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HYDROXYLAMINE OXIDOREDUCTASE, AN ALTERNATIVE FUNCTIONAL AND PHYLOGENETIC MARKER FOR ANALYZING AUTOTROPHIC AMMONIA OXIDIZING BACTERIA

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ABSTRACT: In aerobic autotrophic ammonia oxidizing bacteria (AOB), an octaheme enzyme, hydroxylamine oxidoreductase (*hao*) catalyzes the conversion of hydroxylamine to nitrite. For *hao* to be considered as a functional and phylogenetic marker, sequence based analysis was carried out. Sequence diversity in *hao* gene fragment was assessed and compared with 16S rRNA and ammonia monooxygenase (*amo*A) gene fragments from 21 AOB. Phylogenetic analysis of AOB based on 16S rRNA, *hao*, and *amo*A gene fragments showed similar tree topologies. The diversity of the studied AOB was found to be similar in both richness and evenness for the three genes and the minor differences observed in the Shannon's diversity index were not found to be significant according to t-test and p-values. Co-evolution of *hao* and *amo*A genes was established based on the congruence observed amongst *amo*A and *hao* gene fragments phylogenetic trees, similarities in the rates of synonymous and non-synonymous mutations, correlation plots of *amo*A and *hao* gene fragments (r^2 - 0.9) and their percentage sequence similarity. These evidences suggest high potential of *hao* gene fragment to be used as a functional and phylogenetic marker for the evolutionary evaluation of AOB.

KEYWORDS: Co-evolution, phylogenetic marker, autotrophic ammonia oxidizing bacteria, ammonia monooxygenase, hydroxylamine oxidoreductase.

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1. INTRODUCTION

In autotrophic ammonia oxidizing bacteria (AOB), oxidation of ammonia, is the rate limiting step in carrying out nitrification and hence the most important reaction of the global nitrogen cycle [1, 2]. The 16S rRNA gene has been conventionally used as a phylogenetic marker to study the diversity and phylogeny of AOB [1, 3-5] but it does not necessarily correlate with the physiology of the microbes, and close sequence similarities in the 16S rRNA gene may misplace the identified organism in phylogenetically related but physiologically and ecologically unrelated taxa [6, 7]. Additionally, detection of microorganisms present in very low number is difficult with 16S rRNA gene. These limitations can be easily overcome by using genes unique to AOB and reduce the possibility of detecting non-target organisms [8-10]. Oxidation of ammonia to hydroxylamine, the first step of aerobic ammonia oxidation is catalyzed by ammonia monooxygenase (AMO). The α subunit of the enzyme (*amo*A) is the active site of the enzyme and is an established fine scale molecular marker used frequently for studying the diversity of AOB [11-14]. Existence of *amo*A ortholog is also documented in autotrophic ammonia oxidizing archaea (AOA) through metagenomic studies [15] and has been used in several studies to check the abundance and diversity of both AOB and AOA [8, 16-17]. However, *amo*A gene is relatively short, highly conserved and has been reported to provide lesser resolution than the 16S rRNA gene [5, 7, 18], it is therefore a required study and establish an alternate unique marker for the phylogenetic analysis of AOB. Hydroxylamine oxidoreductase (HAO), is an octahaem enzyme that catalyzes the second step in the reaction converting hydroxylamine to nitrite in AOB [19]. The use of *hao* as a molecular marker for identifying AOB was suggested by Shinozaki and Fukui [20], and subsequently Schmid et al. [21] used it to detect AOB from environmental samples. More recently, grouping of AOB through *hao* gene SSCP analysis has been shown to follow the same pattern as 16S rRNA and *amo*A gene based analysis [22]. *hao,* like *amo*A, is unique to this group of organisms and hence its sequence based analysis as a phylogenetic marker in comparison with *amo*A and 16S rRNA needs detailed investigation. Proteins, which interact with one another or are involved in the same pathway are reported to co-evolve in order to retain their functional relationships [23, 24]. Recent studies have evidently shown high correlation in branch length of phylogenetic trees of proteins involved in translation and ribosomal activities [24]. Evolutionary relationship between AMO and HAO, involved in the same reaction, has not been studied till date. Holmes et al. [25] showed evidences of evolutionary relationship of AMO with particulate methane monooxygenase (pMMO) and Klotz and Norton [26] later showed that the concerted relationship between them occurred under AT/GC mutational pressure. HAO, on the other hand has been reported to have evolved from octahaem cytochrome *c* nitrite reductase [27]. To establish *hao* gene as an alternative functional and phylogenetic marker, one needs to compare the evolutionary relationship of *hao* with 16S rRNA gene, a conventional phylogenetic marker and *amo*A gene fragment as a functional marker.

