Community analysis of betaproteobacterial ammonia-oxidizing bacteria using the *amoCAB* operon

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Abstract The genes and intergenic regions of the *amoCAB* operon were analyzed to establish their potential as molecular markers for analyzing ammonia-oxidizing beta-proteobacterial (beta-AOB) communities. Initially, sequence similarity for related taxa, evolutionary rates from linear regressions, and the presence of conserved and variable regions were analyzed for all available sequences of the complete *amoCAB* operon. The gene *amoB* showed the highest sequence variability of the three *amo* genes, suggesting that it might be a better molecular marker than the most frequently used *amoA* to resolve closely related AOB species. To test the suitability of using the *amoCAB* genes for community studies, a strategy involving nested PCR was employed. Primers to amplify the whole *amoCAB* operon and each individual gene were tested. The specificity of the products generated was analyzed by denaturing gradient gel electrophoresis, cloning, and sequencing. The fragments obtained showed different grades of sequence identity to *amoCAB* sequences in the GenBank database. The nested PCR approach provides a possibility to increase the sensitivity of detection of *amo* genes in samples with low abundance of AOB. It also allows the amplification of the almost complete *amoA* gene, with about 300 bp more sequence information than the previous approaches. The coupled study of all three *amo* genes and the intergenic spacer regions that are under different selection pressure might allow a more detailed analysis of the evolutionary processes, which are responsible for the differentiation of AOB communities in different habitats.

Keywords Ammonia-oxidizing betaproteobacteria ·
PCR primers · *amoC* · *amoA* · *amoB*

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Introduction

Ammonia oxidation to nitrite, the first step in nitrification, is primarily mediated by chemolithoautotrophic micro-organisms belonging to beta- and gammaproteobacteria (Prosser 1989; Head et al. 1993; Teske et al. 1994; Kowalchuk and Stephen 2001) and archaea (Konneke et al. 2005; Treusch et al. 2005). Ammonia-oxidizing bacteria (AOB) are difficult to culture, and therefore, several molecular techniques, including the analysis of the 16S rRNA gene, have been developed to study their diversity in the environment without cultivation (Bothe et al. 2000; Kowalchuk and Stephen 2001; Prosser and Embley 2002).

Although the 16S rRNA gene is a good phylogenetic marker, it is not necessarily related to the physiology of the target organisms (Kowalchuk and Stephen 2001; Calvo and Garcia-Gil 2004), and a slight lack of specificity may shift the spectrum of sequences amplified by PCR toward phylogenetically related but physiologically and ecologically different organisms (Bothe et al. 2000; Junier et al. 2008a, b). Therefore, functional markers such as the genes encoding for key enzymes involved in ammonia oxidation provide an alternative in ecological studies (Rotthauwe et al. 1997). Particularly in the case of AOB, one of these genes, amoA, has been traditionally used to study AOB in environmental samples (Rotthauwe et al. 1997; Alzerreca et al. 1999; Aakra et al. 2001a, b; Norton et al. 2002).

The gene amoA, together with the genes amoC and amoB, codify for the different subunits of the ammonia monooxygenase (AMO). The AMO is a membrane-bound, multiple-subunit enzyme responsible for the oxidation of ammonia to hydroxylamine, which is further converted to nitrite by the hydroxylamine oxidoreductase (Hyman and Arp 1992). In AOB, these genes are organized in the amoCAB operon (Norton et al. 2002). Homologs of amoA, B, and C were found in archaea (Konneke et al. 2005; Treusch et al. 2005), but these genes have low similarity to their bacterial counterparts.

Despite the potential of the amoCAB genes, the portion of the gene amoA generally used as a molecular marker to study the diversity of AOB (Rotthauwe et al. 1997) is relatively short and highly conserved, providing less resolution than the 16S rRNA gene (Koops et al. 2003; Purkhold et al. 2003). Therefore, it seemed advantageous to have alternative targets that could offer additional information on the diversity, evolution, and function of this group in the environment. All the genes on the amoCAB operon offer this possibility. For example, amoC and amoB can be alternative markers for molecular studies of AOB, considering their size (amoC is 800 bp, and amoB, the longest of the three genes, is around 1,200 bp) and the functional role of the protein subunits coded by amoC and amoB.

In this study, we have analyzed the suitability of amoC, amoB, and almost the whole of amoA as molecular markers for AOB. Initially, sequence analysis was carried out with sequences from cultured strains of AOB and related taxa to evaluate the phylogenic relationships, the evolutionary rates, and the presence of conserved or variable regions in the amoCAB genes. Based on these results, a strategy was developed allowing the retrieval and analysis of amoCAB sequences from environmental samples. The feasibility of a nested approach to amplify the amoCAB genes was tested. The results demonstrate the high potential of the amoCAB genes for studying AOB in the environment and the feasibility of a nested PCR approach to amplify environmental sequences of the three genes.

Materials and methods

Sequences and phylogenetic analysis

All amoCAB sequences deposited in GenBank, as well as the sequences from the clones of this study, were integrated into the phylogenetic software ARB (Ludwig et al. 2004). Phylogenies were calculated based on alignments of nucleotide and deduced amino acid sequences using the PHYLIP subroutine by applying the Fitch–Margoliash method to a substitution matrix calculated with the Jones–Taylor Thornton model. Genetic distances were calculated with the program distmat from EMBOSS (Rice et al. 2000), using the Tamura correction method. Similarity was calculated based on the genetic distance. For those species for which the complete sequence of all amoCAB genes (Nitrosomonas europaea, Nitrosomonas sp. TK794, Nitrosomonas sp. ENI11, Nitrosospira sp. NpAV, Nitrosospira multiformis, and Nitrosococcus oceani) or pmoCAB (Methylococcus capsulatus, Methylocystis sp. M, Methylocystis sp. SC2, and Methylosinus trichosporium) was available, the similarity values were plotted using in each axis the similarity for one of the genes. Correlation plots between amoA/amoC, amoA/amoB, amoC/amoB, and their respective pmoCAB were calculated in this way. Plots of sequence similarity along the alignment were calculated using the program plotcon from EMBOSS (Rice et al. 2000).

Samples and DNA extraction

The following strains of beta-AOB were used as positive controls for PCR: N. europaea ATCC 19718, Nitrosomonas eutropha C-71, Nitrosospira briensis C-128, Nitrosospira tenuis Nv1, Nitrosomonas sp. L13. In order to test the specificity of the primers for beta-AOB, the following negative controls were also assayed: N. oceani ATCC 19707 (gamma-AOB) and the type I and II methane-

oxidizing bacteria (MOB) strains *M. capsulatus* Bath NCIMB 11853 and *M. parvus* OBBP. Samples were obtained from rhizospheric soil of pea cultures in northern Germany, the Jordan River (Israel), Lake Schöhsee (Germany), Lake Plußsee (Germany), and the water column and water–sediment interface at Boknis Eck out of the Kiel Fjord at the German coast of the Baltic Sea. For DNA extraction, water samples were filtered through 0.2-μm pore size filters (Supor-200, PALL Life Sciences) and frozen at −18°C. DNA was extracted using the UltraClean Soil DNA kit (MoBio), following the manufacturer's guidelines. Concentration and quality of the DNA were checked by electrophoresis on 0.8% agarose gels stained with ethidium bromide.

