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Laia Calvó[1]
Martí Cortey[2]
Jose-Luís García-Marín[2]
L. Jesús Garcia-Gil[1*]

[1]Laboratory of Molecular Microbiology, Institute of Aquatic Ecology, University of Girona, Spain
[2]Unit of Genetics, Department of Biology, University of Girona, Spain

Polygenic analysis of ammonia-oxidizing bacteria using 16S rDNA, *amoA*, and *amoB* genes

Summary. Finding a unique molecular marker capable of quickly providing rigorous and useful phylogenetic information would facilitate assessing the diversity of ammonia-oxidizing bacteria in environmental samples. Since only one of several available markers can be used at a time in these kinds of studies, the 16S rDNA, *amoA* and *amoB* genes were evaluated individually and then compared in order to identify the one that best fits the information provided by the composite dataset. Distance-based neighbor-joining and maximum parsimony trees generated using the sequences of the three mentioned genes were analyzed with respect to the combined polygenic trees. Maximum parsimony trees were found to be more accurate than distance-based ones, and the polygenic topology was shown to best fit the information contained in the sequences. However, the taxonomic and phylogenetic information provided by the three markers separately was also valid. Therefore, either of the functional markers (*amoA* or *amoB*) can be used to trace ammonia oxidizers in environmental studies in which only one gene can be targeted. [**Int Microbiol** 2005; 8(2):103-110]

Key words: ammonia-oxidizing bacteria · 16S rDNA · *amoA* · *amoB* · polygenic analysis

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* Corresponding author:
L.J. Garcia-Gil
Institut d'Ecologia Aquàtica
Universitat de Girona
Campus de Montilivi
17071 Girona, Spain
Tel. +34-972418175. Fax +34-972418150
E-mail: jesus.garcia@udg.es

Introduction

Environmental and biotechnological interest in ammonia-oxidizing bacteria (AOB) has increased tremendously in recent years. However, their slow growth and the difficulty to be cultured have necessitated the development of a variety of culture-independent techniques for carrying out ecologic and taxonomic studies [18,19,21,28,48,53,54]. These techniques include the use of 16S rDNA and protein-encoding genes to characterize natural AOB populations [4,7,25,42,46] and to analyze their taxonomic and phylogenetic features [1,2,6,37,38]. Nonetheless, although 16S rDNA sequences are suitable for providing a comprehensive long-term evolutionary view of prokaryotic taxonomy, they fail to discriminate among close relatives, such as species within a given group or genus [39]. In addition, considerable variability can be found among organisms with almost identical 16S rDNA genes [3]. Thus, while 16S rDNA has proven useful in the discrimination between nitrosococci and nitrosomonads [5,55], the outcome is confusing when examining a single genus, such as *Nitrosospira* [41]. For this reason, protein-encoding genes, such as *amoA*, have been added to the collection of comparative tools used by taxonomists and molecular ecologists for diversity studies [14,46]. Gene *amoA* codes for the active site of ammonia monooxygenase [30], and it has been extensively used for the detection and study of ammonia oxidizers, particularly in natural environments [1,15,21]. According to Rotthauwe et al. [41], *amoA* is more useful at a fine-scale than 16S rDNA. By contrast, Ludwig and Schleifer [27] stated that the 16S rDNA gene is the best marker to infer phylogenetic relationships, since the topologies derived from 16S rDNA are in accordance with those obtained using markers with rather diverse functions. This has been recently supported by Purkhold et al. [38], who showed higher resolution using 16S rDNA than *amoA* within the tradi-

tional classification. Recently, *amoB* has been shown to be a suitable molecular marker for the study of AOB, as it has a high capacity of resolution within genera. In addition, its phylogeny is highly consistent with the current taxonomic outline [6].

The reconstruction of phylogenetic relationships between closely related species requires the use of markers with significant mutation rates; however, the accumulation of recurrent mutations results in the incorporation of large amounts of mutational homoplasy into the molecular data [47]. In addition, when mutations occur repeatedly at the same site, those that occurred later mask the previous ones, rendering the sequences useless for phylogenetic purposes. This phenomenon, known by geneticists as substitution saturation, should be taken into account before proceeding with any type of phylogenetic analysis [12]. It should also be noted that polymorphisms detected in the sequences of a given population reveal not only the mutations experienced by the ancestors but also the consequences of evolutionary forces, such as genetic drift and natural selection. It is therefore essential to check whether the molecular dataset has been affected by evolutionary pressures, especially since the neutral theory has become the standard null hypothesis in the study of molecular evolution [13,22].

In the present work, the 16S rDNA, *amoA*, and *amoB* genes were used to determine whether or not a polygenic or single-marker analysis was more suitable for taxonomic studies of AOB. These three markers were evaluated independently in a panel of genetic tests to compare the amount of useful information contained in their respective sequences. The phylogenetic trees constructed from each gene were then weighted against the composite sequence dataset to identify the marker that best reproduced the information resulting from the polygenic tree.

Materials and methods

Sequences. 16S rDNA, *amoA*, and *amoB* partial gene sequences from a total of 20 AOB strains of the β- and γ-subclasses of Proteobacteria were obtained from the databases and used in this study (Table 1). The sequences of two methane oxidizers, *Methylocystis* sp. and *Methylosinus trichosporium*, from the β- and γ-subclass of Proteobacteria, respectively, were also included and used as outgroups for phylogenetic reconstruction.

Mutational model. Multiple sequence alignments were performed with CLUSTAL W [51] and refined manually. The proportions of variable and conserved positions were calculated with DNAsp v4 [43]. Silent and effective mutations in the protein-encoding genes *amoA* and *amoB* were manually checked by comparing the DNA sequences with the translated amino-acid sequences.

