* DSM 21299^T, *Sphingobium yanoikuyae* DSM 7462^T (type species of this genus) and *Sphingobium scionense* DSM 19371^T were the closest neighbors (98.8%, 98.0%, 97.9% and 97.4% similarity, respectively). Since DNA-DNA hybridization has at the species level a greater resolution than 16S rRNA gene sequences (Tindall *et al.*, 2010), the DDH were done among the strain 301^T and other type strains of the other 4 species which showed more than 97% similarity of 16S rRNA gene sequences. DNA-DNA relatedness between

strain 301^T and *Sphingobium amiense* DSM 16289^T, *Sphingobium scionense* DSM 19371^T, *Sphingobium yanoikuyae* DSM 7462^T and S*phingobium vermicomposti* DSM 21299^T were 43.15%, 36.85%, 35.3% and 12.05% (mean value of measurements in duplicate) respectively, which were much lower than the recommendations of a threshold value of 70% DNA-DNA relatedness for the definition of bacterial species by the *ad hoc* committee (Wayne *et al.*, 1987). So, strain 301^T did neither belong to the species *Sphingobium amiense*, nor to any of the species *Sphingobium scionense*, *Sphingobium yanoikuyae* or *Sphingobium vermicomposti*. The genomic G+C content of strain 301^T was 63.4 mol%

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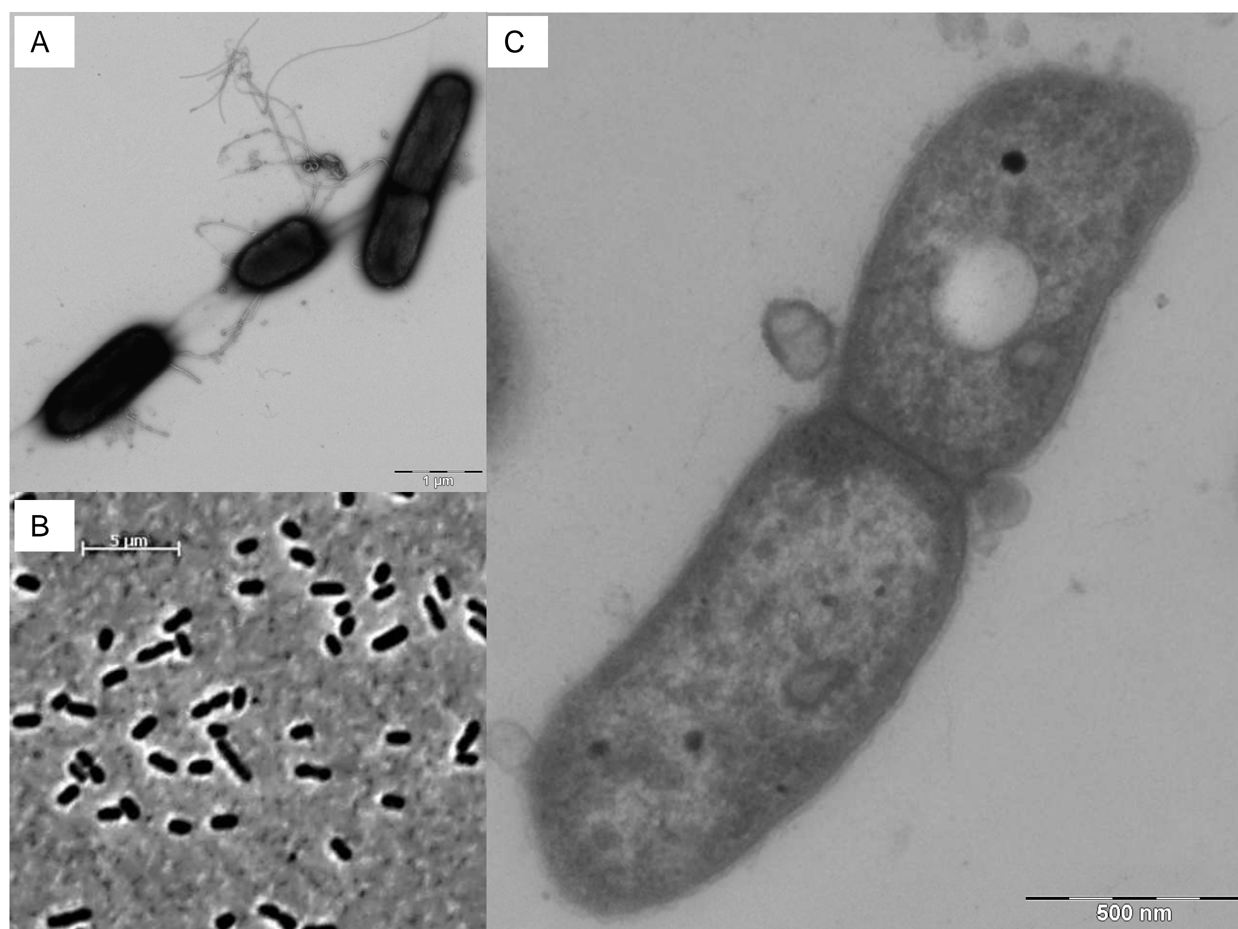

Fig. 18. Phase-contrast micrograph and transmission electron micrographs of strain 301^T. (**A**), TEM-image of negative staining cells, bar, 1 µm; (**B**), phase-contrast image, bar, 5 µm; (**C**), TEM-image of thin sectioned cells, bar, 500nm.

Cells were aerobic, catalase- and oxidase- positive, Gram-negative. The isolate could grow at pH 5-10 (optimum pH 7) and temperature 10-40°C (optimum approx. 28°C) (Table 7). Nitrate is not reduced; acid phosphatase, alkaline phosphatase, α-chymotrypsine, α-glucosidase,

β-glucosidase, β-galactosidase, leucine arylamidase, naphthol-phosphohydrolase, valine arylamidase are hydrolysed. Amygdalin, arabinose, L-arabinose, D-fucose, galactose, D-glucose, lactose, D-lyxose, malic acid, maltose, α-methyl-D-glucoside, melibiose, D-raffinose, rhamnose, starch, sucrose, D-turanose and D-xylose are utilized. Cells are sensitive (inhibition zones >30 mm) to aztreonam (30 μg per disk), chloramphenicol (30), tetracyclin (30), imipenem (10), amikacin (30), vancomycin (30), erythromycin (15), ofloxacin (5), norfloxacin (10), bacitracin (10), nitrofurantoin (100), kanamycin (30), doxycycin (30), moxifloxacin (5), quinupristin/dalfopristin (15), piperacillin/tazobactam (40), teicoplanin (30); resistant to oxacillin (5), cefalotin (30), mezlocillin (30), lincomycin (15) and nystatin (100). Other characteristics of strain 301^T are given in species description and Table7, 8.

Table 7. Differential phenotypic and physiological characteristics among strain 301^T, 469^T and closely related species.

Strains: 1, strain 301^T; 2, strain 469^T; 3, *Sphingobium suberifacien* DSM 7465^T; 4, *Sphingobium yanoikuyae* DSM 7462^T;5, *Sphingobium amiense* DSM 16289^T; 6, *Sphingobium scionense* DSM 19371^T; 7, *Sphingobium vermicomposti* DSM 21299^T; 8, *Sphingobium japonicum* DSM 16413^T. +, positive; -, negative; V, weak reaction; ?, questionable reaction; CY, creamy yellow; Y: yellow; W, white, GW, greyish-white; ND, not detected.

All strains tested negative for: N-acetyl-β-glucosamminidase, arginine dihydrolase, α-fucosidase, α-galactosidase,β-glucuronidase, Lipase (C14), α-mannosidase, protease, urease, fermentation of glucose, Gram-staining, indole prodcution, nitrate to N_2, adipic acid, D-arabitol, L-arabitol, dulcitol, erythritol, L-fucose, β-gentiobiose, gluconate, glycogen, inositol, inulin, 2-keto-gluconate, 5-keto-gluconate, mannitol, α-methyl-D-mannopyranoside, phenylacetic acid, ribose, sorbitol, L-sorbose, D-tagatose, L-xylose, xylitol.
All strains tested positive for: aerobic growth, catalase, alkaline phosphatase, leucine arylamidase, D-glucose.

Substrate or test	1	2	3	4	5	6	7	8
Cell width	0.4-0.5	0.35-0.45	0.4-0.5	0.4-0.5	0.5	0.5-0.6	0.4-0.5	0.4-0.5
Cell length (μm)	1.3-2.2	0.7-1.2	0.9-1.4	1-3	1.1-1.7	1.6-2.0	0.7-1.2	1.5-2
motility	+	+	+	+	+	-	+	-
G+C content (mol%)	63.4	64.6	59	61.7	66.4	63.8	62	ND
Pigmentation of colonies	Y	CY	W	GW	Y	Y	Y	Y
Growth in 4% NaCl	+	-	-	+	-	+	+	+
Nitrate to Nitrite	-	-	+	-	-	+	-	-
Acid phosphatase	+	+	-	+	+	+	-	+
α-chymotrypsin	+	-	-	+	+	-	-	+

Cystine arylamidase	-	-	-	V	-	-	-	V
Esterase (C4)	V	V	-	V	V	-	-	V
Esterase Lipase(C8)	V	V	V	V	-	-	V	V
β-galactosidase	+	+	+	+	+	+	+	-
α-glucosidase	+	-	-	+	+	V	-	+
β-glucosidase	+	+	+	+	-	+	+	+
Naphthol-AS-BI-phosphohydrolase	V	+	-	-	V	V	V	+
Oxidase	+	+	+	-	+	-	+	+
Trypsine	-	-	-	+	-	-	-	-
Valine arylamidase	+	-	-	V	V	V	V	+
D-arabinose	-	-	-	+	-	-	-	-
L-arabinose	+	+	-	+	+	+	-	+
Cellobiose	-	+	-	+	-	+	+	V
D-fructose	-	-	-	-	-	-	-	-
D-fucose	+	?	-	+	V	+	V	+
Galactose	+	-	-	-	+	-	V	-
Lactose	+	-	-	+	+	+	V	-
D-lyxose	+	?	-	-	-	?	V	+
Maltose	-	+	-	-	+	+	-	-
D-maltose	+	+	-	+	+	+	-	-
D-mannitol	-	-	-	-	+	-	-	-
D-mannose	-	-	-	+	-	-	-	-
Melezitose	-	-	-	+	-	-	-	-
Melibiose	+	-	-	+	-	V	-	-
D-raffinose	+	-	-	+	-	-	-	-
Rhamnose	+	-	-	+	+	+	+	-
Starch	+	-	-	+	-	-	-	-
Sucrose	+	-	-	+	-	-	-	-
Trehalose	-	+	-	+	-	V	-	-
D-turanose	+	-	-	V	-	?	-	-
D-xylose	+	+	-	+	-	+	-	+
Adonitol	-	-	-	-	-	-	+	-
Glycerol	-	-	-	+	-	-	-	-
Amygdalin	+	-	-	+	-	+	+	V
Arbutin	-	-	+	-	+	-	-	?
α-methyl-D-glucoside	+	-	-	+	-	+	-	-
β-methyl-D-xyloside	-	-	-	-	-	-	+	-
Salicin	-	V	-	-	-	-	-	-
N-acetyl-glucosamine	-	-	-	+	-	+	-	-
capric acid	-	+	-	+	-	-	-	+
malic acid	+	+	-	-	+	+	-	-
potassium gluconate	?	+	-	+	+	+	-	-
trisodium citrate	?	?	-	+	-	+	-	-