PCR

Several combinations of PCR primers (Holmes et al. 1995; Rotthauwe et al. 1997; Norton et al. 2002; Calvo and Garcia-Gil 2004; Junier et al. 2008a, b) were used to amplify different regions of the *amoCAB* operon in beta-AOB (Table 1). For amplification of the almost complete *amoCAB* operon, the forward primer amoC58f and the reverse primer amoB1179r (Table 1) were used. PCR was carried out with the Expand High Fidelity PCR system (Roche) in 25 μl containing 1× PCR buffer, 200 μM of each dNTP, 200 mM of each primer, 2.5 mM $MgCl_2$, 1% formamide, 0.1% BSA, and 2.6 U of enzyme mix. The temperature program consisted of 94°C for 2 min and 30 cycles of 94°C for 15 s, 56°C for 1 min, and 68°C for 5 min. The *amoCAB* products (approximately 3,000 bp) were purified to remove primer excess (Multiscreen, Millipore), resuspended in HPLC water, and used as template for further nested amplifications indicated in

Fig. 1. Nested PCR was carried out as above, but 1 U of *Taq* DNA polymerase (Roche) was used instead of the Expand High Fidelity PCR system. The temperature program consisted of initial denaturation at 94°C for 5 min, 25 cycles of denaturation at 94°C for 1 min, annealing at 57°C for 1 min, and extension at 72°C for 1.5 min.

DGGE

For denaturing gradient gel electrophoresis (DGGE) of *amoA*, the *amoCAB* products were re-amplified with the primer pair amoA121f with a 40 bp GC-clamp attached (Muyzer et al. 1993) and amoA359rC in a touchdown temperature program with annealing temperatures decreasing from 65°C to 55°C (Muyzer et al. 1993). DGGE was performed according to the manufacturer's guidelines with the D-Gene System (BioRad) in polyacrylamide gels (7.5% of 37:1 acrylamide–bisacrylamide mixture in 0.5× TAE buffer, 0.75 mm thick, 16×10 cm) with a gradient of 35% to 75% of denaturants (100% denaturants contained 420 g/l urea and 400 ml/l deionized formamide). Gels were run in 0.5× TAE buffer at 200 V and constant temperature of 60°C for 10 h. To recover the DGGE bands for sequencing, DGGE gels were stained with SYBR gold (Molecular Probes). The most prominent bands were punched with a sterile pipette tip, transferred to 100 μl HPLC water, and maintained overnight at 4°C for DNA diffusion. The eluted DNA was used as template for PCR with the primers amoA121f/amoA359rC, under the conditions described above. The PCR products were purified with a Multiscreen plate (Millipore) and used for templates for sequencing with the primer amoA359rC. The BigDye Terminator Cycle Sequencing Kit v.1.1 (Applied Biosystems), recommended

Table 1 Primers used to amplify fragments from *amoCAB* genes in betaproteobacterial ammonia-oxidizing bacteria

Primer	Position	Sequence 5′–3′	Reference
amoC58f	58–72	CTA YGA CAT GTC RCT GTG G	Junier et al. 2008a, b
305F	763–786	GTG GTT TGG AAC RGI CAR AGC AAA	Norton et al. 2002
amoA34f	34–57	GCG GCR AAA ATG CCG CCG GAA GCG	Molina et al. 2007
amoA121f	121–137	ACC TAC CAC ATG CAC TT	Junier et al. 2008a, b
amoA-1F	332–349	GGG GTT TCT ACT GGT GGT	Rotthauwe et al. 1997
amoA349r	332–349	ACC ACC AGT AGA AAC CCC	This study
amoA359rC	340–359	GGG TAG TGC GAC CAC CAG TA	Junier et al. 2008a, b
amoA664f	664–681	GCS TTC TTC TCN GCS TTC	This study
A682	664–681	GAA SGC NGA GAA GAA SGC	Holmes et al. 1995
amoA802f	802–822	GAA GAA GGC TTT SCM GAG GGG	This study
amoA-2R	802–822	CCC CTC KGS AAA GCC TTC TTC	Rotthauwe et al. 1997
amoBMf	160–177	TGG TAY GAC ATK AWA TGG	Calvo and Garcia-Gil 2004
amoBMr	643–660	RCG SGG CAR GAA CAT SGG	Calvo and Garcia-Gil 2004
amoB1179r	1164–1179	CCA AAR CGR CTT TCC GG	Junier et al. 2008a, b
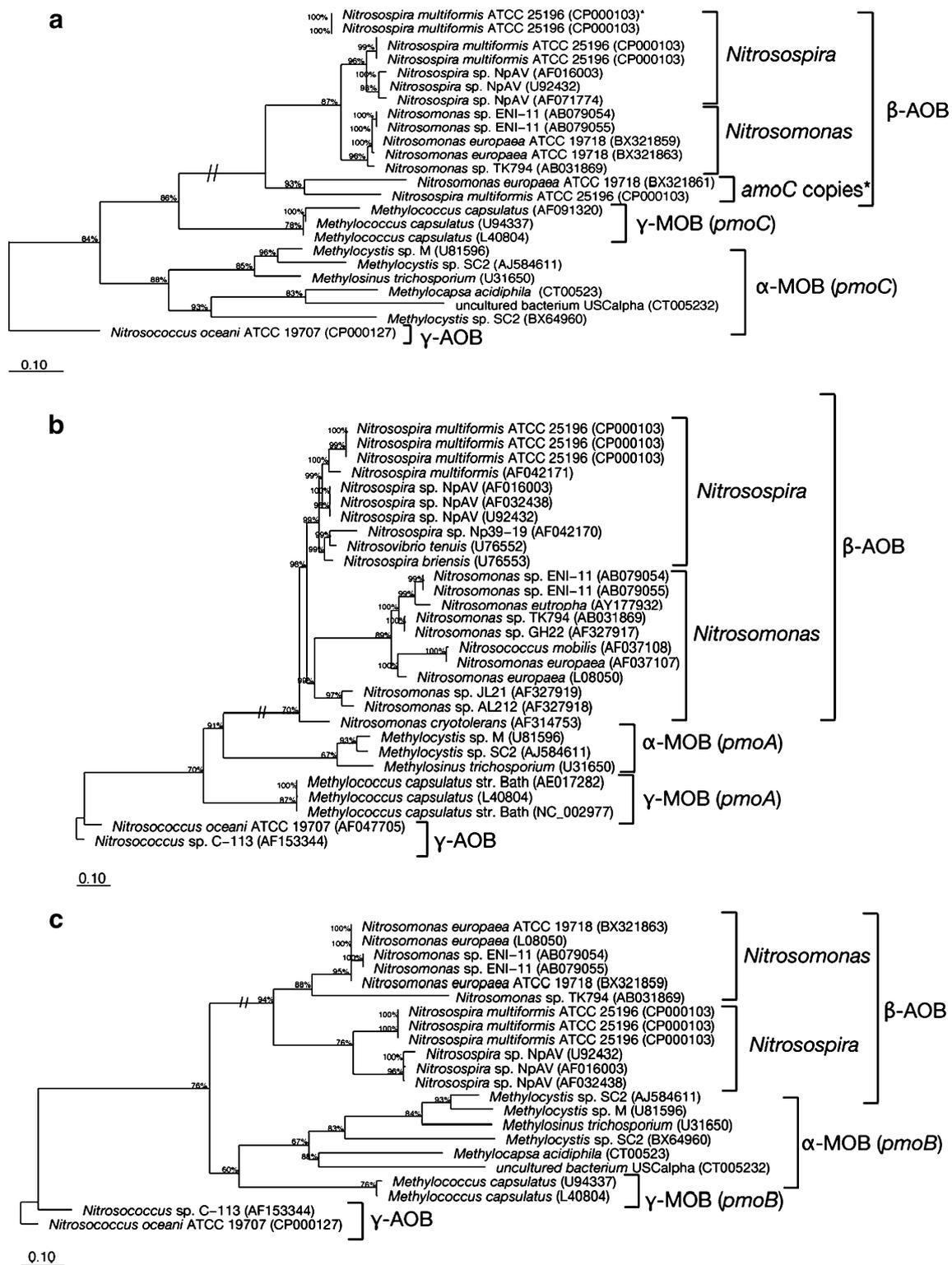
Fig. 1 Phylogenetic tree based on AmoC (**a**), AmoA (**b**), and AmoB (**c**) sequences using all complete sequences of cultured representatives of ammonia-oxidizing bacteria (*AOB*). Sequences of the related PmoC, PmoA, and PmoB from methane-oxidizing bacteria (*MOB*) were also included. Phylogenies were constructed with the PHYLIP subroutine by Fitch-Margoliash in ARB. Bootstraps values are indicated. *Asterisk* AmoC copies outside the *amoCAB* operon in *N. europaea* ATCC 19718 and *N. multiformis* ATCC 25196