The hypothesis of neutrality in nucleotide substitution was tested using Tajima's D test [50], included in the software MEGA v.2.1 [26]. The test was independently performed for every marker and for the three positions of the codons from the *amoA* and *amoB* sequences. Substitution saturation was

Table 1. Source of the sequences used in this study

	16S rDNA		*amoA*		*amoB*	
	Accession number	Reference	Accession number	Reference	Accession number	Reference
Nitrosomonas europaea Nm50	M96399	[17]	AJ298710	[1]	AJ555508	[6]
Nm. aestuarii Nm36	AJ298734	[2]	AJ298707	[1]	AJ555504	[6]
Nm. eutropha Nm57	AY123795	[37]	AJ298713	[1]	AJ555506	[6]
Nm. europaea L08050	AB070982	[45]	L08050	[30]	L08050	[30]
Nm. sp. K794	AB031960	Yokoyama et al., unpublished	AB031869	Yokoyama et al., unpublished	AB031869	Yokoyama et al., unpublished
Nitrospira sp. NpAV – copy1	Y10127	[28]	AF032438	[23]	AF032438	[32]
Ns. sp. NpAV – copy2	Y10127	[28]	AF016003	[23]	AF016003	[23]
Ns. sp. Nsp2	AY123802	[38]	AY123822	[38]	AJ555494	[6]
Ns. sp. 40KI	X84656	[52]	AJ298687	[1]	AJ555496	[6]
Ns. sp. Ka4	AJ012106	Aakra et al., unpublished	AJ298697	[1]	AJ555497	[6]
Ns. sp. B6	X84657	[52]	AJ298690	[1]	AJ555498	[6]
Ns. sp. Nv6	AY123805	[38]	AY123826	[38]	AJ555499	[6]
Ns. sp. Nsp1	AY123808	[38]	AY123828	[38]	AJ555500	[6]
Ns. multiformis Nl13	AY123807	[38]	AJ298702	[1]	AJ555501	[6]
Ns. sp. AF	X84658	[52]	AJ298689	[1]	AJ555502	[6]
Ns. sp. Nsp17	AY123804	[38]	AY123825	[38]	AJ555503	[6]
Ns. sp. AHB1	X90820	[40]	X90821	[40]	X90821	[40]
Nitrosococcus sp. AF153344	AF153343	[32]	AF153344	[32]	AF153344	[32]
Nc. oceani C-107	M96395	[17]	AF047705	[32]	AF047705	[32]
Nc. halophilus Nc4	AF287298	[37]	AF272521	[37]	AJ555509	[6]
Methylocystis sp. M	U81595	[29]	U81596	[29]	U81596	[29]
M. trichosporium OB3b	Y18947	[8]	U31650	[16]	U31650	[16]

determined with the index developed by Xia et al. [58] which is included in the DAMBE software [57]. This test is based on the notion of entropy in information theory and yields a critical value permitting the saturation degree of a given set of aligned sequences to be assessed. The same saturation index was calculated for the first, second, and third codon positions in *amoA* and *amoB*. In addition, the entire sequences of the three markers were tested individually for saturation.

The nucleotide substitution model best fitting the variations observed in the 16S rDNA, *amoA*, and *amoB* partial sequences was determined using the software MODELTEST 3.04 [36]. This program allows the most appropriate among 56 models of nucleotide substitution to be chosen.

Phylogenetic analysis. Neighbor-joining (NJ) trees for 16S rDNA, *amoA*, and *amoB* genes were generated from the corresponding matrix of nucleotide divergence between sequences using the program MEGA2 [26]. Maximum parsimony (MP) trees were also constructed for each marker using the software PAUP 4.0b [49]. To reduce the computational time required by the parsimony algorithm when carried out with a heuristic search, a TBR branch-swapping value of 100 was used. Confidence in the branching points was obtained with 1000 bootstrap replicates. The incongruence length difference test (ILD) [10] was conducted as implemented in the PAUP 4.0b software package (partition homogeneity test) and used to determine whether 16S rDNA, *amoA*, and *amoB* sequence datasets provided similar phylogenetic information. The overall NJ and MP trees including the polygenic composite sequences were constructed applying the same variables used for the construction of individual gene trees.

Tree topologies were compared using maximum likelihood, minimum evolution, and parsimony criteria. First, the topologies were analyzed according to the modified Kishino and Hasegawa test [44], computing the log-likelihoods per site for each tree and comparing the total log-likelihoods among topologies [11]. Minimum evolution (ME) scores were then compared for each topology. Finally, the number of steps and both the consistency and retention indices of the parsimony analysis for each tree were computed.

Results

Quantitative aspects of gene variation. Sequences of *amoA* contained the highest proportion of polymorphic sites. Of the 399 sites in this gene, 203 (62.15%) were found to be variable; of these, 11.27% were silent and 50.88% effective. In *amoB*, 180 (45.80%) out of 393 genes were variable and all of them were effective. Of the 1014 16S rDNA genes analyzed, 278 (27.42%) were variable. There were 231 parsimony-informative sites in 16S rDNA, 232 in *amoA*, and 172 in *amoB*. Approximately 50% of the nucleotide substitutions in *amoA* affected the third position of the codon (Table 2), while in the case of *amoB* the variable positions were evenly distributed. Furthermore, 44.27% of the polymorphisms detected in the third base-pair of *amoB* were silent substitutions, i.e., they had no effect on the amino-acid sequence.