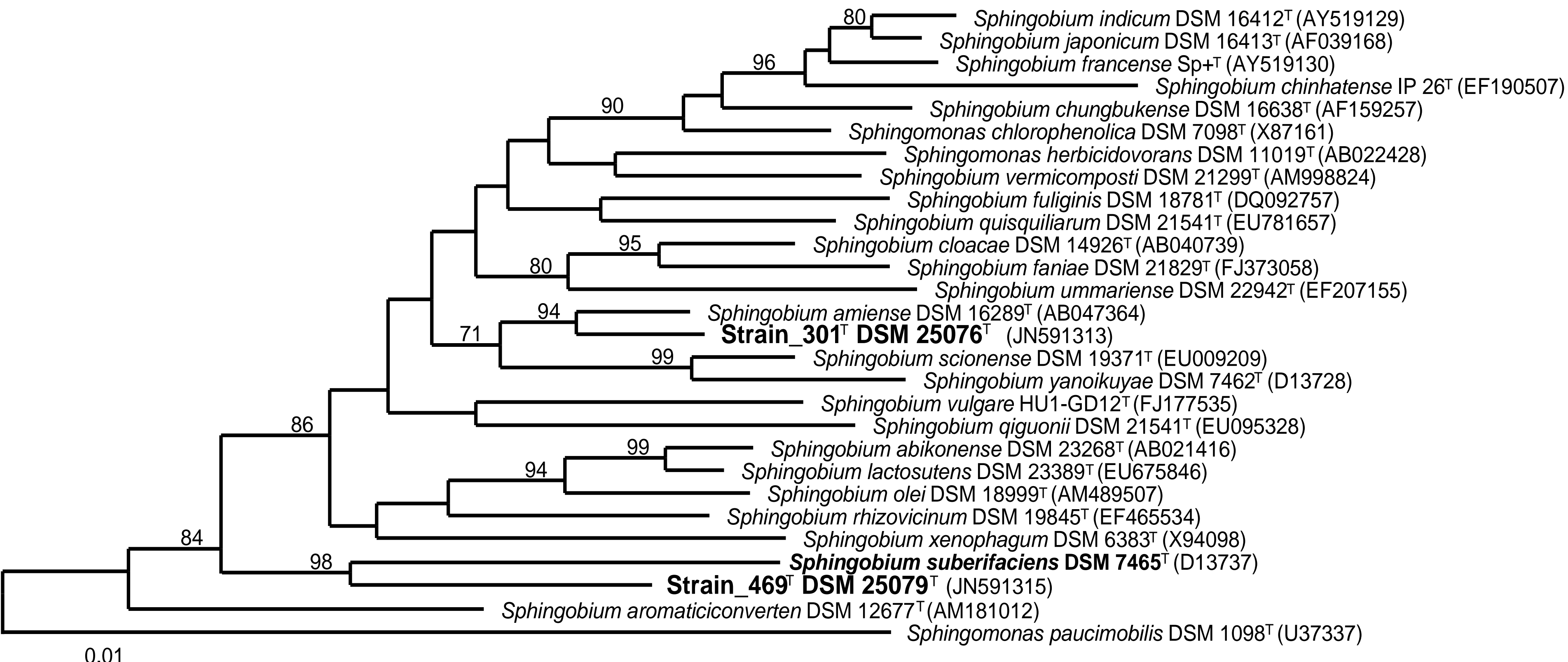

Fig. 19. Rooted neighbor-joining phylogenetic tree, based on 16 rRNA gene sequences showing the relationships among strain 301^T, 469^T, DSM 7465^T and other type species of *Sphingobium*. The tree was constructed using ARB software package (Ludwig et al., 2004). Numbers at nodes indicated the level (%) of bootstrap support based on 1000 re-samples dataset. *Sphingomonas paucimobilis* DSM 1098^T was used as the outgroup.

Table 8. Susceptibilities of strain 301^T, 469^T and related strains against 36 antibiotic agents.

Strains: 1, strain 301^T; 2, strain 469^T; 3, *Sphingobium suberifacien* DSM 7465^T; 4, *Sphingobium yanoikuyae* DSM 7462^T; 5, *Sphingobium amiense* DSM 16289^T; 6, *Sphingobium scionense* DSM 19371^T; 7, *Sphingobium vermicomposti* DSM 21299^T; 8, *Sphingobium japonicum* DSM 16413^T. 9, *Sphingomonas paucimobilis* DSM 1098^T. R, resistant; IM, intermediate; S, susceptible. All data were generated in this study.

antibiotic	1	2	3	4	5	6	7	8	9
Penicillin G	IM	IM	IM	IM	IM	R	S	IM	R
Oxacillin	R	R	IM	R	R	R	IM	R	R
Ampicillin	IM	IM	IM	IM	IM	R	S	R	IM
Ticarcillin	IM	IM	S	IM	IM	IM	S	IM	IM
Cefalotin	R	R	IM	R	R	R	IM	R	IM
Mezlocillin	R	R	IM	R	R	R	S	R	R
Cefazolin	IM	R	IM	R	R	R	S	R	IM
Cefotaxim	IM	IM	S	IM	S	IM	S	IM	S
Aztreonam	S	R	IM	R	R	R	R	R	R
Chloramphenicol	S	S	S	S	IM	IM	IM	S	S
Tetracyclin	S	S	S	S	S	S	R	S	S
Imipenem	S	S	S	S	S	S	S	S	S
Gentamycin	IM	IM	IM	IM	IM	IM	IM	IM	IM
Amikacin	S	S	IM	IM	S	IM	S	S	IM
Vancomycin	S	S	S	IM	S	IM	S	S	S
Erythromycin	S	S	S	IM	IM	S	S	S	S
Lincomycin	R	R	R	R	R	R	R	R	R
Ofloxacin	S	IM	S	IM	IM	IM	S	S	S
Colistin	IM	IM	IM	IM	IM	IM	IM	IM	IM
Norfloxacin	S	IM	S	IM	IM	IM	S	S	IM
Pipemidic	IM	R	S	R	R	R	S	R	R
Bacitracin	S	IM	S	IM	IM	R	S	S	IM
Polymyxin B	IM	IM	IM	IM	IM	IM	IM	IM	IM
Nitrofurantoin	S	R	IM	R	R	R	R	R	R
Neomycin	IM	S	IM	IM	IM	IM	IM	IM	IM
Kanamycin	S	S	S	IM	S	S	S	S	IM
Doxycycin	S	S	S	S	S	S	S	S	S
Clindamycin	IM	IM	R	IM	IM	R	IM	IM	IM
Ceftriaxone	IM	R	S	R	IM	R	S	R	IM
Fosfomycin	IM	IM	IM	IM	S	IM	S	IM	IM
Nystatin	R	R	R	R	R	R	R	R	R
Linezolid	IM	R	IM	R	IM	R	R	R	IM
Moxifloxacin	S	S	S	IM	S	S	S	S	S
Quinupristin/Dalfopristin	S	IM	S	IM	S	IM	S	S	IM
Piperacillin/tazobactam	S	R	S	IM	R	R	R	S	IM
Teicoplanin	S	IM	IM	IM	IM	IM	S	S	IM

Table 9. Cellular fatty acids profiles of strain 301T, 469T and the related species of the genus *Sphingobium*

Strains: 1, strain 301T; 2, strain 469T; 3, *Sphingobium suberifacien* DSM 7465T; 4, *Sphingobium yanoikuyae* DSM 7462T; 5, *Sphingobium amiense* DSM 16289T; 6, *Sphingobium scionense* DSM 19371T; 7, *Sphingobium vermicomposti* DSM 21299T; 8, *Sphingobium japonicum* DSM 16413T; 9, *Sphingomonas paucimobilis* DSM 1098T. All data were generated in this study. Values shown are percentages of the total fatty acids. -, not detected; tr, trace (<1%).

Fatty acids	1	2	3	4	5	6	7	8	9
$C_{14:0}$	-	tr	1.32	-	-	-	tr	tr	tr
$C_{14:0}$ 2OH	10.78	15.23	10.6	13.59	9.63	8.91	7.89	4.82	6.9
$C_{15:0}$	-	tr	-	-	tr	-	-	-	-
$C_{15:0}$ 2OH	-	tr	-	-	tr	-	tr	-	-
Summed feature 3	22.87	32.03	12.4	16.51	19.61	18.3	19.8	11.11	4.71
$C_{16:1}\omega5c$	1.25	1.48	1.44	1.44	1.77	1.71	1.07	1.53	tr
$C_{16:0}$	7.09	10.11	8.88	6.29	7.32	9.29	12.7	9.4	8.75
$C_{16:0}$ 2OH	tr	-	-	1.91	-	tr	tr	tr	-
$C_{16:1}$ 2OH	tr	-	-	-	tr	-	tr	-	-
$C_{16:0}$ ISO 3OH	tr	tr	tr	-	tr	-	-	tr	-
$C_{17:1}\omega8c$	-	-	-	-	tr	-	-	-	-
$C_{17:1}\omega6c$	1.35	1.92	1.37	tr	5.07	tr	1.29	tr	tr
$C_{18:1}\omega7c$	53.29	34.4	58.5	55.26	52.35	56.1	50.9	66.78	75.29
$C_{18:1}\omega5c$	tr	tr	tr	-	tr	tr	tr	1.48	1.77
$C_{18:0}$	-	-	-	tr	-	-	tr	tr	tr
11-Methyl $C_{18:1}\omega7c$	tr	1.81	3.53	2.6	1.26	2.84	3.72	2.93	-
$C_{19:0}$ cyclo $\omega8c$	-	-	tr	1	-	tr	tr	tr	-

Summed features represent groups of two or more fatty acids that could not be separated by gas chromatography with the MIDI system. Summed feature 3 contains $C_{16:1}\omega7c$ and/or $C_{15:0}$ ISO 2OH.

The predominant respiratory quinone was Q-10 (83.08% for strain 301T, and 16.92% were detected as Q-9). The major fatty acids were $C_{18:1}\omega7c$ (53.29%), summed feature 3 (contains $C_{16:1}\omega7c$ and/or C_{15} ISO 2OH) (22.87%), $C_{16:0}$ (7.09%), and $C_{14:0}$ 2OH (10.78%) was the major 2-hydroxy fatty acids, additionally, $C_{16:1}\omega5c$ (1.25%), $C_{17:1}\omega6c$ (1.35%) were identified as minor peaks (Table 9). The presence of $C_{14:0}$ 2OH and absence of any 3-hydroxy acids suggested that strain 301T belongs to the genus *Sphingobium*. And the quantitative differences of the fatty acids profiles among strain 301T to closely related species indicated that strain 301T represents a distinct species in the genus *Sphingobium*. The major polar lipids were diphosphatidylglycerol, phosphatidylglycerol, phosphatidylmonomethylethanolamine, phosphatidylethanolamine, phosphatidyldimethylethanolamine, phosphatidylcholine and two sphingoglycolipids (Fig. 20). The presence of the sphingoglycolipid was the characteristic components reported for member of

Sphingobium. Spermidine is the major polyamine and accompanied by smaller amounts of spermine and trace of putrescine and diaminopropane. This also a characteristic feature of the genus *Sphingobium*.

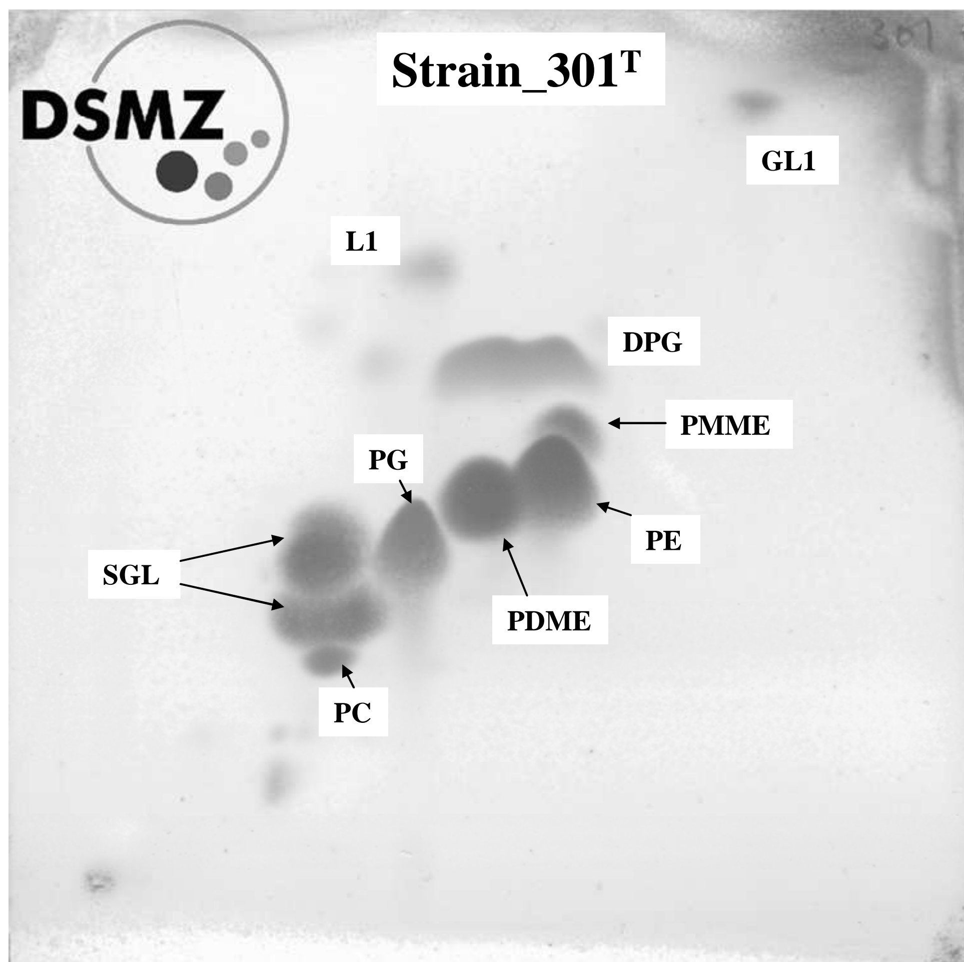

Fig. 20. Polar lipid pattern of strain 301^T after separation by two-dimensional thin-layer chromatogram (TLC), detected with anis aldehyde (for all lipids), ninhydrin (for amino groups), cis-aconitinacid-anhydride (for phosphate-containing lipids), meta-periodate /schiff (for vicinal hydroxy groups), molybdenum blue (for phosphate groups).

DPG, diphosphatidylglycerol; PG, phosphatidylglycerol; PE, phosphatidylethanolamine; PC, phosphatidylcholine; PMME, phosphatidylmonomethylethanolamine; PDME, phosphatidyldimethylethanolamine; GL1, glycolipids; SGL： sphingoglycolipids; L1, unidentified lipid.