for short readings, was used. Sequences were analyzed in an ABI3100 automated sequencer (Applied Biosystems). BLAST search (Altschul et al. 1997) was used to determine similarity with sequences in the GenBank database.

Cloning and sequencing

Pfu DNA polymerase (Promega) was used to minimize PCR errors in the amplification of products for cloning. Triplicate PCR reactions were combined and concentrated in a Multiscreen plate (Millipore) for cloning with the Zero Blunt PCR Cloning Kit (Invitrogen) according to the manufacturer's guidelines. Twelve clones were picked and checked for inserts of the appropriate size by PCR with the vector-specific primers M13F/M13R. Sequencing of both strands was performed with M13F and M13R primers using the BigDye Terminator Cycle Sequencing Kit v3.1 and ABI 3100 capillary sequencer (Applied Biosystems). The sequences were checked using BLASTX (Altschul et al. 1997). Sequences from this study have been deposited in GenBank under the accession numbers EF204958–EF204996 and EF219168–EF219191.

Results

AOB phylogeny inferred from *amoC*, *amoA*, and *amoB* sequences

Sequences from the complete *amoC*, *amoA*, and *amoB* genes were used to reconstruct the phylogenetic relationships of culture strains of AOB. Additionally, sequences from the evolutionary equivalent *pmoCAB* genes, which code for the particulate methane monooxygenase in MOB, were included in the analysis.

In the phylogenetic trees from the deduced amino acidic AmoA/PmoA, AmoB/PmoB, and AmoC/PmoC sequences, AmoCAB sequences from betaproteobacterial AOB (comprising the *Nitrosospira* and *Nitrosomonas* lineages) always formed a cluster separated from gammaproteobacterial AOB and PmoCAB in MOB (Fig. 1). The separation of the betaproteobacterial AOB from the other sequences was supported by bootstrap values of 91% (AmoA), 87% (AmoC), and 60% (AmoB). Sequences from betaproteobacterial AOB were further separated into two groups, one corresponding to the *Nitrosomonas* lineage (including *Nitrosomonas mobilis*) and the second to the *Nitrosospira* lineage (Fig. 1). The separation of the sequences from the two lineages of betaproteobacterial AOB (*Nitrosospira* and *Nitrosomonas*) was supported by high bootstrap values for all the three genes (94–99%).

Single *amoC* copies that are located elsewhere in the complete genome sequence of *N. europaea* ATCC 19718

(Chain et al. 2003) and *N. multiformis* ATCC 25196 were also included in the analysis. One of the two additional AmoC copies in *N. multiformis* ATCC 25196 and the only one of *N. europaea* ATCC 19718 clustered together with other beta-AOB AmoC (Fig. 1a). However, they formed an independent cluster separated from the other AmoC copies in these species.

Co-evolution of the different genes in the *amoCAB* operon

To establish whether the evolution rate of individual *amoCAB* genes was correlated, the sequence similarity derived from evolutionary distance between pairs of species was analyzed. Sequence similarity values were represented in correlation plots (Fig. 2) in which each axis represents the distance for one of the genes (e.g., *amoA* in axis *X* versus *amoB* or *amoC* in axis *Y*). A linear regression was applied to the plots. A high r^2 value will be an indication of co-evolution between pairs of genes, while the slope of the curve will indicate if, despite being co-evolving, one of the genes evolves faster than the other. This analysis was carried out with species for which the complete sequence of all *amoCAB* genes (*N. europaea*, *Nitrosomonas* sp. TK794, *Nitrosomonas* sp. ENI11, *Nitrosospira* sp. NpAV, *N. multiformis*, and *N. oceani*) and *pmoCAB* (*M. capsulatus*, *Methylocystis* sp. M, *Methylocystis* sp. SC2, and *M. trichosporium*) were available. A significant linear correlation was obtained for the comparisons of *amoA/pmoA* versus *amoB/pmoB* (r^2=0.96; Fig. 2a), *amoA/pmoA* versus *amoC/pmoC* (r^2=0.95; Fig. 2c), and *amoC/pmoC* versus *amoB/pmoB* (r^2=0.95; Fig. 2e), suggesting that the genes encoding the different subunits of the AMO and pMMO are co-evolving. The same analysis was conducted only with the *amoCAB* sequences of beta-AOB (Fig. 2b, d, and f), obtaining even higher r^2 values (*amoA/amoB*=0.98; *amoA/amoC*=0.98; *amoC/amoB*=0.97), confirming the co-evolution of the different genes in the *amoCAB* operon.