Table 2. Variable positions in *amoA* (n = 133) and *amoB* (n = 131) for each codon position

	1st position	2nd position	3rd position	
	Number (%)	Number (%)	Number	
			Silent (%)	Effective (%)
amoA	72 (54.14)	51 (38.35)	125 (93.98)	0 (0.00)
amoB	61 (46.56)	57 (43.51)	58 (44.27)	3 (2.29)

Neutrality and substitution saturation. The dataset fit the model of neutral molecular evolution. In fact, the results of Tajima's D test indicated no significant skew in the entire sequences of the three markers in the case of *amoB* (0.505, 0.421, and 0.623 for the positions 1, 2, and 3, respectively). However, this test revealed a significant excess of polymorphisms in the third position of the codons in *amoA* (3.131 in contrast with the values 1.091 and 0.315 for the positions 1 and 2, respectively). These results agreed with measurements of substitution saturation, which produced a strong signal in the third position of *amoA* codons (Fig. 1). The persistent accumulation of changes in these specific sites in *amoA* may produce a loss of phylogenetic information. No substitution saturation was detected in the other two markers.

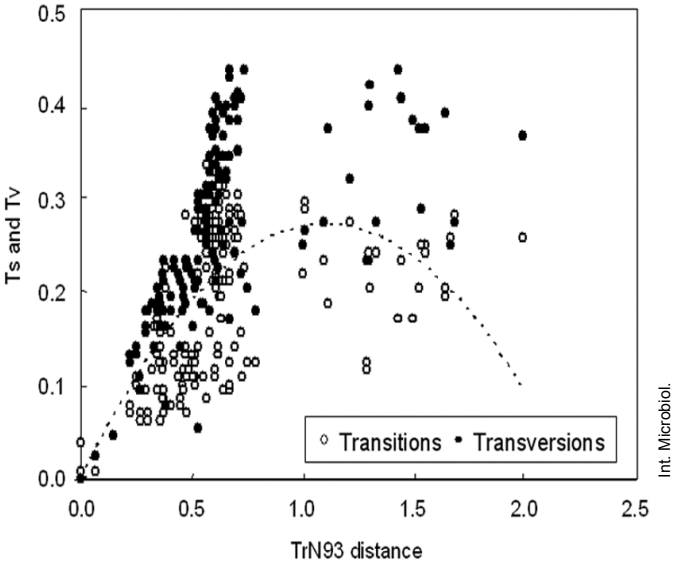

Fig. 1. Saturation diagram for the third position of the codon in *amoA*, showing the rate of transitions and transversions versus the Tamura-Nei 1993 distance.

Phylogenetic and topology analyses. The evolution of each gene can be described by a distinct substitution model (Table 3). Tamura-Nei 1993 (TrN93) is the nucleotide substitution model including the greatest number of parameters, and the one best fitting the combined dataset. The models Hasegawa-Kishino-Yano 1985 (HKY) and Felsenstein 1981 (F81), obtained for *amoA* and *amoB*, respectively, can be considered simplifications of TrN93. TrN93 was applied to all trees based on genetic distances, with a single transition

Table 3. Nucleotide substitution models, obtained base frequencies, and γ-shape distribution values for 16S rDNA, *amoA*, and *amoB* genes, and for the polygenic dataset [36]. Abbreviations: TrN, Tamura Nei 1993; HKY, Hasegawa-Kishino-Yano 1985; F81, Felsenstein 1981; I, proportion of invariable sites; G, γ-shape parameter

		16S rDNA	*amoA*	*amoB*	Polygenic
Model selected		TrN + I + G	HKY + G	F81 + G	TrN + I + G
Base frequencies	A	0.2668	0.1820	0.2321	0.2258
	C	0.2074	0.2990	0.2502	0.2491
	G	0.3108	0.2546	0.3148	0.3026
	T	0.2150	0.2644	0.2029	0.2225
γ-Shape distribution values		0.5645	0.3546	0.1026	0.3824

type and a single substitution rate when the selected models were HKY and F81, respectively.

For each marker, an NJ tree was constructed using the appropriate nucleotide substitution model (Table 3, Fig. 2). The trees constructed by MP showed topologies similar to those of their NJ counterparts (data not shown). In all cases, the *Nitrosomonas* and *Nitrosospira* radiations grouped together, and the γ-proteobacterial nitrosococci branched separately. This agrees with the classical phylogenetic topology of AOB. Likewise, two different clusters were distinguishable within the β-subgroup of ammonia oxidizers, as *Nitrosomonas* and *Nitrosospira* clearly formed two separate clades. Nevertheless, the allocation of *Nitrosomonas aestuarii* Nm36 was uncertain, since it grouped within the *Nitrosospira* cluster when using *amoB* as a molecular marker but fell within the *Nitrosomonas* group when using 16S rDNA or *amoA*. The ILD test corroborated ($P < 0.001$) the incongruence between the phylogenetic information provided by the three markers. However, since under some circumstances combining sequences with different phylogenetic histories can improve the accuracy of phylogenetic analysis [56], polygenic trees were constructed.

The consensus polygenic trees generated by MP and NJ are presented in Fig. 3. The topologies of the two trees were similar and consistent with both the standard classification of AOB and the results previously obtained with each of the three markers. In this polygenic analysis, *Nm. aestuarii* Nm36 was considered to be the most divergent *Nitrosomonas*.

All topological evaluations (likelihood, minimum evolution, and parsimony criteria) indicated that the MP tree obtained from the composite dataset displayed the most probable topology (Table 4). Similar values were obtained for the rest of the trees, which indicated that they were not significantly worse than the best-supported tree.