Accordingly, in the present study, efficiency of *hao* as a phylogenetic marker was evaluated and compared with *amo*A and 16S rRNA genes based phylogenies. The study was further extended towards analyzing the co-evolution of the functional genes (*amo*A and *hao*).

2. MATERIALS AND METHODS

2.1 Sequence data

Sequences of 16S rRNA, *amo*A and *hao* genes of 21 AOB (9 AOB strains belonging to ß and γ – subclass of proteobacteria and 12 uncultured AOB such that all the three genes sequences were available) were examined in this study (Table 1). Sequences for the AOB strains were obtained from GenBank, amongst which 12 uncultured AOB sequences were obtained indigenously by the authors in previous studies [22]. The size of *hao, amo*A and 16S rRNA genes ranged from 654bp - 850bp, 453bp - 883bp and 1207bp - 1529bp respectively. Accession numbers of all the genes used in the study are provided in Table 1.

Table 1. Accession numbers of 16S rRNA, *amo*A and *hao* genes analyzed in this study

	16S rRNA	*amo*A	*hao*
Nitrosomonas sp. ENI 11	AB079053	AB079054	AB030387
Nitrosomonas europaea	AB070983	JN099309	U04053
Nitrosomonas nitrosa	AJ298740	AF272404	FM163624
Nitrosomonas oligotropha	AJ298736	AF272422	FM163625
Nitrosomonas sp. Nm143	AY123794	AY123816	FM163622
Nitrosomonas eutropha C91	NR027566	U51630	CP000450
Nitrosococcus oceanus	M96398	U96611	AY858555
Nitrosospira multiformis	AY123807	DQ228454	AB070980
Nitrosospira briensis	AY123800	U76553	FM163621
Uncultured Bacterium (UN) DnrA	JN099273	JN099285	JN099297
UN DnrB1	JN099274	JN099286	JN099298
UN DnrB2	JN099275	JN099287	JN099299
UN CETP	JN099276	JN099288	JN099300
UN DN1	JN099277	JN099289	JN099301
UN DN2	JN099278	JN099290	JN099302
UN DN3	JN099279	JN099291	JN099303
UN N1	JN099280	JN099292	JN099304
UN N2	JN099281	JN099293	JN099305
UN N3	JN099282	JN099294	JN099306
UN PF1	JN099283	JN099295	JN099307
UN PF2	JN099284	JN099296	JN099308

2.2 Mutation and phylogenetic analysis

Multiple sequence alignments were performed using ClustalW. Maximum likelihood estimate of the nucleotide substitution patterns and the mean diversity of the entire population were calculated independently for the three genes using MEGA 4 software. Neighbor Joining trees for 16S rRNA, *amo*A and *hao* gene fragments were generated, based on the nucleotide sequence divergence, using MEGA 4 software. The trees were drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. All positions containing alignment gaps and missing data were eliminated through pair wise sequence comparisons and deletion option. Codon-based evolutionary diversity for the entire population was calculated for *amoA* and *hao* gene fragments. Total synonymous and non-synonymous mutations and their rates of substitution were calculated using MEGA 4.0 software. Correlation plots based on percentage sequence similarity between species were plotted for 16S rRNA, *amo*A and *hao* genes where simple linear regression was applied. Pearson's correlation coefficient was determined using SigmaStat version 3.5 for the sequence similarity between the three genes.

2.3 Statistical analyses

The number of groups obtained in the phylogenetic trees based on 16S rRNA, *amo*A and *hao* gene sequences were used to determine Shannon's diversity index (*H'*) and Shannon's evenness (E) using Abundance Curve calculator by Dr. James A. Danoff-Burg and X. Chen. Shannon's diversity index was calculated according to the following equation [28].

$$H' = -\sum_{i=1}^{s} (\mathrm{Pi}) \log (\mathrm{Pi})$$

Where S is the number of groups obtained in AOB using the three gene fragments and Pi are the number of species in that group. AOB having ≥ 98% sequence similarities were considered in one group. Statistical significance of the diversity of AOB calculated through Shannon's diversity index was evaluated as per Shannon's t-test and *p*-values. All calculations were carried out according to Abundance Curve calculator by Dr. James A Danoff-Burg and X. Chen.