Detection of conserved and variable regions in the *amoCAB* operon

The average similarity values obtained for the full-length *amoCAB* genes for the different lineages of beta- and gamma-AOB were calculated (Table 2). In beta-AOB, the lowest similarity values were obtained for *amoB*, followed by *amoA* and *amoC*. However, within the *Nitrosomonas* lineage, *amoA* is considerably more variable than *amoC* and *amoB*, while in *Nitrosospira*, *amoA* and *amoB* are both more variable than *amoC*. Sequence similarity of the extra *amoC* copies of *N. multiformis* ATCC 25196 and of *N. europaea* ATCC 19718 with other *amoC* sequences from beta-AOB is very low (66%). Sequence similarity was higher in gamma-AOB for the three genes compared to

Fig. 2 Correlation plots of DNA–DNA sequence similarity between *amoCAB* and *pmoCAB* genes. **a** *amoA/pmoA* versus *amoB/pmoB*. **b** Beta-AOB *amoA* versus beta-AOB *amoB*. **c** *amoA/pmoA* versus *amoC/pmoC*. **d** Beta-AOB *amoA* versus beta-AOB *amoC*. **e** *amoC/pmoC* versus *amoB/pmoB*. **f** Beta-AOB *amoC* versus beta-AOB *amoB*
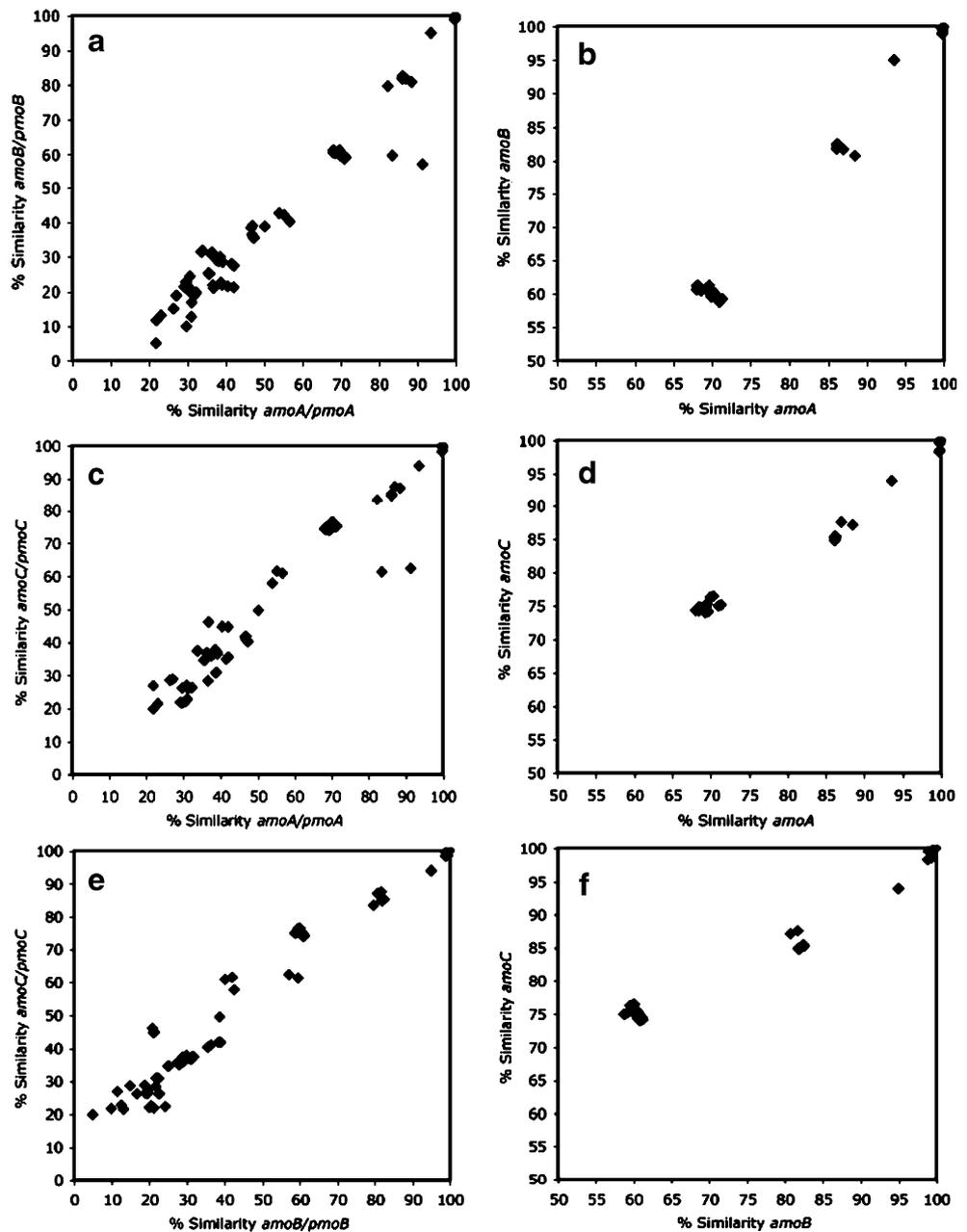
beta-AOB although this could be an artifact due to the smaller number of complete *amoCAB* sequences from different *Nitrosococcus* species.

Local changes in sequence conservation were inspected throughout the alignments to identify regions with less conservation in the different genes of the *amoCAB* operon. The variable regions were indicated by a decrease in the average sequence similarity in the alignments, which were represented graphically (Fig. 3). The gene containing the highest number of the variable regions was *amoB* (Fig. 3c), followed by amoC (Fig. 3a). Interestingly, the fewer number of variable regions was observed in *amoA* (Fig. 3b), which is the gene traditionally used for molecular studies in AOB.

PCR amplification of *amoCAB* in environmental samples

The primers amoC58f and amoB1179r were designed in a previous study (Junier et al. 2008a, b) to amplify the largest segment possible of the operon *amoCAB*, including the three genes and the intergenic regions, in betaproteobacterial AOB. The amplification of *amoCAB* with the primers amoC58f/amoB1179r produced a band of approximately 3 kb with DNA from the following beta-AOB: *N. europaea* ATCC 19718, *N. eutropha* C-71, *N. briensis* C-128, *N. tenuis* Nv1, and *Nitrosomonas* sp. L13 (supplementary Fig. 1). The size of the *amoCAB* products was variable due to the variability of the intergenic region between *amoC* and *amoA* (Norton et al. 2002). Amplification with the

Table 2 Percent average sequence similarity for *amoCAB* in gamma-AOB, beta-AOB, *Nitrosomonas*, and *Nitrosospira*

	Gamma-AOB	Beta-AOB	*Nitrosomonas*	*Nitrosospira*
amoA	89	78	82	89
amoB	90	74	92	89
amoC	100[a]	83	93	91
Extra *amoC*	N.D.	66	N.D.	N.D.