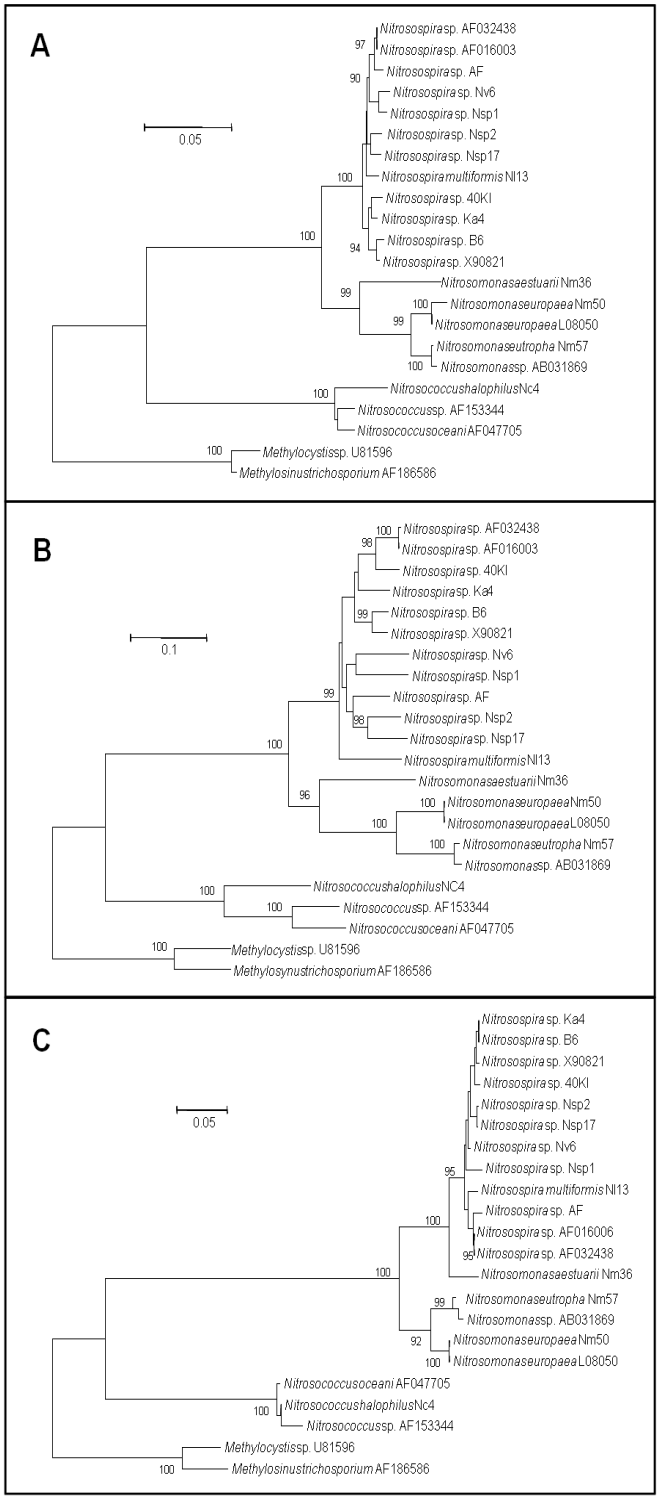

Fig. 2. Neighbor-joining (NJ) tree from alignments of (**A**) 16S rDNA, (**B**) *amoA*, and (**C**) *amoB* sequences. The model of nucleotide substitution used in every case is specified in Table 2. Bootstrap values above 75% are shown; the scale bar represents the number of estimated changes per nucleotide. 16S rDNA, *amoA*, and *amoB* genes sequences from *Methylocystis* sp. U81596 and *M. trichosporium* AF047705 were used as outgroups in (**A**), (**B**), and (**C**).

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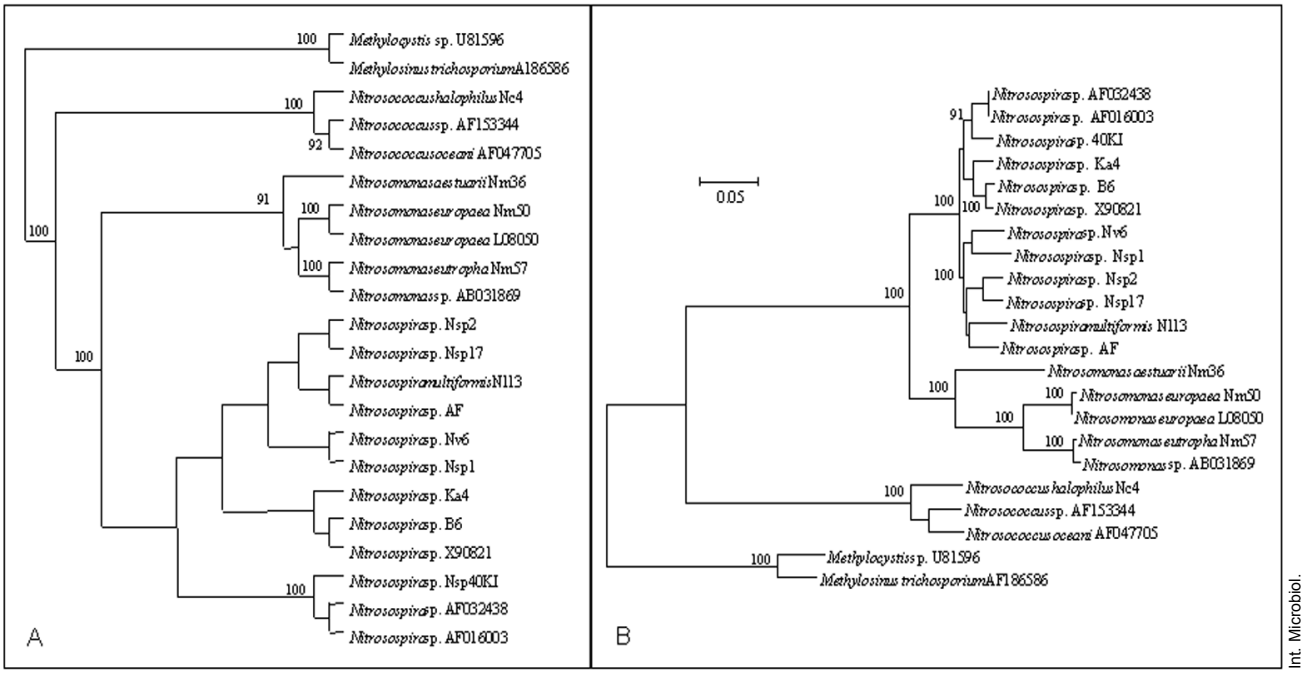