Further, congruence amongst the pair of phylogenetic trees (a) *amo*A and 16S rRNA, b) *amo*A and *hao*, c) 16S rRNA and *hao* genes) was statistically analyzed according to Arnaoudova et al. [29]. Statistical hypothesis accordingly were as follows:

H_0: Phylogenetic trees T_1 and T_2 are congruent. H_0: $\| v(T_1) - v(T_2) \| = 0$

H1: Phylogenetic trees T_1 and T_2 are incongruent. H_1: $\| v(T_1) - v(T_2) \| > 0$

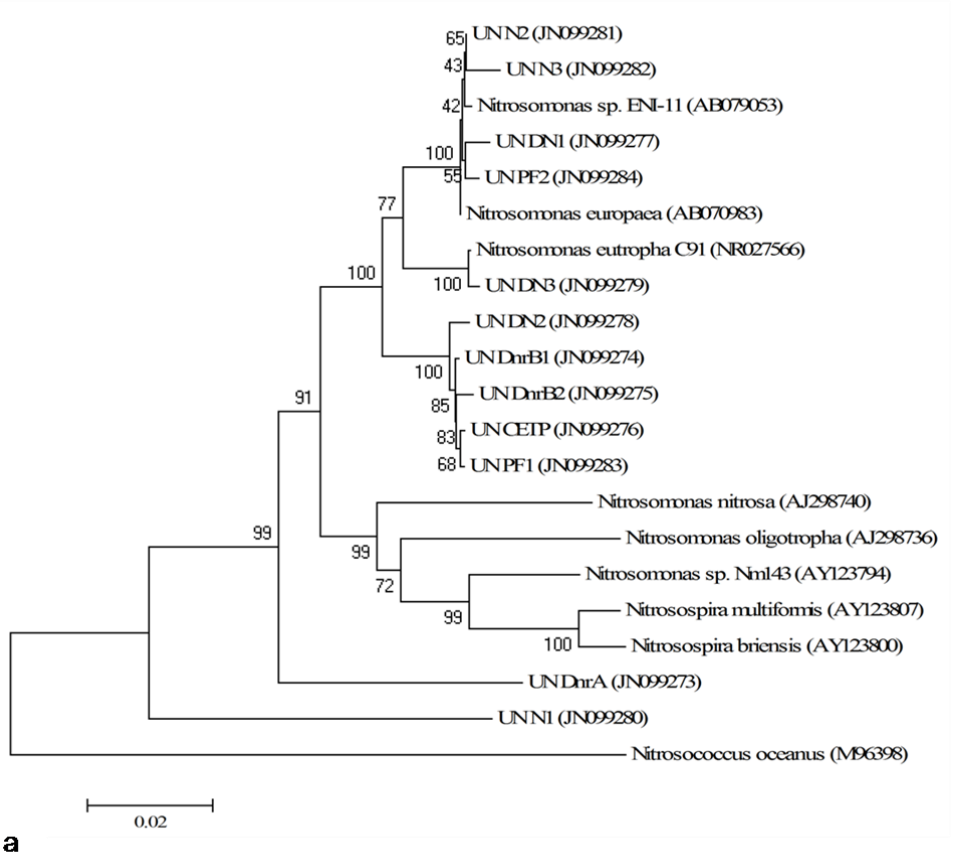
Where, $\| v(T_1) - v(T_2) \| = \sqrt{(d_{1,2}^{T_1} - d_{1,2}^{T_2})^2 + \ldots + (d_{n-1,n}^{T_1} - d_{n-1,n}^{T_2})^2}$ and $d_{i,j}^{T}$ is the pair wise distance between the leaves i and j in tree T.