Description of *Sphingobium limneticum* sp. nov *Sphingobium limneticum* (lim. ne. ti. cum Gr. n. *limnê*, pool of standing water, lake; L. neut. suff. -*ticum*, suffix denoting made of or belonging to; N.L. neut. adj. *limneticum*, from or belonging to a lake).

Colonies are yellow, circular, domed, convex on R2A agar, 1:10 diluted HD agar after 24 hours incubation. Cells are Gram-negative, asporogenous, rod-shaped (1.3-2.2 μ m long and 0.4-0.5 μ m wide), motile by peritrichous flagella, aerobic, catalase- and oxidase- positive. The isolate can grow at pH 5-10 (optimum pH 7) and temperature 10-40°C (optimum approx. 28°C).

Cells can not reduce nitrate; acid phosphatase, alkaline phosphatase, α-chymotrypsine, α-glucosidase, β-glucosidase, β-galactosidase, leucine arylamidase, naphthol-phosphohydrolase, valine arylamidase are hydrolysed. Amygdalin, arabinose, L-arabinose, D-fucose, galactose, D-glucose, lactose, D-lyxose, malic acid, maltose, α-methyl-D-glucoside, melibiose, D-raffinose, rhamnose, starch, sucrose, D-turanose and D-xylose are utilized. Cells are sensitive (inhibition zones >30 mm) to aztreonam (30 μg per disk), chloramphenicol (30), tetracyclin (30), imipenem (10), amikacin (30), vancomycin (30), erythromycin (15), ofloxacin (5), norfloxacin (10), bacitracin (10), nitrofurantoin (100), kanamycin (30), doxycycin (30), moxifloxacin (5), quinupristin/dalfopristin (15), piperacillin/tazobactam (40), teicoplanin (30); resistant to oxacillin (5), cefalotin (30), mezlocillin (30), lincomycin (15) and nystatin (100).

The Q-10 (83.08%) is the major respiratory quinone. The major fatty acids are C18:1ω7c (53.29%), Summed feature 3 (contains C16:1ω7c and/or C15 ISO2-OH) (22.87%), C16:0 (7.09%), and C14:0 2OH (10.78%) is the major 2-hydroxy fatty acids. The major polar lipids are diphosphatidylglycerol, phosphatidylglycerol, phosphatidylmonomethylethanolamine, phosphatidylethanolamine, phosphatidyldimethylethanolamine, phosphatidylcholine and two sphingoglycolipids. The major polyamine was spermidine. The genomic DNA G+C content of strain 301^T was 63.4 mol%. Based on these results, it is proposed that strain 301^T represents a novel species of the genus *Sphingobium*, which is named as *Sphingobium limneticum*. The type strain is 301^T (=DSM 25076^T=LMG 26659^T) isolated from fresh lake water of Starnberger See, Germany.

Description of **Sphingobium boeckii** *sp. nov., and reclassification of* **Sphingomonas suberifaciens** *as* **Sphingobium suberifaciens** *comb.nov.*

Strain 469^T formed yellow, circular, domed, convex colonies on R2A agar, PYE agar and 1:10 diluted HD agar after 48 hours incubation. Cells were Gram-negative, asporogenous, rod-shaped (0.7-1.2 μ m long and 0.35-0.45 μ m wide), motile by one or two subpolar monotrichous flagella (Fig. 21), aerobic, catalase- and oxidase- positive. The isolate could grow at pH 5-10 (optimum pH 7) and temperature 10-40°C (optimum approx. 28°C). Other phenotypic characteristic of strain 469^T and the closely related type strains are listed in species

description and Table 7, 8.

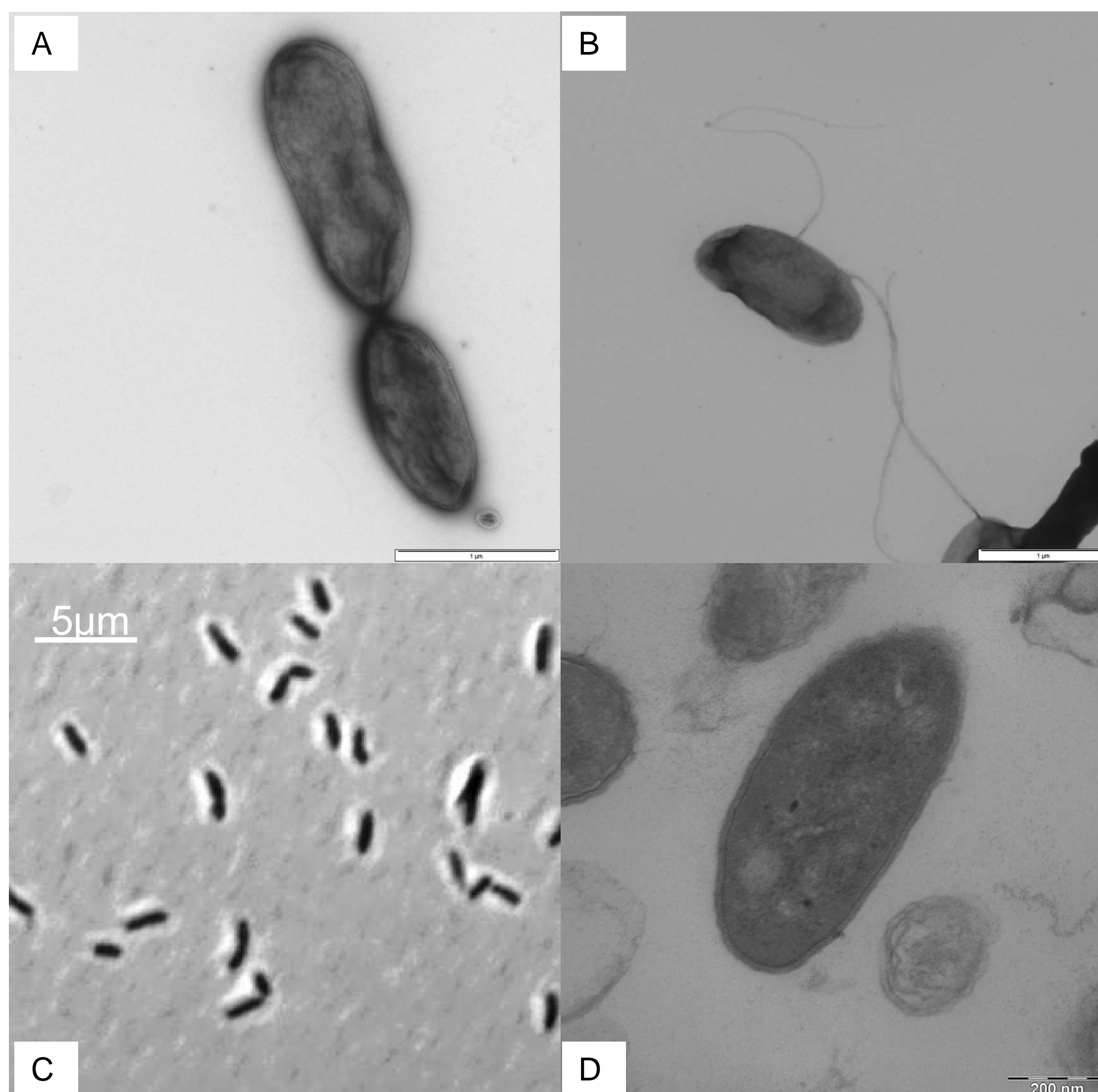

Fig. 21. Phase-contrast micrograph and transmission electron micrographs of strain 469^T. (**A, B**), TEM-image of negative staining cells, bar, 1 μm; (**C**), phase-contrast image, bar, 5 μm; (**D**), TEM image of thin sectioned cells, bar, 200 nm.

The predominant fatty acids were $C_{18:1}\omega7c$ (34.4%), summed feature 3 (contains $C_{16:1}\omega7c$ and/or C_{15} ISO2-OH) (32.03%), $C_{16:0}$ (10.11%), $C_{14:0}$ 2OH (15.23%) was major 2-hydroxy fatty acids. In addition, 11-Methyl $C_{18:1}\omega7c$ (1.81%), $C_{17:1}\omega6c$ (1.92%), $C_{16:1}\omega5c$ (1.48%) were detected as minor peaks (Table 9). The presence of $C_{14:0}$ 2OH and absence of any 3-hydroxy acids suggested that strain 469^T belongs to the genus *Sphingobium*. And the quantitative differences of the fatty acids profiles among strain 469^T to closely related species indicated that strain 469^T represents a distinct species in the genus *Sphingobium*. For strain 469^T, nearly 100%

of respiratory quinone was detected as lipiquinon-10 (Q-10), Q-9 was detected as trace. The major polar lipids were diphosphatidylglycerol, phosphatidylglycerol, phosphatidylethanolamine, phosphatidyldimethylethanolamine, phosphatidylcholine, phosphatidylmonomethylethanolamine and sphingoglycolipids. Image of the TLC polar lipid profiles are shown in Fig. 22. The major polyamine was spermidine acocompanied by small amount of spermine and putrescine and trace of diaminopropane and cadaverine. The chemotaxonomical results of strain 469^T supported its assignment to the genus *Sphingobium*.

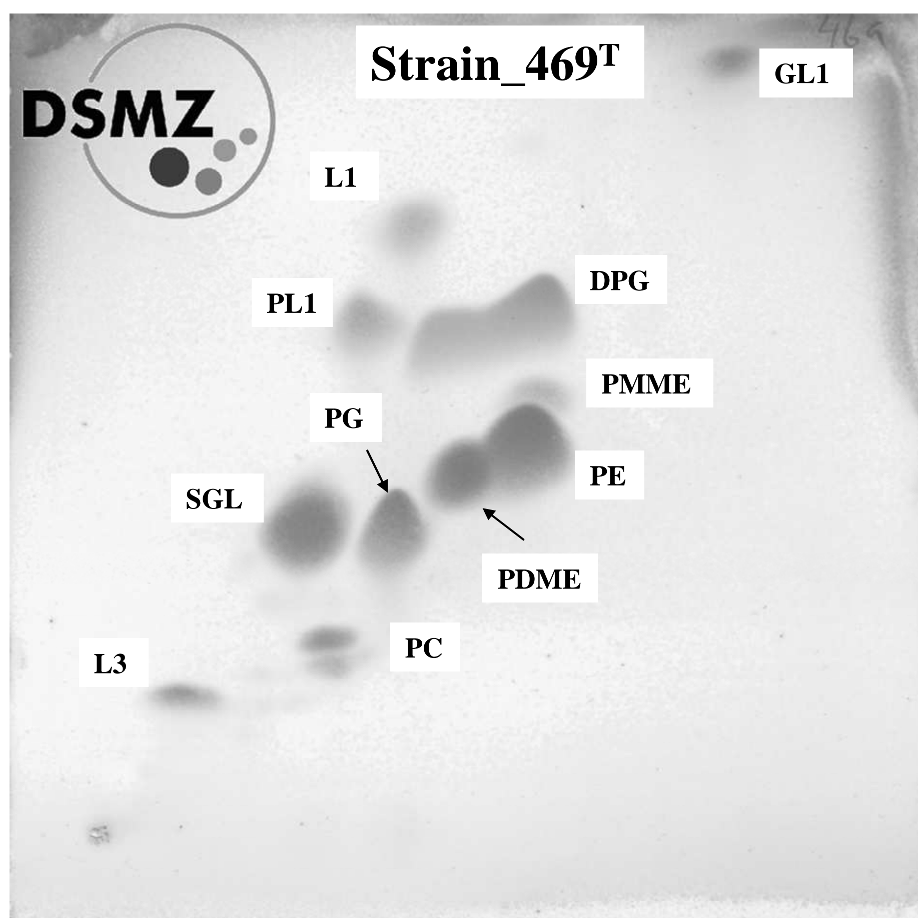

Fig. 22. Polar lipid pattern of strain 469^T after separation by two-dimensional thin-layer chromatogram (TLC), detected with anis aldehyde (for all lipids), ninhydrin (for amino groups), cis-aconitinacid-anhydride (for phosphate-containing lipids), meta-periodate/schiff (for vicinal hydroxy groups), molybdenum blue (for phosphate groups).

DPG, diphosphatidylglycerol; PG, phosphatidylglycerol; PE, phosphatidylethanolamine; PC, phosphatidylcholine; PMME, phosphatidylmonomethylethanolamine; PDME, phosphatidyldimethylethanolamine; GL1, glycolipids; SGL：sphingoglycolipids; L1, L3, unidentified lipid; PL1, unidentified phospholipids.