Fig. 3. Maximum parsimony (MP) consensus tree (**A**), and neighbor-joining (NJ) distance tree (**B**) generated from an alignment of partial *amoA*, *amoB*, and 16S rDNA sequences from ammonia-oxidizing bacteria (AOB). The distance matrix for the NJ tree was constructed based on the Tamura-Nei 1993 substitution rate. Bootstrap values (in percentage) are indicated. Partial 16S rDNA, *pmoA*, and *pmoB* gene sequences from *Methylocystis* sp. U81596 and *M. trichosporium* AF047705 were used as outgroups.

Discussion

Correct classification of any bacterial group requires the input of different genetic and phenetic characters, which is not possible when using uncultured bacteria from natural environments [39]. Alternatively, a polygenic approach leads to more accurate estimations of the diversity and composition of natural populations [34,35], although care must be taken when combining datasets from different markers [9,56]. Gene *amoA* encodes the active site of ammonia monooxygenase [20], which makes it difficult for effective mutations to occur in this gene. In *amoA*, 50% of the mutations were detected in the third base-pair (see Table 2), which showed a large accumulation of nucleotide changes at this position. As a consequence, the third position of the codon in *amoA* is strongly saturated (see Fig. 1) and deviates from neutrality, suggesting that this position has experienced selective pressures different from those of the other two positions [24]. The persistent accumulation of changes in these specific sites in *amoA* may produce a loss of phylogenetic information. By contrast, in the case of *amoB*, ca. 55% of the conserved sites were detected in all codon positions (Table 2). Moreover, ca. 70% of the amino-acid variations observed in the deduced partial amino acid sequences of AmoB proteins are conservative (data not shown).

The general topologies of the constructed trees were almost identical, with one exception. Although taxonomically

Table 4. Comparisons among tree topologies. KHT, Shimodaira and Hasegawa (1999) likelihood analysis (likelihood parameters used are described in Table 3); ME, minimum evolution scores reported by PAUP 4.0b [49]; parsimony length, CI and RI, respectively, the number of steps, the consistency index, and the retention index of the parsimony analysis for each topology as obtained using PAUP 4.0b

	Polygenic NJ + TN93	Polygenic PARS	*amoA* NJ + TN93	*amoA* PARS	*amoB* NJ + TN93	*amoB* PARS	16S rDNA NJ + TN93	16S rDNA PARS
KHT	–12764.5	–12755.1*	–12783.0	–12786.5	–12996.1	–13039.6	–12884.7	–12872.7
ME	1.67865	1.67904*	1.69236	1.75645	1.79980	1.79070	1.69853	1.71277
Parsimony length	2170	2168*	2176	2184	2260	2264	2206	2201
Parsimony CI	0.633	0.633*	0.631	0.629	0.608	0.606	0.622	0.624
Parsimony RI	0.771	0.772*	0.770	0.767	0.746	0.744	0.761	0.762

* Statistically significant best tree.

classified into the genus *Nitrosomonas*, the strain *Nm. aestuarii* Nm36 showed significant phylogenetic distances, supported by high bootstrap values, from the central cluster of *Nitrosomonas* when using 16S rDNA and *amoA* gene sequences. By contrast, *Nm. aestuarii* Nm36 grouped together with the *Nitrosospira* lineage when using *amoB*, but a considerable phylogenetic distance also distinguished this strain from the rest of the nitrosospiras. In the polygenic tree, *Nm aestuarii* Nm36 again grouped with the *Nitrosomonas* cluster. Purkhold et al. also reported the ambiguous phylogenetic arrangement of this species depending on the treeing method employed and the type of sequences used [37]. Therefore, this strain should be further studied in order to clarify its phylogenetic affiliation. Moreover, it would be of interest to determine whether *amoB* of *Nm. aestuarii* Nm36 has followed a different pattern of evolution and represents the ancestral state within the *Nitrosomonas* cluster, or whether it is a case of lateral gene transfer.

The composite dataset, consisting of 16S rDNA, *amoA*, and *amoB* sequences, provided more information than any of the three markers alone, and therefore resulted in the most accurate classification. Thus, the marker leading to the tree best-fitting the information of the entire dataset should be the one chosen for taxonomic and diversity studies. As expected, results of a comparison between all of the trees and the data obtained using the likelihood, minimum evolution, and parsimony criteria showed that the polygenic MP tree was the best. However, phylogeny could be inferred using any of the markers. Although our results may be biased due to both the sequence sizes of the markers (16S rDNA: 1014 bp; *amoA*: 399 bp; *amoB*: 393 bp) and the number of parsimonic informative sites, they support 16S rDNA as a good phylogenetic marker, especially concerning the avoidance of ambiguous classifications. Several authors have recently reaffirmed the potential of 16S rDNA sequences for drawing phylogenetic inferences [3,27,38]. Nonetheless, obtaining the 16S rDNA gene from environmental samples is time-consuming and tedious. It requires the cloning of all 16S rDNA genes present in the sample and then distinguishing the 16S rDNA genes belonging to AOB from the rest.