3. RESULTS AND DISCUSSION

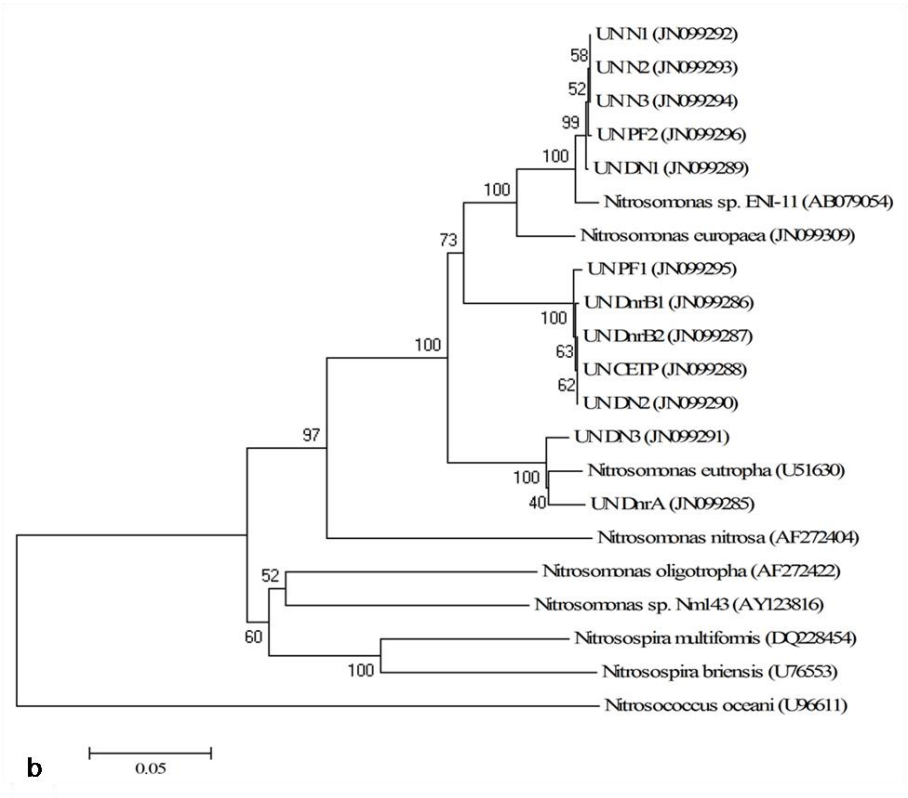
Oxidation of ammonia to nitrite in AOB is a two-step process, wherein the first reaction, oxidation of ammonia to hydroxylamine, is catalyzed by ammonia monooxygnase and the second reaction, oxidation of hydroxylamine to nitrite, is catalyzed by hydroxylamine oxidoreductase [30]. Gene coding for the active site of ammonia monoxygenase (*amo*A) has been widely used as a marker for studying the diversity and phylogenetic analysis of AOB [11], but *hao* has not been studied in depth as a functional and phylogenetic marker. Availability of primers for *hao* led to amplification of quite a few *hao* genes sequences [21], which are accessible in the databanks. With the aim to compare *hao* as a marker with *amo*A (a well-established functional marker) and 16S rRNA gene (a conventional phylogenetic marker) the study was initiated by obtaining *hao* sequences from AOB whose *amo*A and 16S rRNA gene sequence data were also available from GenBank (Table 1). Two strains *Nitrosospira* sp. 40KI and *Nitrosospira* sp. III7 were not considered in this study as their *hao* sequences were too short (243bp).

3.1 Phylogenetic analysis of AOB with respect to 16S rRNA, *amo*A and *hao* genes

Phylogeny of AOB was deduced using 16S rRNA, *amo*A and *hao* gene fragments. Similar tree topologies were observed in the phylogenetic trees constructed for the three genes, clustering *Nitrosomonas* and *Nitrosospira* lineages (betaproteobacterial AOB) together, separated from *Nitrosococcus* lineage (gammaproteobacterial AOB) (Fig 1). *Nitrosomonas* lineage in the present study constituted of three stable branches (100 bootstrap value) excluding *Nitrosomonas nitrosa* and *Nitrosomonas oligotropha* in the trees derived from all the three genes. *Nitrosmonas nitrosa* clustered in *Nitrosospira* lineage according to 16S rRNA phylogeny but was clustered with *Nitrosomonas* lineage according to the functional genes (*amo*A and *hao*) based phylogenies. Similarly, UN DnrA and UN N1 formed separate branches according to 16S rRNA gene phylogeny but were included in the *Nitrosomonas* lineage according to both the functional genes (Fig 1). *Nitrosomonas oligotropha* in *amo*A and 16S rRNA genes based phylogenies separated from the *Nitrosomonas* lineage whereas in the *hao* phylogeny it was included in the *Nitrosomonas* lineage indicating that its *hao* sequence was more closely related with other members of the *Nitrosomonas* lineage than 16S rRNA and *amo*A genes.