A nearly complete (1493 bp) 16S rDNA gene sequence of strain 469^T was determined. From the comprehensive phylogenetic analysis of 16S rRNA gene sequences which included sequences from the type strains of species in the genera *Sphingomonas* and *Sphingobium*, strain 469^T was clustered within the genus *Sphingobium*. *Sphingomonas suberifaciens* DSM 7465^T and *Sphingobium scionense* DSM 19371^T were the closest neighbors (97.1%, 96.5% similarity, respectively). *Sphingobium yanoikuyae* DSM 7462^T (type species of this genus *Sphingobium*) exhibited 95.9% similarity and *Sphingomonas paucimobilis* DSM 1098^T (type species of genus *Sphingomonas*) exhibited 94.1% similarity to strain 469^T (Fig. 19). DNA-DNA relatedness between strain 469^T and *Sphingomonas suberifaciens* DSM 7465^T was 17.9% (mean value of measurements in duplicate), which was much lower than the recommendations of a threshold value of 70% DNA-DNA relatedness for the definition of bacterial species by the *ad hoc* committee (Wayne *et al.*, 1987). So, strain 469^T did not belong to the species *Sphingomonas suberifaciens*, and this strain probably represents to a novel species. The DNA G+C content of strain 469^T was 64.6%.

For strain 469^T, even the characteristics of the 16S rRNA signature of strain 469^T were neither the same as genus *Sphingomonas* or as genus *Sphingobium*, but nevertheless showed higher similarity with cluster *Sphingobium* than *Sphingomonas*. Also, all of the closely related species belonged to the genus *Sphingobium*. Since also typical physiological and phylogenetic characteristics were characteristic for the genus *Sphingobium*, strain 469^T was affiliated to the genus *Sphingobium*.

The closest species to the strain 469^T was *Sphingomonas suberifaciens*. However, according to the phylogenetic, chemotaxonomic and physiological analysis results (Fig. 18, S3, S4; Table 7, 8, 9), *Sphingomonas suberifaciens* seems to be misnamed. From the newest ARB database of 16S rRNA gene sequences, the representatives of the species *Sphingomonas suberifaciens* clustered within the genus *Sphingobium* (Fig. 19). The characteristic 16S rRNA signature of this species was the same as *Sphingobium*. Similar support came from the polyamine analysis; the major polyamine of *Sphingomonas suberfacien* was spermidine but not homospermidine. All other characteristics detected from this strain fitted to the description of genus *Sphingobium*, so this species was reclassified as *Sphingobium suberfaciens*.

Description of *Sphingobium boeckii* sp. nov *Sphingobium boeckii* (boe. ckii N.L. gen. masc. n. *boeckii*, of Böck, in honour of August Böck (born 1937), a renowned German microbiologist, for his contributions to general microbiology and microbial biochemistry).

Colonies are yellow, circular, domed, convex on R2A agar, 1:10 diluted HD agar after 48 hours incubation. Cells are Gram-negative, asporogenous, rod-shaped, 0.7-1.2 μ m long and 0.35-0.45 μ m wide, motile by one or two subpolar monotrichous flagella, aerobic, catalase- and oxidase- positive. The isolate can grow at pH 5-10 (optimum pH 7) and in a temperature range of 10-40(C (optimum approx. 28(C). Nitrate is not reduced; produces acid phosphatase, alkaline phosphatase, esterase lipase (C8), β-galactosidase, β-glucosidase, leucine arylamidase, naphthol-phosphohydrolase are hydrolysed. Arabinose, capric acid, cellobiose, esculin, N-acetyl-glucosamine, D-glucose, malic acid, maltose, potassium gluconate, trehalose, D-xylose are utilized and L-arabinose, β-gentiobiose, D-lyxose, D-fucose and salicin are weakly utilized. Cells are sensitive (inhibition zones >30 mm) to chloramphenicol (30 μ g per disk), tetracyclin (30), imipenem (10), amikacin (30), vancomycin (30), erythromycin (15), neomycin (30), kanamycin (30), doxycycin (30), moxifloxacin (5); resistant to oxacillin (5), cefalotin (30), mezlocillin (30), cefazolin (30), aztreonam (30), lincomycin (15), pipemidic (20), nitrofurantoin (100), ceftriaxone (30), nystatin (100), linezolid (10) and piperacillin/tazobactam (40).

The Q-10 (nearly100%) is the major respiratory quinone. The predominant fatty acids are $C_{18:1}\omega7c$ (34.4%), summed feature 3 (contains $C_{16:1}\omega7c$ and/or C_{15} ISO2-OH) (32.03%), $C_{16:0}$ (10.11%), $C_{14:0}$ 2OH (15.23%) is major 2-hydroxy fatty acids. The major polar lipids are diphosphatidylglycerol, phosphatidylglycerol, phosphatidylethanolamine, phosphatidyldimethylethanolamine, phosphatidylcholine, phosphatidylmonomethylethanolamine and sphingoglycolipids. The major polyamine is spermidine. The genomic DNA G+C content of strain 469^T is 64.6mol%. Based on these results, strain 469^T is proposed to represent a new species of the genus *Sphingobium*, which named as *Sphingobium boeckii*. The type strain is 469^T (=DSM 25079^T=LMG $xxxxx^T$) isolated from fresh lake water of Walchensee, Germany.

Description of *Sphingobium suberifaciens* comb. Nov *Sphingobium suberifaciens* (su.be.ri.fa'ci.ens. L. gen. n. *suberis,* of cork, corky; L. part. adj. *faciens,* making, producing; M.L.part. adj. *suberifaciens,* corky making).

The description is identical to the description given for *Sphingomonas Suberifacien* by Van Bruggen *et al.* (1990) and amended by Yabuuchi *et al.* (1999) and Takeuchi *et al.* (2001). The type strain is strain $Ca1^T$ = EY 2404^T = ATCC 49355^T = CIP 105429^T = DSM 7465^T = ICMP 12535^T = IFO (now NBRC) 15211^T = JCM 8521^T = LMG 17323^T = NCPPB 3629^T isolated from corked lettuce roots in California.

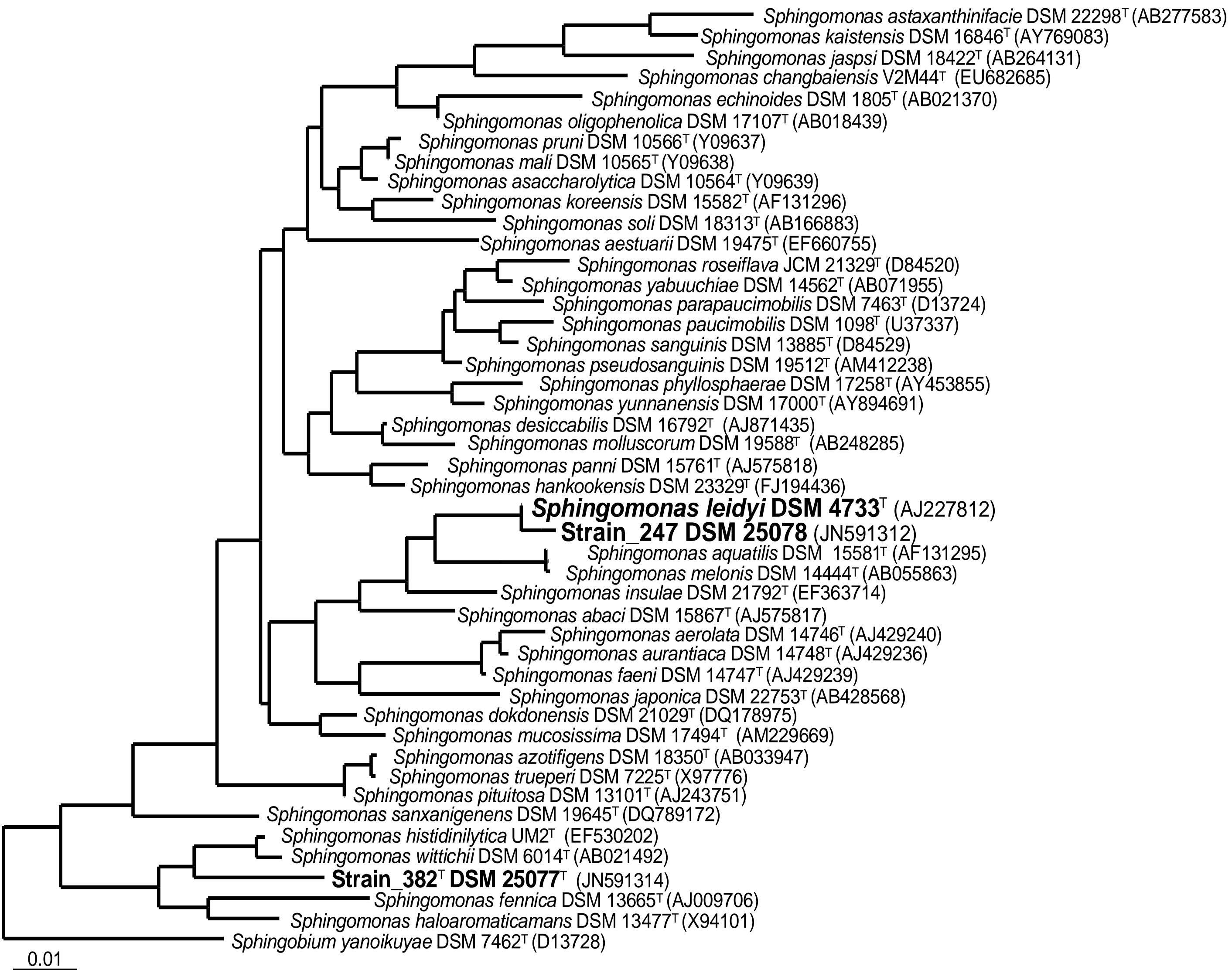

Fig. S1. Maximum-likelihood tree, based on 16 rRNA gene sequences showing the relationships among strain 247, 382^T, *Sphingomonas leidyi* DSM 4733^T and other type species of *Sphingomonas*. The tree was constructed using ARB software package (Ludwig *et al.*, 2004). *Sphingobium yanoikuyae* DSM 7462^T was used as the outgroup.

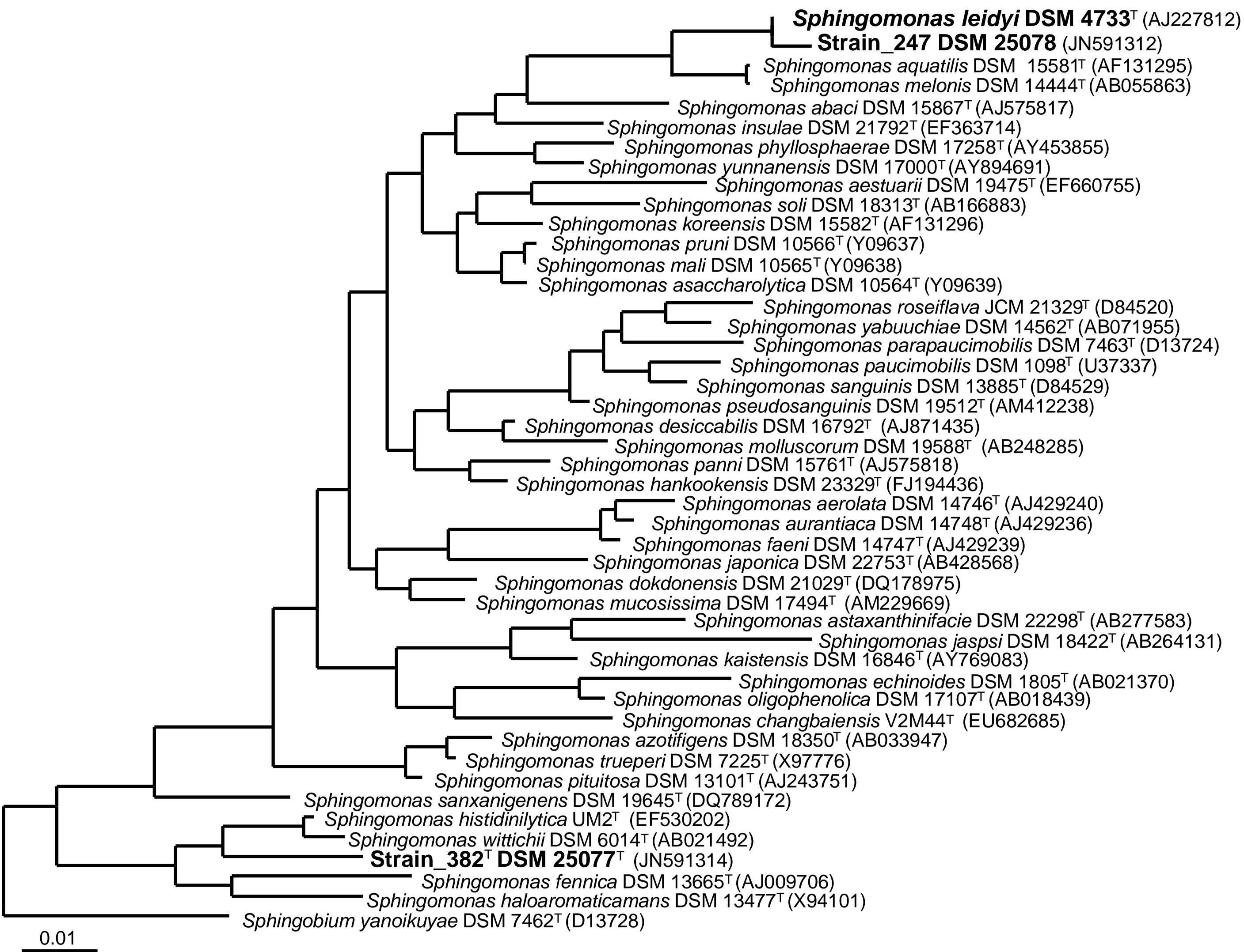

Fig. S2. Maximum-parsimony tree, based on 16 rRNA gene sequences showing the relationships among strain 247, 382^T, *Sphingomonas leidyi* DSM 4733^T and other type species of *Sphingomonas*. The tree was constructed using ARB software package (Ludwig *et al.*, 2004). *Sphingobium yanoikuyae* DSM 7462^T was used as the outgroup.