By contrast, environmental population studies based on the analysis of *amoA* or *amoB* present some significant advantages: the genes are AOB-specific, are large enough to allow quick fingerprinting of natural communities, and provide a phylogeny consistent with the current taxonomic outlines. Nevertheless, Oved et al. [33] and Nicolaisen and Ramsing [31] reported the amplification of non-AOB sequences when using *amoA* sequences in a PCR–denaturing gradient gel electrophoresis (DGGE) approach. Our experiments based on *amoB* amplification combined with DGGE resulted in the establishment of a sensitive and reliable screening method to detect and identify AOB in environmental samples (data not shown). Additionally, the benefit of using *amoB* in ecophysiology studies is the ability to distinguish methane-oxidizing bacteria from AOB on a simple agarose gel [6].

Based on the results reported here, for taxonomic purposes we strongly recommend sequencing16S rDNA, *amoA* and *amoB* genes, and to construct a polygenic tree. Since the third position of the codon in *amoA* is saturated, and due to the non-AOB sequences retrieved by other authors when using this gene [31,33], the use of *amoB* is recommended when carrying out environmental ecophysiology studies. *amoB* allows fingerprinting techniques, such as terminal restriction fragment length polymorphism (tRFLP) and DGGE, to be performed, and results in a reliable phylogenetic profile. Moreover, when using *amoB* as a marker, the methane-oxidizers present in the sample can be quickly and easily distinguished from AOB, which may be of great help in analyzing complex samples.

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References

1. Aakra A, Utåker JB, Nes IF (2001a) Comparative phylogeny of the ammonia monooxygenase subunit A and 16S rRNA genes of ammonia-oxidizing bacteria. FEMS Microbiol Lett 205:237-242
2. Aakra A, Utåker JB, Pömmerening-Röser, A, Koops, H. P, Nes, I.F (2001b) Detailed phylogeny of ammonia-oxidizing bacteria determined by rDNA sequences and DNA homology values. Int J Syst Evol Microbiol 51:2021-2030
3. Béjà, O, Koonin, EV, Aravind L, Taylor LT, Seitz H, Stein JL, Bensen DC, Feldman RA, Swanson RV, DeLong EF (2002) Comparative genomic analysis of archaeal genotypic variants in a single population and in two different oceanic provinces. Appl Environ Microbiol 68:335-345
4. Bothe H, Jost G, Schloter M, Ward BB, Witzel K (2000) Molecular analysis of ammonia oxidation and denitrification in natural environments. FEMS Microbiol Rev 24:673-690
5. Bruns MA, Stephen JR, Kowalchuk GA, Prosser JI, Paul EA (1999) Comparative diversity of ammonia oxidizer 16S rRNA gene sequences in native, tilled, and successional soils. Appl Environ Microbiol 65:2994-3000
6. Calvó L, Garcia-Gil LJ (2004) Use of *amoB* as a new molecular marker for ammonia-oxidizing bacteria. J Microbiol Methods 57:69-78
7. Calvó L, Vila X, Abella CA, Garcia-Gil LJ (2004) Use of the ammonia-oxidizing bacterial-specific phylogenetic probe Nso1225 as a primer for fingerprint analysis of ammonia-oxidizer communities. Appl Microbiol Biotechnol 63:715-721
8. Dedysh SN, Liesack W, Khmelenina VN, Suzina NE, Trotsenko YA, Semrau JD, Bares AM, Panikov NS, Tiedje JM (2000) *Methylocella palustris* gen. nov, sp. nov., a new methane-oxidizing acidophilic bacterium from peat bogs, representing a novel subtype of serine-pathway methanotrophs. Int J Syst Evol Microbiol 3:955-969