a



b

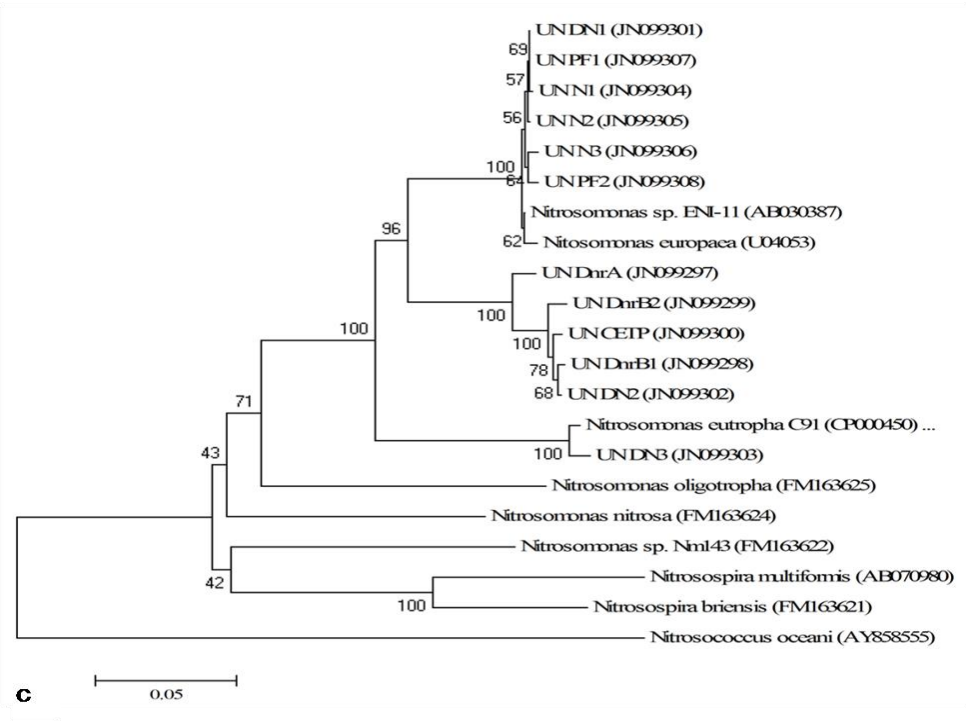


Figure 1: Phylogenetic tree reflecting the relationships of the 21 AOB with respect to the three genes a) 16S rRNA b) *amo*A and c) *hao* using the Neighbor Joining method. The sum of branch length were 0.452 for 16S rRNA, 1.18 for *amo*A and 1.073 for *hao*. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. Bar indicates 2% estimated sequence divergence in case of 16S rRNA and 5% estimated sequence divergence for *amo*A and *hao* trees.

Shannon's index of diversity (H') was calculated using the three genes individually and were estimated to be 2.02, 1.99 and 1.92 using 16S rRNA, *amo*A and *hao* gene fragments respectively. Evenness (E) in the population calculated for 16S rRNA, *amo*A and *hao* genes were 0.88, 0.86 and 0.83 respectively. The extent of diversity of the studied AOB with respect to both richness and evenness in the population was found to be similar using the three genes. Shannon's t-tests was therefore applied find out the significance of the minor differences observed in the Shannon's index of diversity. Shannon's t-tests and p-values calculated for a) 16S rRNA and *amo*A gene pair, b) *amo*A and *hao* gene pair and c) 16S rRNA and *hao* gene pair were 0.146, 0.317, 0.452 and 0.88, 0.65, 0.57 respectively. The obtained t-test values for the pairs of genes were less than the table value 2.021 and p-values of the two-tailed test were more than 0.05. This indicated that there was no significant difference in the diversity of AOB observed through 16S rRNA, *amo*A and *hao* gene fragments. Although overall phylogeny of 16S rRNA, *amo*A and *hao* genes showed similarity in tree topology but distinct difference was observed in the branch lengths of the functional genes (*amo*A and *hao*; 1.18 and 1.073 respectively) compared to the 16S rRNA gene (0.452) (Fig 1). This suggests that there were differences in the evolutionary rates of the functional genes compared to 16S rRNA. Since, the branch lengths of *amo*A and *hao* gene (functional genes) based phylogenetic

trees showed similarity, statistical analysis was carried out to test congruence between the trees based on the vector of the pair wise distance between the leaves according to Arnaoudova et al. [29]. $\|v(T_1)-v(T_2)\|$ for the phylogenetic trees based on the gene pairs a) *amo*A and *hao,* b) *amo*A and 16S rRNA and c) *hao* and 16S rRNA were 0.6, 2.2 and 1.8 respectively. The value for *amo*A and *hao* gene pair being close to zero and complied with our hypothesis that *amo*A and *hao* gene based phylogenetic trees were congruent whereas when the functional genes were compared to the phylogenetic marker i.e. *amo*A and 16S rRNA and *hao* and 16S rRNA, the values were much higher than zero and were suggestive on their incongruent nature. Transitions and transversions form the base in plotting phylogenetic trees and as congruence was observed amongst the *amo*A and *hao* genes based phylogenetic trees, it led us to study the magnitude of genetic variation in AOB based on transitions and transversions.