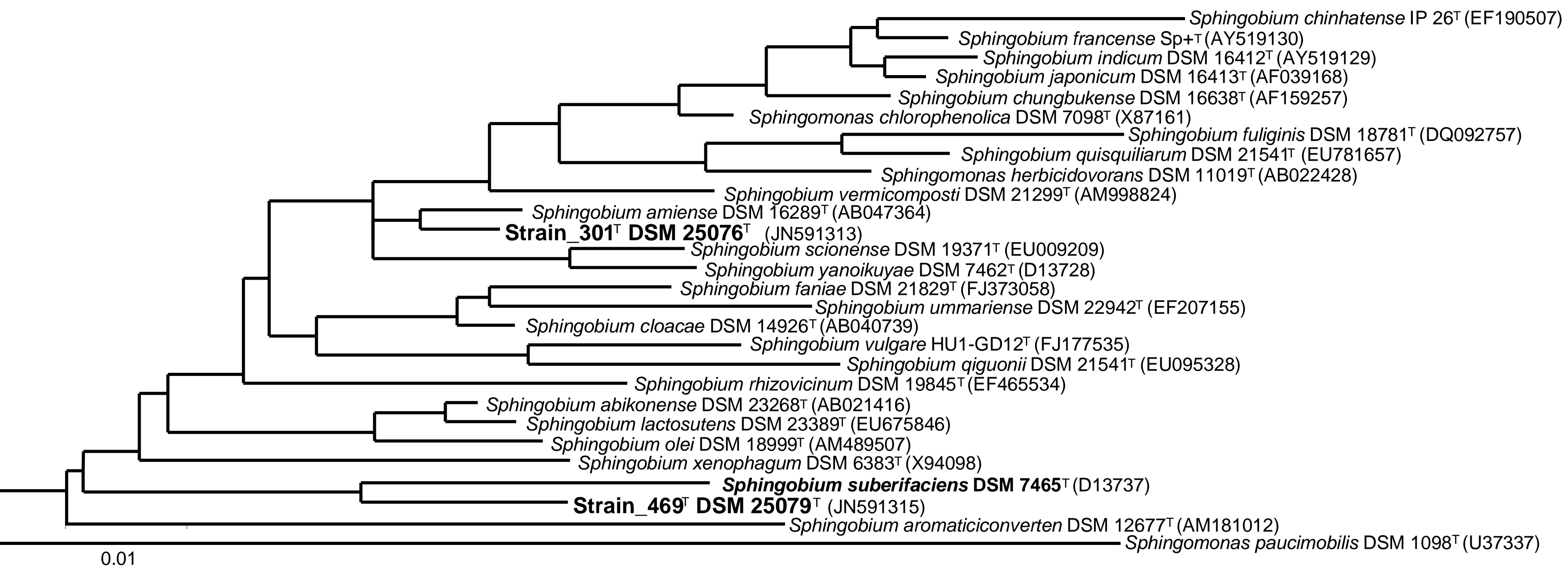

Fig. S3. Maximum-likelihood tree, based on 16 rRNA gene sequences showing the relationships among strain 301^T, 469^T, *Sphingobium suberifaciens* DSM 7465^T and other type species of *Sphingomonas*. The tree was constructed using ARB software package (Ludwig *et al.*, 2004). *Sphingomonas paucimobilis* DSM 1098^T was used as the outgroup.

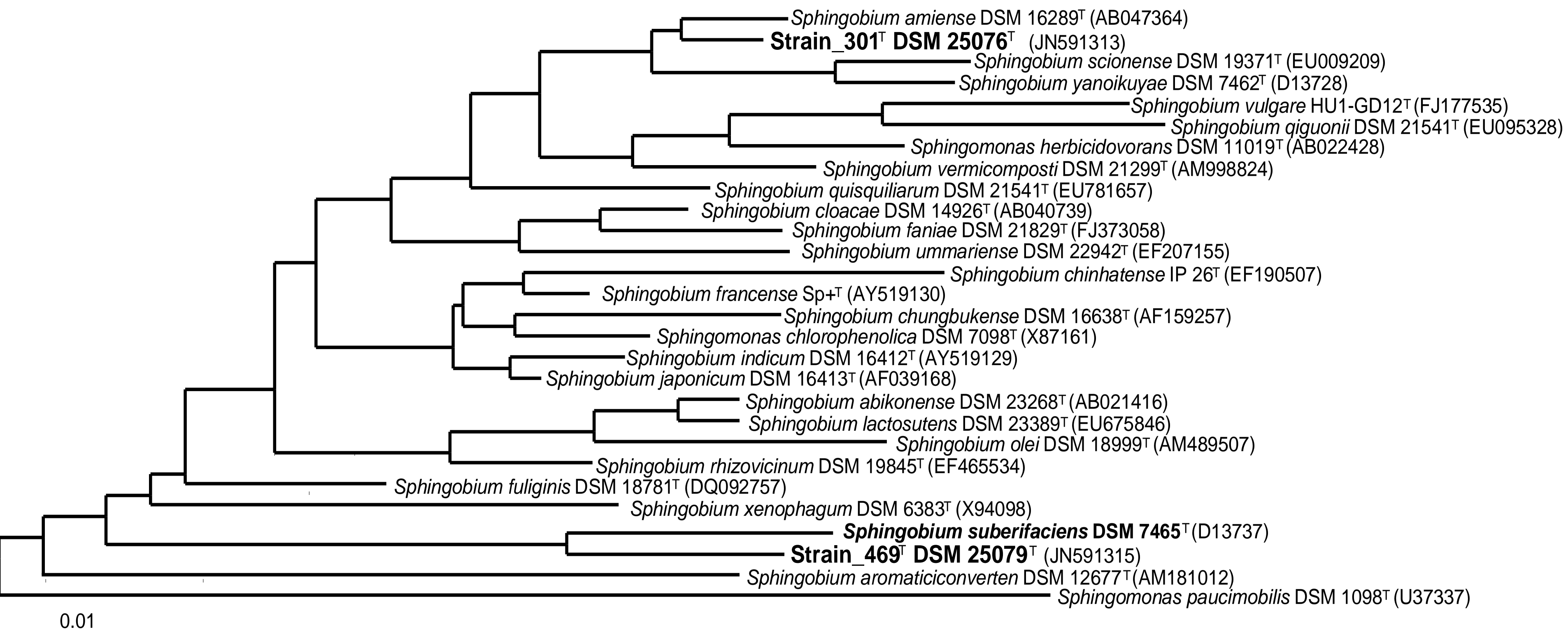

Fig. S4. Maximum-parsimony tree, based on 16 rRNA gene sequences showing the relationships among strain 301^T, 469^T, *Sphingobium suberifaciens* DSM 7465^T and other type species of *Sphingomonas*. The tree was constructed using ARB software package (Ludwig *et al.*, 2004). *Sphingomonas paucimobilis* DSM 1098^T was used as the outgroup.

Chapter 5

Discussion

Complex Population structure of freshwater Sphingomonadaceae

Sphingomonadaceae represent the typical constituent of freshwater bacterioplankton communities and can be recovered by using low nutrient liquid media (Gich *et al.*, 2005). In the 16S rRNA sequence analysis of the 95 isolates of *Sphingomonadaceae*, 6 phylotypes were identified with multiple isolates. G1A was the dominant group, as was proved by a parallel study (Jogler *et al.*, 2011), culture independent analyses of 16S rRNA genes and 16S rRNA demonstrated that this particular phylotype G1A in fact dominates the natural population of *Sphingomonadaceae in situ* and is metabolically active throughout all seasons.

As populations of certain marine planktonic bacteria have been demonstrated to harbor considerable DNA polymorphisms (Thompson *et al.*, 2005; Vergin *et al.*, 2007; Hunt *et al.*, 2008) the 16S rRNA can not distinguish closely related isolates. The phylogenetic analysis based on the concatenated sequences of nine housekeeping genes from 95 strains of *Sphingomonadaceae* isolated from Walchensee and Starnberger See here also reveled high DNA polymorphisms. The genetic diversity detected for G1A strains sharing the same 16S sequence is unusually high and significantly surpasses that of the marine oligotrophic `*Candidatus* Pelagibacter ubique´ (π=0.004-0.056; Vergin *et al.*, 2007), of *Synechococcus* populations in an alkaline siliceous hot spring microbial mat (0-7.8% sequence divergence; Melendrez *et al.*, 2011), of the soil bacterium *Bacillus simplex* (π=0-0.05; Sikorski and Nevo, 2005), of the human pathogens *Vibrio cholerae* (0-5.9% sequence divergence for concatenated sequences; Keymer and Boehm, 2011) and *Neisseria meningitidis* (*S*=3.4-27.1%; Jolley *et al.*, 2005), and of the plant pathogen *Pseudomonas syringae* (π=0.042-0.100; Sarkar and Guttman, 2004, θ_S = 0.0032-0.0145 $bp^{-1} generation^{-1}$; Yan *et al.*, 2008). Bacteria with identical 16S rRNA phylotypes in some cases have been suggested to harbour ecologically distinct subgroups that differ with respect to morphology, carbon substrates, maximum growth rate, and internal transcribed spacer (ITS) sequence types (Jaspers and Overmann, 2004), with respect to fatty acid composition, growth rates under temperature stress, and carbon substrates (Sikorski and Nevo, 2007; Koeppel *et al.*, 2008; Sikorski *et al.*, 2008a,b), or genomic structure, metabolomes and phage susceptibility (Peña *et al.*, 2010). It has previously been suggested that populations that have not yet been

purged by periodic selection or drift may hold up to 1-2% sequence divergence in concatenated housekeeping genes (Koeppel *et al*., 2008). The significantly higher genetic diversity of G1A prompted us to conduct a detailed analysis of the population substructure of the freshwater *Sphingomonadaceae*, of the underlying evolutionary forces and the associated physiological diversity.

Theoretically, the population substructure of the freshwater *Sphingomonadaceae* isolates suggested by MLSA could be the result of some unknown selection bias during cultivation. The splitstree analysis of the *gyrB* gene from environmental DNA was compared with the *gyrB* from isolates. The results proved that the inclusion of cultivation-independent clones did not provide evidence for a continuum of divergence and supports the conclusion of a distinct population substructure of the freshwater *Sphingomonadaceae* in Walchensee and Starnberger See.

Evolutionary forces underlying the population structure

MLSA revealed the presence of distinct subgroups among individual 16S rRNA sequence types, suggesting different selection pressure between subclusters and the existence of distinct evolutionary units despite the identical or very similar 16S rRNA gene sequences. The high divergence suggests these strains experienced marked evolutionary events.

The presence of numerous polytomies in the consensus tree indicated a significant effect of recombination on the population structure. Indeed, an initial phi test for recombination (Bruen *et al*., 2006) also revealed statistically significant evidence for recombination for the entire dataset. The population recombination rates calculated from the individual genes for all strain or only from G1A strains are comparable with the gene-specific values that are available for *Bacillus simplex* (0-0.042 $bp^{-1}generation^{-1}$; Sikorski and Nevo, 2005) *Pseudomonas syringae* (0.0012-0.155 $bp^{-1}generation^{-1}$; Yan *et al*., 2008), and *Neisseria meningitidis* (0.003-0.034 $bp^{-1}generation^{-1}$; Jolley *et al*., 2005). This result makes the presence of extraordinarily effective recombination barriers between the different *Sphingomonadaceae* genomes rather unlikely. The population mutation rate surpassed the values reported. All the data from phylogenetic analysis place the freshwater *Sphingomonadaceae* isolates in the middle rank list of homologous recombination rates determined for approximately 48 species (Vos and Didelot, 2008). Most notably, the relative impact of recombination on population structure in the freshwater *Sphingomonadaceae* isolates is markedly lower than in most of the other free-living aquatic bacteria investigated, including `*Candidatus* Pelagibacter ubique´, *Vibrio parahaemolyticus*, *V. vulnificus* and *Microcystis aeruginosa* for which *r/m* values between 18.3 and 63.1 were

determined (Vos and Didelot, 2008). Based on the phylogenetic analysis, the ρ/θ_S values from every gene of all strains and G1A strains were smaller than 0.25 except the *tuf* gene. Normally, the clonal bacterial populations has this value below and sexual populations above the values of 0.25 - 2.0 (Fraser *et al*., 2007). Additionally, the estimate of θ_S and N_e are larger than those used in these simulations. So, the low ratios ρ/θ determined for freshwater *Sphingomonadaceae* still suggest that their population structure is clonal rather than sexual.