9. Dolphin K, Belshaw R, Orme CDL, Quicke DLJ (2000) Noise and incongruence: interpreting results of the Incongruence Length Difference Test. Mol Phyl and Evol 17:401-406
10. Farris JS, Kallersjo M, Kluge AG, Bult C (1994) Testing significance of incongruence. Cladistics 10:315-319
11. Felsenstein J (2004) Inferring phylogenies. Sinauer Associates, Sunderland, MA
12. Ford MJ (2002) Applications of selective neutrality tests to molecular ecology. Mol Ecol 11:1245-1262
13. Fu YX, Li WH (1993) Statistical tests of neutrality of mutations. Genetics 133:693-709
14. Garcia-Gil LJ, Gich FB, Fuentes-Garcia X (2003) A comparative study of *bchG* from green photosynthetic bacteria. Arch Microbiol 179:108-115
15. Gieseke A, Purkhold U, Wagner M, Amann R, Schramm A (2001) Community structure and activity dynamics of nitrifying bacteria in a phosphate-removing biofilm. Appl Environ Microbiol 67:1351-1362
16. Gilbert B, McDonald IR, Finch R, Stafford GP, Nielsen AK, Murrell JC (2000) Molecular analysis of the *pmo* (particulate methane monooxygenase) operons from two type II methanotrophs. Appl Environ Microbiol 66:966-975
17. Head IM, Hiorns WD, Embley TM, McCarthy AJ, Saunders JR (1993) The phylogeny of autotrophic ammonia-oxidizing bacteria as determined by analysis of 16S ribosomal RNA gene sequences. J Gen Microbiol 139:1147-1153
18. Hermansson A, Lindgren PE (2001) Quantification of ammonia-oxidizing bacteria in arable soil by real-time PCR. Appl Environ Microbiol 67:972-976
19. Hiorns WD, Hastings RC, Head IM, McCarthy AJ, Saunders JR, Pickup RW, Hall GH (1995) Amplification of 16S ribosomal RNA genes of autotrophic ammonia-oxidizing bacteria demonstrates the ubiquity of Nitrosospiras in the environment. Microbiology 141:2793-2800
20. Hyman MR., Arp DJ (1992) $14C_2H_2$- and $14CO_2$-labeling studies of the *de novo* synthesis of polypeptides by *Nitrosomonas europaea* during recovery from acetylene and light inactivation of ammonia monooxygenase. J Biol Chem 267:1534-1545
21. Juretschko S, Timmermann G, Schmid M, Schleifer KH, Pömmerening-Röser A, Koops HP, Wagner M (1998) Combined molecular and conventional analyses of nitrifying bacterium diversity in activated sludge: *Nitrosococcus mobilis* and *Nitrospira*-like bacteria as dominant populations. Appl Environ Microbiol 64:3042-3051
22. Kimura M (1983) The neutral theory of molecular evolution. Cambridge University Press, Cambridge, MA
23. Klotz MG, Norton JM (1995) Sequence of an ammonia monooxygenase subunit A-encoding gene from *Nitrosospira* sp. NpAV. Gene 1:159-160
24. Klotz MG, Norton JM (1998) Multiple copies of ammonia monooxygenase (*amo*) operons have evolved under biased AT/GC mutational pressure in ammonia-oxidizing autotrophic bacteria. FEMS Microbiol Lett 168:303-311
25. Kowalchuk GA, Stienstra AW, Heilig GH, Stephen JR, Woldendorp JW (2000) Molecular analysis of ammonia-oxidizing bacteria in soil of successional grasslands of the Drentsche A (The Netherlands). FEMS Microbiol Ecol 31:207-215
26. Kumar S, Tamura K, Jakobsen IB, Nei M (2001) MEGA2: Molecular Evolutionary Genetics Analysis software, 2.1 ed. Arizona State University, Tempe, Arizona, USA
27. Ludwig W, Schleifer KH (1999) Phylogeny of bacteria beyond the 16S rDNA standard. ASM News 65:752-757
28. McCaig AE, Embley TM, Prosser JI (1994) Molecular analysis of enrichment cultures of marine ammonia oxidizers. FEMS Microbiol Lett 120:363-367
29. McDonald IR, Murrell JC (1997) The particulate methane monooxygenase gene *pmoA* and its use as a functional gene probe for methanotrophs. FEMS Microbiol Lett 156:205-210
30. McTavish H, Fuchs JA, Hooper AB (1993) Sequence of the gene coding for ammonia monooxygenase in *Nitrosomonas europaea*. J Bacteriol 175:2436-2444
31. Nicolaisen MH, Ramsing NB (2002) Denaturing gradient gel electrophoresis (DGGE) approaches to study the diversity of ammonia-oxidizing bacteria. J Microbiol Methods 50:189-203
32. Norton JM, Alzerreca JJ, Suwa Y, Klotz MG (2002) Diversity of ammonia monooxygenase operon in autotrophic ammonia-oxidizing bacteria. Arch Microbiol 177:139-149
33. Oved T, Shaviv A,Goldrath T, Mandelbaum RT, Minz D (2001) Influence of effluent irrigation on community composition and function of ammonia-oxidizing bacteria in soil. Appl. Environ. Microbiol 67:3426-3433
34. Park DH, Kim JS, Kwon SW, Wilson C, Yu YM, Hur JH, Lim CK (2003) *Streptomyces luridiscabiei* sp. nov, *Streptomyces puniciscabiei* sp. nov. and *Streptomyces niveiscabiei* sp. nov, which cause potato common scab disease in 35. Korea. Int J Syst Evol Microbiol 53:2049-2054
35. Pena JA, Li SY, Wilson PH, Thibodeau SA, Szary AJ, Versalovic J (2004) Genotypic and phenotypic studies of murine intestinal *lactobacilli*: species differences in mice with and without colitis. Appl Environ Microbiol 70:558-568
36. Posada D, Crandall KA (1998) MODELTEST: testing the model of DNA substitution. Bioinformatics 14:817-818
37. Purkhold U, Pömmerening-Röser A, Juretschko S, Schmid MC, Koops HK, Wagner M (2000) Phylogeny of all recognized species of ammonia oxidizers based on comparative 16S rRNA and *amoA* sequence analysis: implications for molecular diversity surveys. Appl Environ Microbiol 66:5368-5382
38. Purkhold U, Wagner M, Timmermann G, Pömmerening-Röser A, Koops HP (2003) 16S rRNA and *amoA*-based phylogeny of 12 novel betaproteobacterial ammonia-oxidizing isolates: extension of the dataset and proposal of a new lineage within the nitrosomonads. Int J Syst Evol Microbiol 53:1485-1494
39. Rosselló-Mora R, Amann R (2001) The species concept for prokaryotes. FEMS Microbiol Rev 25:39-67
40. Rotthauwe JH, deBoer W, Liesack W (1995) Comparative analysis of gene sequences encoding ammonia monooxygenase of *Nitrosospira* sp. AHB1 and *Nitrosolobus multiformis* C-71. FEMS Microbiol Lett 133:131-135
41. Rotthauwe JH, Witzel KP, Liesack W (1997) The ammonia monooxygenase structural gene *amoA* as a functional marker, molecular fine-scale analysis of natural ammonia-oxidizing populations. Appl Environ Microbiol 63:4704-4712
42. Rowan AK, Snape JR, Fearnside D, Barer MR, Curtis TP, Head IM (2003) Composition and diversity of ammonia-oxidizing bacterial communities in wastewater treatment reactors of different design treating identical wastewater. FEMS Microbiol Ecol 43:195-206
43. Rozas J, Sanchez-DelBarrio JC, Messeguer X, Rozas R (2003) DnaSP, DNA polymorphism analyses by the coalescent and other methods. Bioinformatics 19:2496-2497
44. Shimodaira H, Hasegawa M (1999) Multiple comparisons of log-likelihoods with applications to phylogenetic inference. Mol Biol Evol 16:1114-1116
45. Shinozaki H, Fukui M (2002) Comparison of 16S rRNA, ammonia monooxygenase subunit A and hydroxylamine oxidoreductase gene, in chemolithotrophic ammonia-oxidizing bacteria. J Gen Appl Microbiol 48:173-176
46. Sinigalliano CD, Kuhn DN, Jones RD (1995) Amplification of the *amoA* gene from diverse species of ammonium-oxidizing bacteria and from an indigenous bacterial population from seawater. Appl Environ Microbiol 61:2702-2706
47. Smouse PE (1998) To tree or not to tree. Mol Ecol 7:399-412
48. Stephen JR, McCaig AE, Smith Z, Prosser JI, Embley TM (1996) Molecular diversity of soil and marine 16S rRNA gene sequences related to beta-subgroup ammonia-oxidizing bacteria. Appl Environ Microbiol 62:4147-4154