Extra *amoC* designates *amoC* copies outside the *amoCAB* operon in *Nitrosomonas europaea* ATCC 19718 and *Nitrosospira multiformis* ATCC 25196

[a] Only one sequence was available

primers amoC58f/amoB1179r did not produce any visible band with gamma-AOB (*N. oceani* ATCC 19707) or with the type I and type II MOB *M. capsulatus* Bath NCIMB 11853 and *M. parvus* OBBP (supplementary Fig. 1). To confirm the specificity of this amplification, PCR products of *amoCAB* were used as template for *amoA* PCR with the primers amoA34f/amoA-2R. A single amplicon of 789 bp was obtained with the beta-AOB products, but no amplification was observed in both gamma-AOB and MOB (supplementary Fig. 1).

Amplification of *amoCAB* with the primers amoC58f/amoB1179r was also tested with environmental samples using DNA from *N. europaea* as positive control. A PCR product was visible in the amplification with genomic DNA from *N. europaea* but not in the environmental samples (data not shown). In this case, the primer combination amoA121fgc–amoA359rC was selected for nested amplification because it produced a fragment that could be directly analyzed by DGGE. A fragment of the expected size (around 230 bp) was obtained from all environmental samples, including the positive control (data not shown).

The AOB community composition in the environmental samples was analyzed by DGGE (Fig. 4a) using *N. europaea*, *N. tenuis*, and *N. briensis* as references. Although more than one band was present on the DGGE gel, each culture produced a dominant band at different positions in the gradient: The band from *N. europaea* was located at around 45%, the band from *N. tenuis* at 55%, and the one from *N. briensis* at around 65% of denaturant concentration.
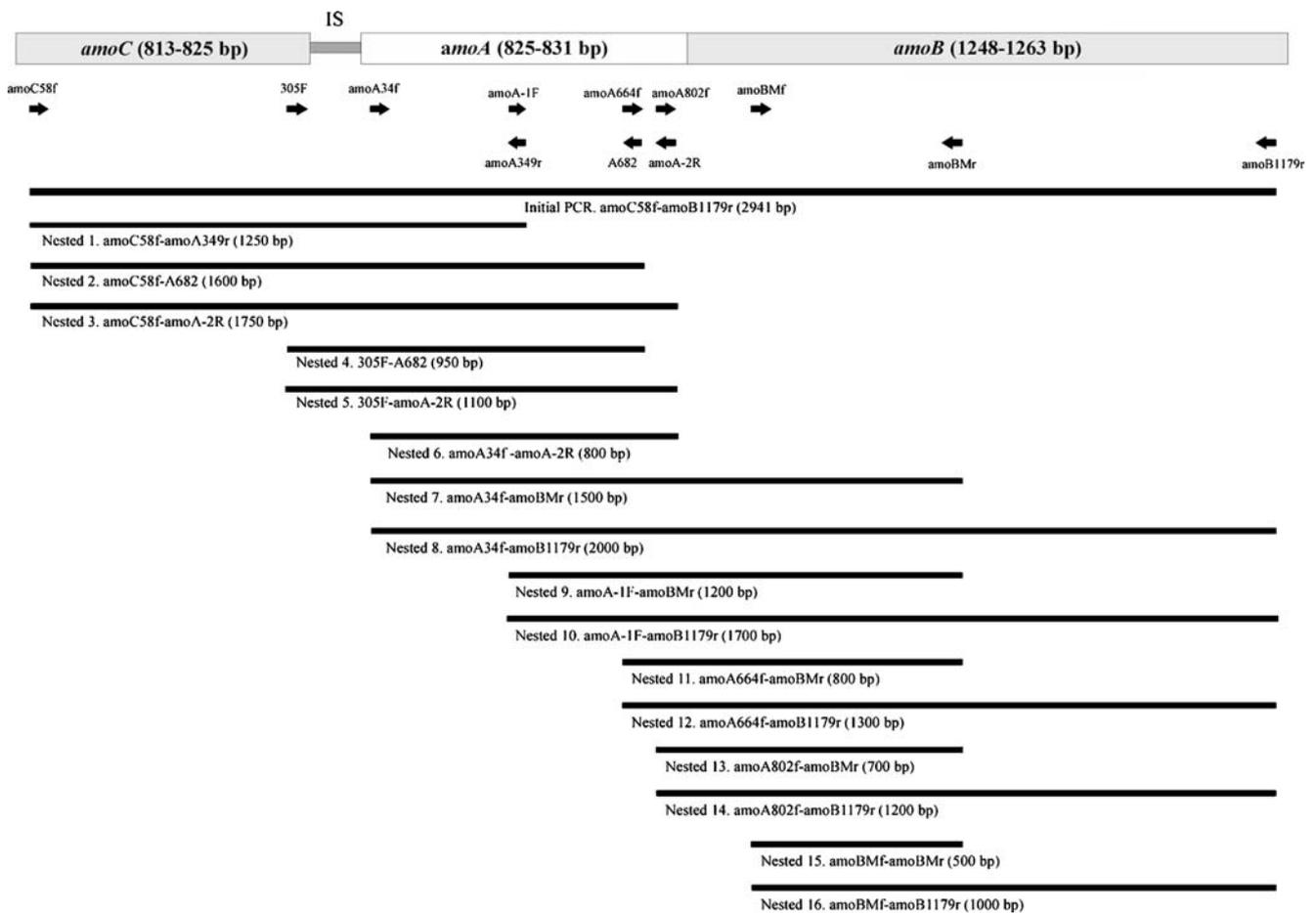
Each environmental sample was characterized by a specific pattern of bands. The most complex patterns were observed in rhizospheric soil and a water sample from Lake Plußsee, whereas in both Lake Schöhsee and the Baltic Sea, just a single band was observed. The most prominent bands from each pattern were excised from the gel and sequenced. All the sequences matched *amoA* when subjected to BLAST, with the first hit corresponding to an identified
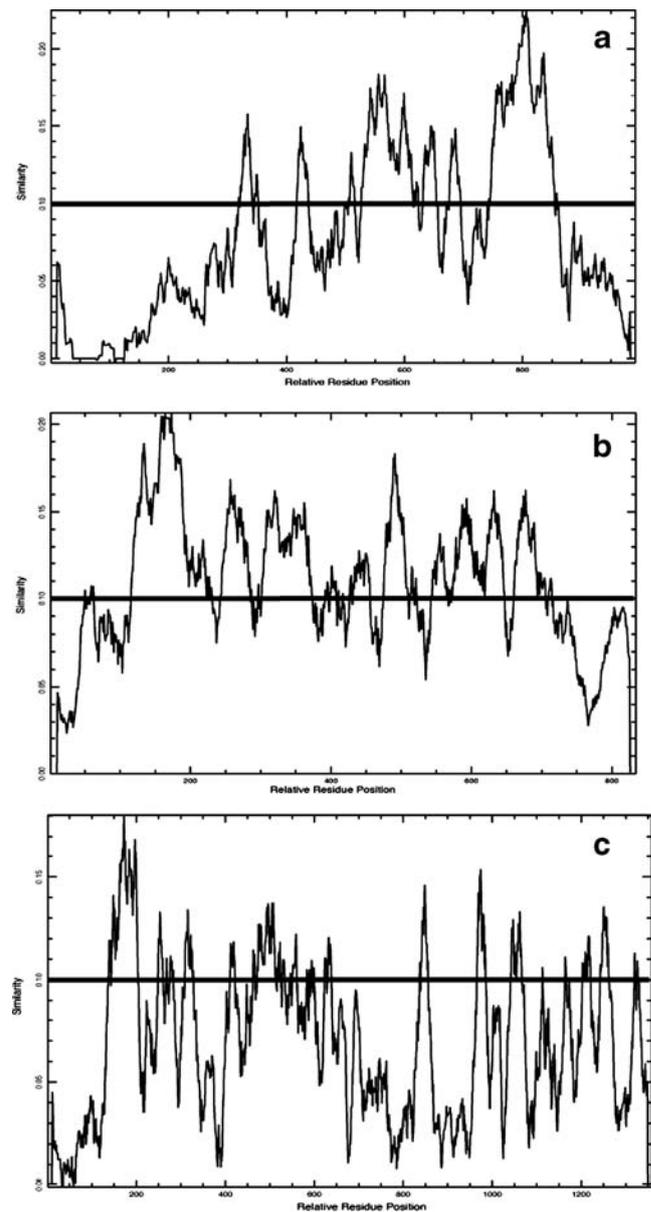
Fig. 3 Conservation plots made with *plotcon*. This program implements a sliding-window method and computes a local measure of similarity over a multiple alignment. Window size was 20 nt. Higher values indicate higher conservation. **a** *amoC*; **b** *amoA*; **c** *amoB*