Linkage equilibrium was used to determine the sufficiency of recombination. The values of the standardized index of association (I_A^S) of eight individual subpopulations were low and not significant, suggesting that the recombination within the eight individual subpopulations is rather free or panmictic. In contrast, all pairwise combinations of eight subpopulations yielded large and significant I_A^S values, suggesting barriers for free recombination between subpopulations (linkage equilibrium). Similar to the freshwater *Sphingomonadaceae*, a significant linkage disequilibrium has been detected in *Vibrio cholerae* despite a considerable frequency of recombination (I_A^S=0.143-0.439; Keymer and Boehm, 2011). With the null hypothesis of free combination between the eight different subpopulations being rejected, the results of our multipronged recombination analysis thus indicate incipient sexual isolation but not a full inhibition of homologous recombination between the eight subpopulations identified by MLSA. This sexual isolation may be the result of niche separation and/or genetic barriers and obviously even exists between the three subpopulations of the identical 16S rRNA phylotype G1A.

The Tajima´s D and MacDonald-Kreitman (MK) tests were used to test selective neutrality. Although both tests can be used to detect deviation from neutral evolution, they address different characteristics of the sequence divergence and may therefore yield different results. The results of Tajima´s D indicate a lack of selection pressure and also a lack of recent demographic events such as bottlenecks or population expansion in generating the nucleotide diversity observed for the nine housekeeping genes. The MK test, in contrast to Tajima´s D test, distinguishes between polymorphic and fixed substitutions at both synonymous and nonsynonymous sites. When the MK test was applied to study whether selection pressure may have been important in separating the observed eight individual subpopulations, neutrality indices did not yield any evidence for selection pressure acting within these clusters. And the deviations from the neutral model detected between the larger clusters G1A, G2, and G3 and only for some of the genes, suggest that any potential selection pressures driving the divergence of the subgroups like G1A1, G1A2, and G1A3 do not act on the nine housekeeping genes studied here.

Natural selection is strong in large populations, whereas random genetic drift increases in

power for small populations. The N_e values detected here for the freshwater *Sphingomonadaceae* belong to the largest values determined in a comparative study for a diverse set of prokaryotes (Lynch and Conery, 2003). Hence, the power of random genetic drift to shape the observed population structure most likely has been low. Recent bottlenecks may create scenarios in which N_e is temporarily reduced, allowing for a larger possibility of drift to fix differentiating nucleotides in cladogenesis. And the Tajima´s D values were generally close to zero, which does not allow to deduce recent bottlenecks but rather suggest a stable long-term population structure. Finally, the *Sphingomonadaceae* dispersed evenly across the subpopulations indicating that at least short-distance geographic barriers did not affect the population structure.

Metabolic diversification and its role in niche invasion

Ecotype Simulation tries to model the evolutionary dynamics of bacterial populations based on sequences, then to thereby identify the ecotypes within the natural community. Here, an ecotype is modeled as an ecologically distinct group, the diversity is limited by cohesion, and genome-wide diversity is purged by periodic selection and genetic drift. Ecotype Simulation (ES) has the advantage to be independent of the availability of environmental data associated with the different MLSA types (Koeppel *et al.*, 2008; Connor *et al.*, 2010). The results of the ES performed for all housekeeping genes suggested that the 8 clonal subpopulations identified each consist of several putative ecotypes. The large numbers of putative ecotypes hypothesized by Ecotype Simulation to exist within the G1A group suggests an enormous ecological diversity within the single 16S phylotype of G1A.

Biolog Gen III microplates were chosen for the phenotypic analysis of 48 strains from all groups. The results of the assessment of phenotypic differences in different levels of population substructure can provide a “phenotypic fingerprint” of the bacteria which can then be used to identify the strains at the species level. Besides addressing the ability of the cell to metabolize as sole carbon source representatives from all major classes of biochemicals, the Gen III microplate also contains 23 chemical sensitivity assays for the detection of the important physiological proterties like pH, salt, lactic acid tolerance, reducing power and chemical sentitivity (Biolog, part 00A 0XX, Rev A 08/2007). There is a difference between G1A strains to G2 and G3 strains in the utilization of several sugars and their monomeric constituent. The inability of monomeric constituents utilization may suggest a lack or inactivity of glycosidic enzymes or of respective disaccharide transporters of strains from G1A group. The phylogenetically closest genomes of *Novosphingobium aromaticivorans* DSM 12444^T (NC_007794), *Novosphingobium* sp. PP1Y

(NC_015580), *Sphingobium japonicum* UT26^T (NC_014006, NC_014013), *Sphingopyxis alaskensis* RB2256^T (NC_008048), and *Sphingomonas wittichii* RW1^T (NC_009511) revealed multiple genes for glycosidic exoenzymes that typically clustered together, whereas suitable transporters for the oligosaccharides substrates were not detected. Possibly, oligosaccharide substrates are hydrolyzed outside of the cell and the monomers imported through a PTS system. Interestingly, *Sphingomonas wittichii* RW1^T has no genes for glycosidic exoenzymes, which could suggest loss of these genes by deletion. In a similar manner, the absence of glycosidic exoenzyme activity in the G1A group may be due to absence of the gene or alternatively, an impaired sec-pathway.

The diversity of the phenotype increases only when all groups were combined, suggesting that the phenotypic similarity in general only reflects the split into the major groups as identified by the 16S rRNA phylogeny, but not the subpopulation within the identical 16S rRNA phylotypes. The G1A strains differ substantially from G2 and G3, but are quite homogeneous, and do not split phenotypically into the observed MLSA subgroups. Whereas our phylogenetic population genetic and ecological modeling analyses are consistent and provide strong evidence for the existence of ecologically distinct groups, the physiological testing clearly demonstrated distinguishing traits between the major groups of *Sphingomonadaceae* that had different 16S rRNA phylotypes, but selective adaptations that may be involved in the specific adaptations and niche invasion of the G1A subpopulations were not found among standard metabolic traits. In sum, the tested phenotypic traits apparently do not account for the split into the G1A MLSA groups, suggesting that other selection pressures must be active.

The application of MLSA to systematics

The 'species' category is regarded to be the fundamental taxonomical unit in the microbial world. MLSA has since long been considered to be a very powerful genotyping tools in the study of the evolution and population structure of microbial organism and also represents a novel standard in microbial molecular systematics. In order to differentiate bacterial strains at the level of the 'species' category, normally at least two genotypic methods are needed. The results of these two methods must be coherent to each other. The first method is comparing 16S rRNA gene sequences to determine the genetic distance between isolates at the level of the 16S rRNA. Distance values of 16S rRNA gene sequences above 3% are thought to be clearly discriminative at the species level, above 5% at the genus level, above 10% at the family/class level (Schloss & Handelsman, 2004; Stackebrandt *et al.*, 2002). In this study, we found that strains of

Sphingomonadaceae were identical in their 16S rRNA gene sequence but nevertheless comprised distinct genotypes at the level of housekeeping genes. The MLSA results of concatenated sequences of nine housekeeping genes was used to infer their phylogeny by means of a distance-matrix. This approach is expected to have an increased resolving power due to the large number of characters analyzed (6972 bp), and a lower sensitivity to the impact of conflicting signals (i.e. phylogenetic incongruence) that result from eventual horizontal gene transfer events (Rokas *et al.*, 2003). Genetic distance of concatenated sequences was compared with whole genomic DNA-DNA hybridization (WDDH). Based on our distance matrix, the similarity value of the concatenated sequences of subpopulations below 0.75 should be different genera, and the value above 0.94 should be the same species. So, further investigations and a broader collection of the bacteria are needed. The MLSA similarity value of 97.9%, therefore, appears to be useful as a cut-off to differentiate *B. burgdorferi* s. L. species. *B. spielmani* appeared to be closely related to one other, while differing from other species by similarity values of 92.1%-94.8% (Richter *et al.*, 2006). For the genus *Bacteroides*, concatenated sequences of three housekeeping genes (*dnaJ*, *gyrB* and *hsp60*) with cut-off value 97.5% similarity were reliable for species delineation (Sakamoto *et al.*, 2011). Studies of *Alteromonas macleodii* (Ivars-martinez *et al.*, 2008) and *Agrobacterium* (Aujoulat *et al.*, 2011) demonstrate that robust phylogeny and species differentiation can be achieved with MLSA of only several marker genes. MLSA has been used successfully to explore clustering patterns among large number of strains assigned to very closely-related species by current taxonomic method (Godoy *et al.*, 2003; Hanage *et al.*, 2005a, b, 2006; Hoshino *et al.*, 2005; Bennett *et al.*, 2007; Kilian *et al.*, 2008; Bishop *et al.*, 2009), to look at the relationships between small numbers of strains within a genus (Martens *et al.*, 2008), or within a broader taxonomic grouping (Sawabe *et al.*, 2007). If performed with a sufficient number of appropriately selected loci, the sequences derived from MLSA could also be used to infer the average nucleotide identity (ANI) of the genomes and also to calculate the genome-to-genome distance (GGD), the latter two methods were suggested to substitute DDH by bioinformatic method in species delineation (Richter and Rossello-Mora, 2009; Auch *et al.*, 2010). The clustering of strains obtained by MLSA is strongly associated with the clustering from DNA-DNA relatedness values. Therefore, MLSA constitutes a valuable alternative to laborious DNA-DNA hybridization (Richter *et al.*, 2006).

Now, an electronic taxonomic way for bacteria using MLSA is available as eMLSA (www.emlsa.net), it is the implementation of the MLSA approach to the assignment of strains to species clusters via the internet (Bishop *et al.*, 2009). eMLSA requires the generation of a large

database and the software for concatenating the sequences and producing a tree to show the clustering pattern of the sequences of the multiple house-keeping loci (typically about seven loci) from multiple strains of a set of related species of interest. By comparing the assignments of the strain within each cluster and the position of the type strain of each species, every strain can be assigned into species clusters. To assign the query strain correctly, a sufficiently large database is needed. At moment, there are only two databases (*Viridans* group streptococci and *Burkholderia* spp.) available, more effort is needed.

Polyphasic analysis in differentiating species

A strain identified as 'Caulobacter leidyi' was used in the MLSA analysis, which revealed that it should be affiliated to *Sphingomonadaceae*. A polyphasic analysis was performed with the strain of this species. The genus *Caulobacter* was originally described in 1935 (Henrici & Johnson, 1935) and later amended to comprise a variety of dimorphic prosthecate bacteria (Poindexter, 1981b, 1989; Schmidt 1981). Prior to nucleotide sequence analysis, species of the genus *Caulobacter* were distinguished based on their cell morphology, salt tolerance, carbon substrate utilization and growth factor requirements (Poindexter, 1989). Subsequently, 16S rRNA gene sequence comparisons revealed that bacteria exhibiting a developmental life cycle with asymmetric binary fission are paraphyletic and form at least 6 phylogenetically distant groups, suggesting that prosthecate morphology developed several times, or was lost repeatedly, during the evolution of the *Alphaproteobacteria*. Besides members of the genus *Caulobacter sensu strictu* (*Caulobacter* group III.; Garrity *et al.*, 2005) that include *Caulobacter crescentus*, *C. fusiformis*, *C. henrici* and *C. vibrioides*, dimorphic representatives are found in the related genus *Brevundimonas* (*B. alba*, *B. aurantiaca*, *B. bacteroides*, *B. intermedia*, *B. subvibrioides*, *B. variabilis*; group IV.), and in the distantly related genera *Asticacaulis* (*A. biprosthecium*, *A. excentricus*; group II.) and *Maricaulis* (*M. maris*; group V.) (Abraham *et al.*, 1997, 1999, 2001; Garrity *et al.*, 2005). More recently, a prosthecate representative of the genus *Phenylobacterium* (*P. conjunctum* FWC21^{T}) was described (Abraham *et al.*, 2008). A sixth lineage is represented by strain CB37^{T} = ATCC15260^{T} = DSM4733^{T} that was originally described as *Caulobacter leidyi* (Poindexter, 1964). 16S rRNA gene sequence analyses showed that this strain is affiliated with the family *Sphingomonadaceae* (Stahl *et al.*, 1992; Garrity *et al.*, 2005). As a consequence, *C. leidyi* was excluded from the genus *Caulobacter*, omitted from Bergey's Manual of Systematic Bacteriology and removed to limbo (Garrity *et al.*, 2005). Although the isolation of additional strains (strains WCP 2020S, CB 296 and an unnamed representative from Lake Baikal) has been

reported (Garrity *et al.*, 2005; Lapteva *et al.*, 2007), no further information on their properties is currently available. Due to the limited physiological and chemotaxonomic information available for strain DSM4733^T and the lack of additional strains, a taxonomic revision of this lineage is so far missing.