49. Swofford DL (1998) PAUP*. Phylogenetic Analysis Using Parsimony (*and other methods). Version 4. Sinauer Associates, Sunderland, Massachusetts.
50. Tajima F (1989) Statistical method for testing the neutral mutation hypothesis by DNA polymorphism. Genetics 123:585-595
51. Thompson JD, Higgins DG, Gibson TJ (1994) CLUSTAL W, improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res 22:4673-4680
52. Utåker JB, Bakken L, Jiang QQ, Nes IF (1995) Phylogenetic analysis of seven new isolates of ammonia-oxidizing bacteria based on 16S rRNA gene sequences. Syst Appl Microbiol 18:549-559
53. Voytek MA, Ward BB (1995) Detection of ammonium-oxidizing bacteria of the beta-subclass of the class Proteobacteria in aquatic samples with the PCR. Appl Environ Microbiol 61:1444-1450
54. Wagner M, Rath G, Amann R, Koops HP, Schleifer KH (1995) In situ identification of ammonium oxidizing bacteria. Syst Appl Microbiol 18:251-264
55. Ward BB, Martino DP, Diaz MC, Joye SB (2000) Analysis of ammonia-oxidizing bacteria from hypersaline Mono Lake, California, on the basis of 16S rRNA sequences. Appl Environ Microbiol 66:2873-2881
56. Wiens JJ (1998) Combining data sets with different phylogenetic histories. Syst Biol 47:568-581
57. Xia X, Xie Z (2001) DAMBE: software package for data analysis in molecular biology and evolution. J Hered 92:371-373
58. Xia X, Xie Z, Salemi M, Chen L, Wang Y (2003) An index of substitution saturation and its application. Mol Phylogenet Evol 26:1-7

Análisis poligénico de cepas de bacterias oxidadoras de amoníaco por medio de los genes 16S rDNA, *amoA* y *amoB*

Resumen. Encontrar un marcador molecular único capaz de proporcionar rápidamente información filogenética rigurosa y útil facilitaría evaluación de la diversidad de las bacterias oxidadoras de amoníaco en muestras ambientales. En esta clase de estudios no se puede utilizar simultáneamente más que uno de los marcadores disponibles. Los genes 16S rDNA, *amoA* y *amoB* se evaluaron individualmente para identificar el que se ajusta mejor a la información proporcionada por el conjunto de datos de los tres genes. Se compararon los árboles de Neighbor-Joining, basados en las distancias, y los árboles de máxima parsimonia basados en las secuencias conocidas de los tres genes mencionados, y se analizaron en relación con los árboles poligénicos construidos con la información combinada proporcionada por los tres genes. Los árboles de máxima parsimonia resultaron más fieles que los basados en las distancias, y la topología poligénica era la que mejor se ajustaba a la información contenida en las secuencias. Sin embargo, la información taxonómica y filogenética proporcionada por los tres marcadores por separado también resultó válida. Por tanto, cualquiera de los dos marcadores funcionales (*amoA* o *amoB*) se puede utilizar para detectar los oxidantes del amoníaco en estudios ambientales en los que solamente puede usarse un gen. [**Int Microbiol** 2005; 8(2):103-110]

Palabras clave: bacterias oxidadoras de amoníaco · 16S rDNA · *amoA* · *amoB* · análisis poligénico

Análise poligénico de cepas de bactérias oxidadoras de amoníaco através dos genes 16S rDNA, *amoA* e *amoB*

Resumo. Encontrar um marcador molecular único capaz de proporcionar rapidamente informação filogenética rigorosa e útil facilitaria avaliação da diversidade das bactérias oxidadoras de amoníaco em amostras ambientais. Nesta classe de estudos não é possível utilizar simultaneamente mais que um dos marcadores disponíveis. Os genes 16S rDNA, *amoA* e *amoB* foram avaliadas individualmente para identificar o que se ajusta melhor à informação proporcionada pelo conjunto de dados dos três genes. Foram comparadas as árvores filogenéticas de Neighbor-Joining, baseadas nas distâncias, e as árvores de máxima parcimônia baseadas nas seqüências conhecidas dos três genes mencionados, e foram analisadas em relação com as árvores poligénicas construídas com a informação combinada proporcionada pelos três genes. As árvores de máxima parcimônia resultaram mais fiéis que as baseadas nas distâncias, e a topologia poligénica foi a que melhor se ajustou à informação contida nas seqüências. No entanto, a informação taxonômica e filogenética proporcionada pelos três marcadores separadamente também resultou válida. Portanto, qualquer dos dois marcadores funcionais (*amoA* ou *amoB*) pode-se utilizar para detectar os oxidantes do amoníaco em estudos ambientais nos quais somente pode-se usar um gene. [**Int Microbiol** 2005; 8(2):103-110]

Palavras chave: bacterias oxidadoras de amoníaco · 16S rDNA · *amoA* · *amoB* · análise poligénico