species within the *Nitrosospira* lineage. The sequences were integrated into ARB and compared to all sequences of the region amplified available in GenBank (Fig. 4b). All sequences but one grouped with sequences from *Nitrosospira* species. The sequence from Schöhssee-10 clustered with sequences from *Nitrosomonas* species. Sequences from soil were closely related to each other. The soil band 2 and the sequence from the Baltic Sea were related to a sequence from uncultured AOB obtained from rhizosperic soil from a pea plantation. Three out of five sequences from Lake Plußsee formed a cluster related to a clone sequence

Fig. 4 Evaluation of nested PCR coupled to DGGE for analysis of AOB communities in environmental samples. **a** DGGE profiles of PCR amplicons obtained with the primers amoA121fgc–amoA359rC after nested PCR amplification from environmental samples. *Lane 1* rhizospheric soil; *lane 2* Jordan River; *lane 3* Lake Schöhsee; *lane 4* Lake Plußsee; *lane 5* Baltic Sea; *lane 6* N. europaea ATCC 19718; *lane 7* N. tenuis Nv1; *lane 8* N. briensis C-128. The bands extracted and re-amplified from the gel are indicated with numbers. **b** Phylogenetic tree based on *amoA* sequences from the bands extracted from the DGGE gel. Phylogeny was constructed with the PHYLIP subroutine by Fitch-Margoliash in ARB using sequences from *N. oceani* ATCC 19707 and PmoA from *M. capsulatus* str. Bath and *Methylocystis* sp. M and *Methylocystis* sp. SC2 as out groups. Clones from this study are shown in *bold*

from grassland at the Sourhope research station in Scotland that had received partial additions of N fertilizers (Webster et al. 2002). Sequences from the Jordan River were very diverse and did not form a cluster. One of the sequences

(Jordan-9) grouped together with a clone from agricultural soil (Avrahami et al. 2003). The band Jordan-8 was distantly related to the cluster of sequences from Lake Plußsee and *Nitrosospira* sp. NpAV. Finally, the band

Fig. 5 Structure of the *amoCAB* operon in beta-AOB, including the intergenic space (*IS*) between *amoC* and *amoA* and indicating the position of the primers and different nested PCR tested (expected length of fragments is given in *parentheses*)

Jordan-7 grouped with clones from agricultural soil (Avrahami et al. 2003) and *N. multiformis*.

Amplification and characterization of additional fragments of the *amoCAB* operon

Because no bands were visible in the initial PCR with the primers amoC58f/amoB1179r in all environmental samples tested, additional primer combinations (Table 1) were used to amplify different regions of the *amoCAB* operon for their analysis. These combinations were tested with genomic DNA from *N. europaea* ATCC 19718 and total DNA extracted from a sample taken at the water–sediment interface at Boknis Eck out of the Kiel Fjord in the Baltic Sea. This sample was selected because a previous characterization using the 16S rRNA gene (Kim et al. 2006) and *amoA* (Kim et al. 2008) showed a diverse community of AOB composed by both *Nitrosomonas* and *Nitrosospira*-like sequences. A total of 16 nested amplifications were applied, but only ten of them produced a band of the expected size in both *N. europaea* and the environmental sample (primer combinations 1, 4, 5, 6, 7, 8, 9, 10, 13, and 14; Fig. 5). With the primer combinations 2, 3, 11, and 12, a product with the expected size was obtained from *N. europaea*, but no-product or a product with an unexpected size was obtained from the environmental sample (data not shown). Although a band with the expected size was observed in PCR with the primer combinations 15 and 16, the amplification was very inefficient in both *N. europaea* and the environmental sample (data not shown).

To better characterize the *amoCAB* products obtained from the environmental sample and to validate the methodological approach, clone libraries were prepared with the following PCR products: amoC58f/amoA349r, 305F/amoA-2R, amoA34f/amoBMr, amoA34f–amoB1179r, amoA-1F/amoB1179r, and amoA802f/amoBMr (primer combinations 1, 5, 7, 8, 10, and 13 in Fig. 5). These combinations were selected because they represent different regions within the *amoCAB* operon, including *amoC* (primer combination 1), the intergenic region (IR) between *amoC* and *amoA* (primer combinations 1 and 5), *amoA* (primer combinations 1, 5, 7, 8, and 10), and *amoB* (primer combinations 7, 8, 10, and 13). Finally, 47 clones were obtained, containing an insert with the expected size, and in all the cases, the cloned products

corresponded to the different genes of the *amoCAB* operon (see below).

In the phylogenetic trees, all *amoC* clones (766 bp) formed a single cluster (cluster C-I) related to *Nitrosomonas* (Fig. 6a). The corresponding *amoA* sequences from the same clones also grouped into a single cluster (cluster A-I, Fig. 6b), which contained sequences from products obtained with the primer combinations 5 (one clone), 7 (three clones), 8 (two clones), and 10 (five clones) in addition. The cluster A-I was related to other sequences from *Nitrosomonas* species, but formed an independent branch from sequences of cultured AOB.