The results of the phylogenetic analysis of 16S rRNA gene sequences indicate that DSM 4733^T and strain 247 represent a novel species within the genus *Sphingomonas* and the family *Sphingomonadaceae*. Both 16S rRNA gene sequences also include all 7 signature sequences of *Sphingomonadaceae* but none of those determined for *Caulobacteraceae* or other Alphaproteobacterial families (Yabuuchi & Kosako, 2005). Aside from 16S rRNA gene sequence similarity, two biochemical characteristics, the fatty acids patterns and polar lipids composition of strain DSM4733^T point towards a distinct taxonomic position of this strain and support an affiliation with the family *Sphingomonadaceae*. The holdfast of this strain does not bind wheat-germ agglutinine and does not contain oligo-N-acetylglucosamine that participates in adhesion as in most *Caulobacter* spp. (Garrity *et al.*, 2005). Whereas hexadecanoic acid ($C_{16:0}$) and octadecenoic acid ($C_{18:1}\omega7c$/$C_{18:1}\omega9t$/$C_{18:1}\omega12t$) represent the dominant non-polar fatty acids in members of the *Caulobacteraceae* and *Sphingomonadaceae* alike, the dominance of hydroxymyristic acid ($C_{14:0}$ 2OH) is diagnostic for *Sphingomonadaceae* since this compound is missing in the *Caulobacteraceae* (containing $C_{12:1}$ 3OH or, $C_{12:0}$ 3OH in the closely related *Brevundimonas* species). Strain DSM4733^T contains 2-hydroxy myristic acid (C14:0 2-OH) as a dominant fatty acid (Abraham *et al.*, 1999). This feature is unique among all known dimorphic prosthecate bacteria yet characteristic for the *Sphingomonadaceae* where glucuronosyl ceramide and 2-hydroxy myristic acid replace the lipopolysaccharides that are present in the cell wall of other bacteria (Yabuuchi & Kosako, 2005). The presence of sphingoglycolipids is the key characteristic that defines the genus *Sphingomonas* and the family *Sphingomonadaceae*, and together with phosphatidylcholine, distinguished both investigated strains from the *Caulobacteraceae* or other prosthecate Alphaproteobacteria (Abraham *et al.*, 1999; Yabuuchi & Kosako, 2005).

The characteristics such as the use of *sym*-homospermidine as the dominant polyamine, the presence of cystine arylamidase (variable in DSMZ 4733^T), the absence of β-galactosidase, and the lack of L-arabinose and sucrose utilization (Table 4; Takeuchi *et al.*, 2011) distinguish strain DSM 4733^T and strain 247 from most other *Sphingomonadaceae*. So far, the dimorphic life cycle that involves a prosthecate and a flagellated stage is unique among the *Sphingomonadaceae*. Based on a detailed comparative phylogenetic analysis of 16S rRNA gene sequences of strain

DSM 4733^T and strain 247 which shows them to be phylogenetic closely related to species *Sphingomonas aquatilis*, both of them showing all seven signature sequences determined for the genus *Sphingomonas*, and based on the physiological and chemotaxonomic characterization of strain DSM4733^T and strain 247, we propose to reclassify as *Sphingomonas leidyi* comb. nov. as species name for this unique lineage of prosthecate dimorphic bacteria within the family *Sphingomonadaceae*.

The genus *Sphingomonas* was named by Yabuuchi *et al.* (1990) because of the presence of a specific sphingoglycolipid, and later emended by Takeuchi *et al.* (1993, 2001), Yabuuchi *et al.* (1999, 2002) and Busse *et al.* (2003). Strains in this genus are strictly aerobic, chemoheterotrphic, Gram-negative, rod-shaped and contain sphingoglycolipid. Until 2001, there were around 30 species affiliated to this genus. The phylogenetic analysis of their nearly complete 16S rRNA gene sequences were clearly divided into four clusters and supported by high levels of bootstrap values. The sequence similarity between different clusters were ranging from 92.6% to 96.6% suggesting each cluster corresponds to a single genus. Simultaneously, their chemotaxonomic and phenotypic analysis results supported this division and provided additional information to differentiate them. Integrated the phylogenetic, chemotaxonomic and physiological differences, genus *Sphingomonas* was divided into four genera: *Sphingomonas sensu stricto*, *Sphingobium*, *Sphingopyxis* and *Novosphingobium* (Takeuchi *et al.*, 2001). Genus *Sphingomonas sensu stricto* differ with other genera by homospermidine as the major polyamine, Major 2-hydroxy fatty acids are 2-OH 14:0 or 2-OH 15:0. Phylogenetic characteristic of 16S rRNA signatures are found at position 52:359 (C: G), 134 (G), 593 (G), 987:1218 (G: C) and 990:1215 (U: G) (Takeuchi *et al.*, 2001). The type species is *Sphingomonas paucimobilis* (Yabuuchi *et al.*, 1990). Genus *Sphingobium* differs with other by Spermidine as the major polyamine, the major 2-hydroxy fatty acids is 2-OH 14:0. Characteristic 16S rRNA signatures are present at positions 52:359 (U:A), 134 (G), 593 (U), 987:1218 (A:U) and 990:1215 (U:G). The type species is *Sphingobium yanoikuyae* (Yabuuchi *et al*. 1990). At the time of writing, these four genera contain 122 species with validly published names. Among these 122 species, 64 strains were affiliated to genus *Sphingomonas*, 24 to *Sphingobium*, 15 to *Sphingopyxis*, and 19 to *Novosphingobium* (Information from List of Prokaryotic names with Standing in Nomenclature (LSPN) http://www.bacterio.cict.fr/, accessed on Aug. 31. 2011).

Based on the newest phylogenetic analysis of 16S rRNA gene sequences (Last update in August 2011), the genera *Sphingobium*, *Sphigopyxis* and *Novosphingobium* were found to form distinct clusters, however, the genus *Sphingomonas* appeared to be paraphylectic as there were

some *Sphingomonas* species affiliated to the other 3 genera like *Sphingomonas macrogoltabidus*, *S. adhaesiva*, *S. terrae* in the genus *Sphingopyxis* and *Sphingomonas chlorophenolica*, *S. suberifaciens* placed in genus *Sphingobium*. The time of the description of these species were checked and all of them were found be described and named before the division of genus *Sphingomonas* in 2001 (Takeuchi *et al.*, 2001). It suggests that these *Sphingomonas* species fall into the genera *Sphingobium*, *Novosphingobium* and *Sphingopyxis* maybe merely misclassified.

In this analysis, the closest species to the strain 469^T was *Sphingomonas suberifaciens*, which seems to be misclassified according to the polyphasic analysis presented here. *Sphingomonas suberifaciens* was first identified as *Rhizomonas suberifaciens* by Van Bruggen *et al.* (1990). Then this species was emended as *Sphingomonas* by Van Bruggen *et al*. (1993) and Yabuuchi *et al*. (1999). All this happened before the division of genus *Sphingomonas* to 4 genera (Takeuchi *et al.*, 2001). Based on the melting temperature of the of the rRNA hybrids of *Rhizomonas suberifaciens* and other *Sphingomonas* species detected by Van Bruggen *et al.*, (1993), *R. suberifaciens* (Later named as *Sphingomonas suberifaciens*) and *Sphingobium yanoikuyae* belong to the same branch but not with *Sphingomonas paucimobilis*. This affiliation was supported by the phylogenetic clustering pattern of 270 bp of the 16S rRNA gene sequences analysis. In the phylogenetic analysis of the the 16S rRNA gene sequence, the species *Sphingomonas suberifaciens* clustered with the genus *Sphingobium* instead of with the genus *Sphingomonas*, and the 16S rRNA gene sequence also revealed all the seven signature characteristics of genus *Sphingobium*. This result was also supported by polyamine and fatty acids analysis, the major polyamine of *Sphingomonas suberfaciens* was spermidine but not homospermidine, the 2-hydroxy fatty acids was only 2-OH 14:0 (Takeuchi *et al.*, 2001). All other phenotypic characteristics of this strain fitted to the description of genus *Sphingobium*, so this species was reclassified as *Sphingobium suberfaciens*.

For the new isolate strain 469^T, even the seven 16S rRNA signature characteristic of strain 469^T is not the same as genus *Sphingobium*, but it also not the same genus *Sphingomonas* (Takeuchi *et al.*, 2001), the phylogenetic analysis showed that it clustered within genus *Sphingobium* not with genus *Sphingomonas*. The most closely related species were *Sphingomonas suberifaciens* with 97.1% similarity of the 16S rRNA gene sequence. But the DDH analysis shown these two species to represent two different species. The polyphasic analysis of the species *Sphingomonas suberifaciens* showed this species to be misclassified, leading to its reclassification as *Sphingobium suberifaciens*. Typical physiological and chemotaxonomic characteristics of strain 469^T are: 2-OH 14:0 as the major 2-hydroxy fatty acids,

spermidine as the major polyamine, and the cells could not reduce nitrate to nitrite. So, concluding from all the characteristics of the polyphasic analysis, strain 469^T was identified as a new member of the genus *Sphingobium*.

Niche adaptation and speciation

Polyamines are compounds existing in all living organisms; their functions are related to the nucleic acids and protein biosynthesis and they are also important constituent of the cell. The distribution pattern of polyamines can also be used in the chemosystematics of some bacteria. In this study, the major polyamines were determined from 4 strains representing 4 subpopulations as revealed by the MLSA analysis. The pattern of polyamines is in good agreement of the clustering patterns deduced from MLSA. Strain 247 and 382 had sym-homosperidine as the major polyamine and were clustered into genus *Sphingomonas*. Strain 301 and 469 had spermidine as the major polyamine and were clustered into genus *Sphingobiu*m. Both of these two polyamines are triamines, homospermidine can be formed from spermidine and their function can be exchangeable in some species. But spermidine is the so called 'common' polyamine and homospermidine is 'uncommon', therefore, they may serve in adaptation of bacteria to some extreme environments. Homospermidine was reported in extremely thermophilic bacteria and nitrogen-fixing cyanobacteria. Also, homospermidine may play a role in the nitrogen cycle (Oshima and Baba, 1981; Hamana *et al.*, 1983; Kaiser *et al.*, 2003). In the comparison of the two genera *Sphingomonas* and *Sphingobium*, the difference in nitrate reduction is correlated to their major polyamine. For the genus *Sphingomonas*, the major polyamine is sym-homospermidine and there were some studies showed strains in this genus was able to reduce nitrate which is also part of the nitrogen cycle (Takeuchi *et al.*, 2001), so maybe there are connections of the existence of homospermidine and the ability of nitrate reduction.

The pattern of antibiotics resistance can be vary between strains from the same species (Baquero and Coque, 2011). In this study, the strain 247 and DSM4733^T, which belong to the same species *Sphingomonas leidyi*, show only small differences in the resistance to the antibiotics colistin, kanamycin and fosfomycin. Inferring from the high diversity of the subpopulation of bacterial species, some of them acquired the ability by adaptating to their own special niches. Bacteria produce antibiotics to compete for nutrient sources. To survive, they must become resistant to the antibiotics around them. The ability of antibiotic resistance comes from mutations or acquiring new genes encoding the resistant mechanism. The different in the ability of rifampin resistance between group G1A, G2 and G3 is mostly due to mutation. Strains

from the G1A group are susceptible to antibiotic rifampicin but strains from other groups are resistant to it. In several organisms, rifampicin resistance is provided by single amino acid changes in the Rif^R clusters at positions 500- 600 of the RpoB polymerase protein (Kim *et al.* 2005). These mutations prevent the binding of the antibiotic to the RpoB polymerase. Since such typical substitutions could not be detected in our isolates, other mechanisms such as altered membrane permeability could acount for the resistance to rifampicin (Abadi *et al.*, 1996) and potentially also for the resistance to several other inhibitory substances (see Fig. 9). The resistant to vancomycin and ampicillin belongs to the second way in which the resistant strains received the gene from others and then changed their cell wall, the strains from G1A group differ to strains from other groups by its resistance to vancomycin. Vancomycin has high affinity to the dipeptide D-Ala-D-Ala C-terminus. This dipeptide is used in the formation of Uracil diphosphate-*N*-acetylmuramyl-pentapeptide (precursor of peptidoglycan). Those strains are resistant to vancomycin because of the acquiring of the new operons, which encoded the enzymes to synthesis a lower vancomycin binding affinity peptidoglycan precursor. The C-terminal D-Ala is replaced by the new synthesizd low affinity D-Lac or D-Ser (Courvalin, 2006). For ampicillin, strain 247 and 382^T cluster in a genus which shows resistance to ampicillin, whereas strain 301^T and 469^T are affiliated to a genus can not resist to it. Strain 247 differs from other three strains by the resistance to kanamycin and erythromycin. The resistance to kanamycin is most probably due to the enzymatic inactivation of the antibiotic. The resistance to erythromycin is probably caused by modification the antibiotic attacking or binding site (Courvalin, 2006; Shaw *et al.*, 1993; Pechere *et al*., 2001; Brown, 2008).