Since many of the *amoA* sequences obtained here include the starting codon of the gene, the use of an alternative starting codon was analyzed in the clones. It has been noted before that the unusual start codon GTG is preferentially found in *Nitrosomonas amoA*, in contrast to the standard ATG start found in *Nitrosospira* strains (Norton et al. 2002). The analysis of the sequences included in the cluster A-I showed the triplet GTG as start codon (data not shown), further supporting their placement in the *Nitrosomonas* lineage.

Two additional *amoA* clusters related to *Nitrosospira* were observed (Fig. 6b). These clusters include most of the clones from the primer combination 5 (nine clones in cluster A-II and two clones in cluster A-III) and some of the clones of the primer combination 7 (two clones in cluster A-III). All these clones have the standard ATG start codon that has been observed in *Nitrosospira* strains (Norton et al. 2002).

The phylogenetic assignment of *amoB* sequences was in agreement with those of *amoA*. The clones in the cluster B-I of *amoB* (Fig. 6c) phylogenetically corresponded to those in the *amoA* cluster A-I, whereas the clones in cluster B-II corresponded to those included in *amoA* cluster A-III. The cluster B-I was related to the *Nitrosomonas* lineage and included clones from the primer combinations 7 (three clones), 8 (two clones), 10 (five clones), and 13 (five clones). The cluster B-II belonged to the *Nitrosospira* lineage and contained sequences from primer combinations 7 (two clones) and 13 (five clones).

Non-coding intergenic regions between *amoC–amoA* and *amoA–amoB*

In all the clones, including sequences from *amoC* and *amoA*, an IR between the two genes was observed that varied from 136 to 428 bp (data not shown).

In all the clones related to *Nitrosospira*, the *amoA* was followed immediately by *amoB*. In contrast, a 1-bp overlap and shift in the open reading frame between *amoA* and *amoB* was observed in the clones related to *Nitrosomonas*. This is in agreement with previous observations of the physical structure of the *amoCAB* operon, which indicate that in beta-AOB, *amoA* is either followed immediately by *amoB* or there is a 1-bp overlap between both genes (Norton et al. 2002). This is in contrast to the situation with gamma-AOB where the *amoA* and *amoB* genes are separated by an intergenic region of approximately 65 bp (Alzerreca et al. 1999; Norton et al. 2002).

Discussion

Traditionally, a fragment of 453 bp from *amoA* has been used to infer the phylogeny (Purkhold et al. 2000, 2003) and to study the diversity of AOB (Rotthauwe et al. 1997). However, this fraction of *amoA* provides less resolution than the 16S rRNA gene because it is relatively short and highly conserved (Koops et al. 2003; Purkhold et al. 2003). Therefore, one of the main challenges for studying AOB in environmental samples is the search for alternative molecular markers with higher variability, but can still be amplified by PCR. In the present study, the three genes forming the *amoCAB* operon are proposed as an alternative to study the phylogeny and diversity of betaproteobacterial AOB in environmental samples.

The general topology of the phylogenetic trees obtained with the full-length *amoCAB* genes agreed with the existing trees for the 16S rRNA gene and the short fragment of *amoA* (Purkhold et al. 2000, 2003; Aakra et al. 2001a, b). Additionally, specific lineages within the betaproteobacterial AOB that have been defined based on the short deduced AmoA peptide fragment and 16S rRNA gene sequences (Purkhold et al. 2000, 2003) were also observed. In the phylogeny based on the deduced amino acid sequence of the complete AmoA, the lineages *N. oligotropha* or *N. europaea/N. mobilis* were identified (Fig. 1a), but the separation of specific lineages within *Nitrosospira* spp. could not be evaluated because all available sequences correspond to strains from the *Nitrosospira* cluster 3. Similarly, because there are fewer *amoC* and *amoB* sequences available, only the lineages *N. europaea/N. mobilis* and the *Nitrosospira* cluster 3 were represented in the phylogenetic trees of AmoC and AmoB, and therefore, a comprehensive comparison with previously reported AmoA and 16S rRNA gene lineages was not possible.

The position of *Nitrosospira cryotolerans* in the phylogenetic tree changed when the whole AmoA sequence was considered. With the complete AmoA sequences, *N. cryotolerans* was related to *Nitrosomonas* sp. JL21 and *Nitrosomonas* sp. AL212, while it stands alone in the phylogenetic tree with the short AmoA sequences (Purkhold et al. 2003).

Interestingly, two single *amoC* copies found in the complete genomes of *N. europaea* ATCC 19718 (Chain et

Fig. 6 Phylogenetic tree based on AmoC (**a**), AmoA (**b**), and AmoB (**c**) sequences obtained from the Baltic Sea water–sediment interface sample. Phylogenies were constructed with the PHYLIP subroutine by Fitch-Margoliash in ARB using sequences from *N. oceani* ATCC 19707 and PmoCAB from *M. capsulatus* str. Bath and *Methylocystis* sp. SC2 as out groups. Bootstraps values are indicated
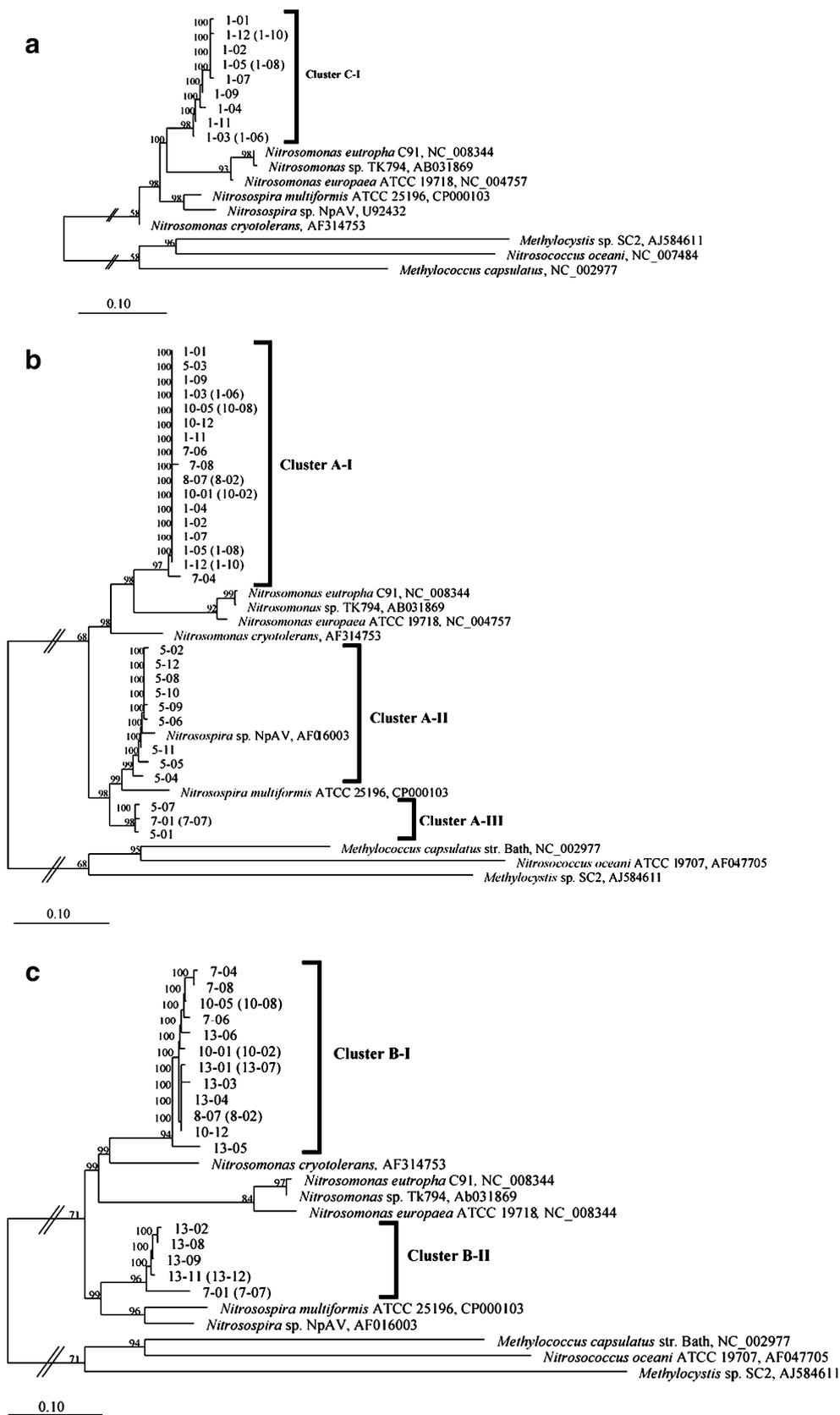
al. 2003) and *N. multiformis* ATCC 25196 formed an independent cluster separated from the other AmoC homologs in these species. The separation of these two *amoC* copies might indicate a different origin and evolutionary background or a less constrictive selective pressure for the evolution of these copies that do not belong to the *amoCAB* operon. Surprisingly, the second copy in *N. multiformis* ATCC 25196 clustered with other *amoC* of the same species.

The analysis of the co-evolution of the different genes in the *amoCAB* operon showed a significant correlation in the evolutionary rates of the individual genes. Although there are evidences of co-evolution between the genes, this does not imply that the evolutionary rates of each individual gene were equal. In fact, evolutionary rates reflected by the slope of the linear regression were highest in *amoB*, followed by *amoA* and *amoC* (Fig. 2b, d, and f). The differences in the evolutionary rates between the *amoCAB* genes points toward differences that might correlate to the function of the subunits coded by these genes. In the homologous particulate methane monooxygenase (pMMO), it has been established that the subunit coded by *pmoB* constitutes the soluble component of the otherwise membrane-bound enzyme complex (Lieberman and Rosenzweig 2005; Balasubramanian and Rosenzweig 2007). By analogy, it can be assumed that *amoB* also codes for a soluble subunit of AMO. If that is the case, *amoB* might be subject to a different selection pressure compared to *amoC* and *amoA*, which code for the subunits involved in the anchoring of the enzyme in the membrane and in formation of the active site.

An additional element that was considered for evaluating whether a gene is a good candidate for a molecular marker is the presence of conserved regions (suitable for primer design) as well as variable regions (e.g., suitable for phylogenetic inference). This study (Fig. 3) shows that *amoB* contains a considerably higher amount of variable regions, compared to the shorter *amoC* and *amoA*. It also shows that the partial *amoA* region traditionally amplified in community studies is highly conserved, reaffirming the limitations previously signaled in other studies (Koops et al. 2003; Purkhold et al. 2003). Amplifying the whole *amoA* allows the inclusion of four additional variable regions, increasing the informative positions that can be used for phylogenetic inference.

The results of the sequence analysis indicate that all the genes *amoCAB* can be equally used as molecular markers for studying AOB. In case all the *amoCAB* genes are included as molecular markers for AOB in environmental samples, *amoC* and *amoA* (being the most conserved) could be recommended for studying less closely related species, whereas *amoB*, due to its lesser sequence conservation, could be a better molecular marker to resolve closely related species.

Conventional PCR often is not sensitive enough to amplify *amoCAB* genes from natural habitats with low abundances of AOB (Beman and Francis 2006; Leininger et al. 2006). Therefore, we have selected a nested PCR approach that could, through successive amplification steps, increase the detection limit of *amoCAB* genes as has been shown previously for the 16S rRNA gene (Ward et al. 1997, 2000; Hastings et al. 1998; O'Mullan and Ward 2005; Kim et al. 2006). Although *amoCAB* products could be obtained after initial amplification in all beta-AOB strains tested, in environmental samples, nested PCR was always required to obtain PCR products.

The two methodologies tested to determine the identity of these environmental *amoCAB* fragments (DGGE and cloning and sequencing) confirmed the obtainment of environmental *amoCAB* sequences. In this case, the sequences analyzed were not intended to fully characterize the community composition, but rather to confirm the specificity of the products obtained. In all cases, the environmental *amoCAB* sequences obtained were different enough to be clustered separately of cultured strains of beta-AOB, but clearly they belonged to the beta-AOB.

An additional advantage of the approach proposed here is that it allows the amplification not only of the coding regions but also of the non-coding parts of the *amoCAB* operon. Sequence analyses of cultured AOB species indicate the existence of an IR between *amoC* and *amoA* (Norton et al. 2002). In the present study, both IR regions between *amoC* and *amoA*, as well as between *amoA* and *amoB*, could be observed in some of the clones sequenced and were informative for the placing of the clones in different phylogenetic groups. Although the intergenic region between the 16S and the 23S rRNA genes has been used to study the phylogeny of AOB (Aakra et al. 2001a, b), the phylogenetic relevance of the IR region between *amoC* and *amoA* has never been considered for this purpose.

In summary, the approach presented here opens new possibilities to study the diversity and evolution of AOB in the environment, for the following reasons: (1) It enables an increase in the sensitivity of detection by nested PCR targeting the different AMO genes; (2) it allows the amplification of the almost complete *amoA* gene, with about 300 bp more sequence information than the previous approaches; (3) as *amoB* turns out to have the highest sequence variability of the three *amo* genes, this might be a better molecular marker than the most frequently used *amoA* to resolve closely related species; (4) the coupled study of all three *amo* genes and the intergenic spacer regions that are under different selection pressure allows a more detailed analysis of the evolutionary processes, which are responsible for the differentiation of AOB communities at different habitats.

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