Antibiotic resistance is linked to the metabolism of the bacteria. Most strains that are resistant to vancomycin also show positive reaction to substrates *N*-acetyl-D-glucosamine. *N*-acetyl-glucosamine (GlcNAc) and *N*-acetyl-muramic acid (MurNAc) are the basic precursors of peptidoglycan. Representative strains from subpopulations G3B (strain 247) and 382^T are resistant to ampicillin, this antibiotic functions similar to vancomycin by binding to peptidoglycan precursors (Sauvage *et al.*, 2008). Strain 301^T from subpopulation G2D and strain 469^T cannot resist to ampicillin, vancomycin and penicillin. This result corresponds well with the clustering patterns. The former two strains are clustered into genus *Sphingomonas*, the latter two are clustered into the genus *Sphingobium*. So, that may be the reason why they exhibit the positive reaction to the substrates *N*-acetyl-glucosamine. It can also be connected to the formation of sphingoglycolipid (SGL). There are five molecular species of Sphingoglycolipid, labelled as SGL-1, SGL-1', SGL-2, SGL-3, SGL-4. For all 26 strains of *Sphingomonadaceae*

used in the SGL analysis, the SGL-1, SGL-2, SGL-3, SGL-4 have been detected from *Sphingomonas* but no SGL-1' (The four species *Sphingomonas herbicidovorans*, *S. subterranea*, *S. ursincola* and '*S. xenophaga'* which contains SGL-1' were not in the genus *Sphingomonas sensu stricto* (Takeuchi *et al.*, 2001)). *S. herbicidovorans* and *S. xenophaga* are in genus *Sphingobium*, and '*S. xenophaga'* now is reclassified as *Sphingobium xenophagum*. *S. subterranea* affiliated to the genus *Novosphingobium* and *S. ursincola* is in the genus *Blastomonas*). SGL-1, SGL-1' have been found in *Sphingobium*. The polar lipids analysis of strain 247 shows two kinds of SGL, and it is affiliated to the genus *Sphingomonas* which means they must have one kind of SGL-2, SGL-3 or SGL-4. The formation of the two kinds of SGL in *Sphingobium* does not need the glucosamine as moiety, but the glucosamine is one of the moieties in the formation of SGL-2 SGL-3 and SGL-4 (Kawahara *et al.*, 1999, 2000; Yabuuchii and Kosako, 2005). Strain 247 needs the moiety glucosamine for the SGL synthesis and it shows the positive reaction to *N*-acetyl-glucosamine in the BiOLOG and API assay. Maybe there are some connections between the utilization of N-acetyl-glucosamine as nutrient sources and the synthesis of glucosamine for the cell wall and membrane, or both of them are parts of the cycle of glucosamine, For details, more studies are needed.

Different species have different number of sphingoglycolipids and kinds of carbohydrates (glucuronic acid, glucosamine, galactose and mannose). In *Sphingomonadaceae* cells, the chemical structure, function, and distribution of SGL have been reported to replace lipopolyaccharide (LPS) in the cell wall of Gram-negative rods. The composition of the SGL in all strains of this family contains a 2-hydroxy acid, makes the detection of 2-hydroxy acid be a systematic character for this family. The carbohydrate of SGL is shorter and simpler than LPS, it makes the cells become more hydrophobic and allows more hydrophobic substrates to get into the cell (Kawahara *et al.*, 1999). That may be the reason for all the four tested strains cannot resistant to the hydrophobic antibiotics erythromycin and polymyxin B. For the fatty acid profiles, respiratory quinone and polar lipids patterns of the four representative strains, even these results could be affected by the growth condition (temperature, light and medium), all results determined in this study supported the phylogenetic relationships of them and fitted to their taxa. The ability to be resistant to antibiotics is affected by different mechanism, and has high diversity inside one species, so it is not useful in systematics but it can provide important information about the described strains, such as the metabolic capability diversity.

The phenotypic divergence determined from the BiOLOG analysis cannot distinguish all strain sharing the same 16S rRNA phylotype. The 33 traits depicted in Fig. 8 shows significant

variation between the larger group G1A, G2 and G3. There are even some significant differences within the subpopulations. Bacteria need the carbohydates as energy sources and use them as substrates to build up their cell structures. Their ability to grow or adapt to different environments also based on their ability to utilize different nutrients. While comparing strains from G1A to other groups, they are not able to utilize some sugars like maltose, sucrose, gelatin which are important for energy metabolism or are the substrates of biosynthesis of their macromolecules. This maybe one reason for the slower growth and reproduce rate, and far more limited growth conditions (narrow range of pH and temperatures) of strains in this group.

The strains in the G1A groups which have the same 16S rRNA gene sequence phylotypes probably derive from the same ancestor. From the MLSA results, many putative ecotypes co-existing in this population were deduced by ecotype simulation. Strains in the same subpopulation or ecotype may use different substrates, they adapted to the resources and competed for it, hence, their metabolic capabilities may give insights of their evolutionary history. Different strain may experience different ecological niches; the supplement of the resource is a kind of driving force of their evolution. Strains of the same species from distinct ecological niches can evolve different metabolic strategies. Studies on the metabolic traits of *Saccharomyces cerevisiae* from different original showed to express different life-history strategies according to their ecological niches (Spor *et al.*, 2009). The metabolic traits of a strain can illustrate the adaptation history to different ecological niches.

Mutation and horizontal gene transfer play important role in bacterial adaptation. By the chance of modifying their genes, bacteria can acquire the ability to adapt. For different strains in an ecotype, initiative mutations and niche-invasion mutations drive some of the strains to explit nutrient resources, to obtain ecological properties and finally become a new ecotype. And they can also acquire the alleles providing higher fitness by homologous recombination or horizontal gene transfer. Most of the changes of the capability of the utilization of different substrates are likely due to gene loss or acquisition. For strain 247, which differs to the other strain by resistance to penicillin, may have acquired this ability by this way. Similar resistance gain has been reported for *Streptococcus* and *Neisseria* (Maynard Smith *et al.*, 1991). For strains in the same subpopulation, they may not differ in their overall ability to utilize the same set of substrates, but they may have tiny difference in the quantitative levels. For the strain 247 and strain DSM 4733^T, both affiliated to *Sphingomonas leidyi*, strain 247 has a strong positive reaction to cystine arylamidase and trypsine, but DSM 4733^T has only a weak positive reaction to these two substrates. This difference is most likely due to the mutation or modifying of the

respective genes. Using the ecotype simulation, several putative ecotypes were deduced based on the housekeeping genes used in MLSA. Different ecotypes most probably occupy different ecological niches. Either by mutations or horizontal transfer of a gene, a strain may gain or increase it ability of utilize a substrate, this mutant can exist together with its progenitor strain but may be more adaptive to the same ecological environment (Cohan, 2004, Feldgarden *et al.*, 2003). For the strains of group G1A, they are slowly growing and have small size of the cells, and they lost the ability to utilize some sugars. In this study, the result showed that they are adapted to the oligotrophic freshwater environment, they can be more tolerant to harsher environmental conditions (low temperature around 4°C in winter) because they are found to be the most abundant group in our isolation and *in situ* natural population (Jogler *et al.*, 2011). They have high genetic diversity and contain lots of putative ecotypes maybe because of the strains evolved by gradual evolution during their long existing histories, which makes them being adapted to the same environment but still forming distinct populations.

For the subpopulations of bacteria species co-existing in the same ecological environment, the periodic selection may play an important role in their diversity level. The periodic selection increases the frequency of the adaptive mutant or even makes others be extinct; it only purges the diversity inside the ecotypes, so the putative ecotypes may be able to contribute to the metabolic and genomic diversity of the population of a species (Cohan, 2002, 2004; Feldgarden *et al.*, 2003). Here we connected the ecotypes simulated from the MLSA data with the metabolism patterns of the same set of strains. They have higher correspondence between big groups than between subpopulations inside these groups. There are several reasons for this phenomenon and demand further studies. Kreimer *et al.* (2008) run the analysis of evolution of the modularity based on the metabolic networks of more than 300 bacterial species, their results showed the modularity is moderately congruent to the phylogenetic tree of life, the evolution of modularity was significantly enhanced by the accommodating to different ecological niches, horizontal gene transfer is an importance force to the metabolic modularity (Kashtan *et al.*, 2007; Kreimer *et al.*, 2008). The same study has been done by comparing the metabolic and genetic diversity of three closely related *Bacillus* taxa, showing that changes in the existing genes play an important role in adaptation (Feldgarden *et al.*, 2003). The metabolism of different nutrient resources is the result of the adaptation, and it is also a kind of driving force of the generating of ecotypes, and finally also for the evolution of novel species. So, the nutrient resources play an important role in speciation, as does the antibiotic resistance. The different patterns of antibiotics can generate different resistant and sensitive bacterial populations in a species. Strains experienced and

adapted to different niches may get the metabolic abilities to different nutrient sources. Strain 382^T which can utilize most kinds of substrates in the API tests could simply be better adapted to the basic medium used in these tests. In the phylogenetic analysis of the 16S rRNA gene sequence, strain 469^T holds a single phylotype and located between G2 and G3 groups. After the MLSA analysis, this strain did not move its position and represents still a singleton. The network splitstree showed that there is little genetic similarity between this strain to others. For the phenotypic and physiological analysis of the four described strains, this one is the slowest growing one, and susceptible to lots of antibiotics. This strain exhibited a low probability of genetic exchanges, thus it may have had less possibilities to acquire the adaptive traits from others.

The complex population structure determined by MLSA is because of their long past evolutionary heritage and their present adaptation to the current niches. Also among the strains in G2 and G3 groups, MLSA has revealed subpopulations within them. The phenotypic analysis and antibiotic test also showed significant variation among subpopulations. The Strains in a subpopulation has the same 16S rRNA gene sequence, and they are more frequently be deduced as a single ecotype (Cohan 2004; 2002). These subpopulations are equal to an ecotype. For the four strains we chosen for representing four separated subpopulations informed from the MLSA results, sometimes these four strains were in the same putative ecotype by ecotype simulation based on some housekeeping genes. But each strain was affiliated to a different species after polyphasic analysis. So, even strains in the same ecotype, they may diverse to each other by mutations and gains or loss genes by horizontal gene transfer. For some bacteria, the amount of the genes they obtain from other different origins can reach up to 16.6 % of its genome (Ochman *et al.*, 2000). These genetic changes, which allow a genotype to escape periodic selection from its previous ecotype, form the basis of bacterial speciation (Cohan, 2004). Occasionally, mutations also allowed a strain to step into new ecological niche. Likewise, antibiotic resistance is because of the mutations. It was also shown by the clones from the same strain that they can adapt to different mediums and finally evolved as different subpopulations (Spor *et al.*, 2009). If clones got enough divergence to differ from each other and to survive from the periodic selections, they eventually become different ecotypes co-existing in the same ecological niches. A typical bacterial species may have several ecotypes, but each ecotype will experience mutations, homologous recombination, horizontal gene transfer and its own periodic selection events, finally surviving ecotypes will form new species differing from each other and also from their original species, So, the speciation is a complex process by integrating evolutionary events.

Conclusion

The population substructure detected within the same 16S rRNA phylotype G1A most likely is a result of natural selection rather than random drift. According to the niche variation hypothesis (Bolnick *et al*, 2007) considerable ecological heterogeneity would therefore be expected in the habitats of aquatic *Sphingomonadaceae*. Initial support comes from a parallel study, in which the seasonal *in situ* dynamics of different G1A subpopulations was followed by pyrosequencing of the internal transcribed spacer sequences (Jogler *et al*. 2011). The analysis of 8,576 sequences revealed strong seasonal shifts in the abundance of 15 different ITS-types whereby most of the ITS-types exhibited a distinct temporal pattern, and hence may occupy different ecological niches. Since the housekeeping genes investigated showed little evidence of positive selection and no indications towards selective pressures were detected for central metabolic pathways, alternative traits may be involved, such as the degradation of refractory high-molecular-weight organic compounds, resistance to UV radiation, a planktonic or sessile life style, or a different susceptibility towards bacteriophages. Possible evolutionary mechanisms comprise the acquisition of novel traits by illegitimate recombination and subsequent niche invasion (Perna *et al*., 2001) by recurrent selective sweeps (Majewski and Cohan, 1999) separating the G1A clades. In any event, the genetic diversity that is maintained within the sphingomonad G1A phylotype is enormous compared to other bacteria investigated so far. This makes this population an especially suitable model system to unveil novel aspects of the bacterial speciation process.

In this study, a population of *Sphingomonadaceae* isolates from two German lakes, Walchensee and Starnberger See, were studied in detail using 16S rRNA sequences, a MLSA analysis on nine housekeeping genes, and the determination of a large variety of phenotypic traits. The study of the much closer related strains, i.e., members of the same 16S rRNA phylotype, allowed for population genetic insights into the formation of subclusters by most probably selective processes, thus giving insight into speciation processes. Given enough time, speciation can result in the formation of distinct species or even distinct genera. Among the strains of the population being already quite distinct to each other, thereby representing the results from a long-term process of generating species, four strains were then consequently chosen for a taxonomic characterisation from the perspective of systematists. Three of the strains were found to represent novel species in the genera *Sphingobium* and *Sphingomonas*. The taxonomic insights into the fourth strain led to the possibility to reclassify a formerly

Caulobacter species into a *Sphingomonas* species. In summary, this study gave insights into incipient speciation among *Sphingomonadaceae* isolates along with a taxonomical description of long-term results of such speciation processes.

Chapter 6

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