3.2 Magnitude of genetic variation in AOB with respect to 16S rRNA, *amo*A and *hao* genes

Patterns of nucleotide substitutions in the three genes showed higher transitions (Ti) than transversions (Tv) (Table 2). Higher transitions are commonly observed for most genes [32, 33]. Amongst the three genes (16S rRNA, *amo*A and *hao*), Ti substitution were more predominant in 16S rRNA than *amo*A and *hao* genes (Table 2). Ti/Tv rate ratios are extremely important in studying DNA sequence evolution, distance and phylogeny reconstruction [34, 35] and the level of transition bias varies in different microorganisms and also in the different genes in a group of microorganisms; thereby it assists in comprehending the patterns of molecular evolution of the genes [36]. High Ti/Tv rate ratios are indicative of low levels of genetic divergence [35]. 16S rRNA gene showed higher Ti/Tv rates ratios for both purines and pyrimidines (K1-3.641 and K2-8.581) than the functional genes *amo*A and *hao* and overall p-distance calculated for 16S rRNA gene was 3 folds lower than *amo*A and *hao* (Table 3) indicating lower sequence divergence in 16S rRNA gene than *amo*A and *hao* genes. Congruence observed in the phylogenetic trees based on *amo*A and *hao* genes and similar genetic divergence observed through Ti/Tv rates paved way to study their co-evolution.

Table 2 Pattern of nucleotide substitution in the 16S rRNA, *amo*A and *hao* gene fragments in the studied AOB

	A			T			C			G		
	16S rRNA	*amo*A	*hao*	16S rRNA	*amo*A	*hao*	16S rRNA	*amo*A	*hao*	16S rRNA	*amo*A	*hao*
A	-	-	-	2.64	6.23	3.72	2.86	4.71	6.54	14.66	10.31	10.16
T	3.39	4.18	6.28	-	-	-	24.5	17.5	21.74	4.03	5.38	5.92
C	3.39	4.18	6.28	22.68	23.17	12.38	-	-	-	4.03	5.38	5.92
G	12.33	8.01	10.79	2.64	6.23	3.72	2.86	4.71	6.54	-	-	-

Table 3 Estimate of the mean diversity of the entire population with respect to the three genes

	16S rRNA	*amo*A	*hao*
Ti/Tv rate (Purines) - K1	3.641	1.918	1.717
Ti/Tv rate (Pyrimidines) - K2	8.581	3.717	3.325
Over all Ti/Tv bias - R	2.867	1.508	1.275
Overall average P distance	0.065	0.184	0.169

3.3 Co-evolution *amo*A and *hao* genes and their correlation with 16S rRNA gene

The percent sequence similarity between all the species was analyzed to examine the correlation between the evolutionary rates of *amo*A and *hao* genes and were compared with 16S rRNA gene. Percentage sequence similarity values were represented in correlation plots (Fig 2). A linear regression was applied to the correlation plots for the three genes where a high r^2 value was indicative of co-evolution between pairs of genes. Amongst the co-evolving genes, comparative evolutionary rates could be determined from the slope of the curve [7]. Significant linear correlation was observed from the comparison between *amo*A and *hao* genes (r^2=0.9, p value < 0.001) whereas lower correlation was found between *amo*A and 16S rRNA and between *hao* and 16S rRNA gene pairs (*amo*A/16S rRNA gene r^2=0.65 and *hao*/16S rRNA gene r^2=0.57, p value < 0.001 in both the case). This indicated co-evolution of *amo*A and *hao* genes (Fig 2). However, slope of the correlation curve between *amo*A and *hao* was 0.843 which suggested that amongst these two co-evolving genes, *amo*A had higher evolutionary rate compared to *hao* gene (Fig 2). The differences in the evolutionary rates between the genes have been reported to correlate with the function of the enzymes coded by the genes [7]. AMO can act on a number of substrates including aliphatic, aromatic and halogenated molecules (methane, diethylether, fluoromethane, chloromethane, benzene, toluene, etc.) thereby supporting AOB to endure heterotrophic conditions [30]. HAO of AOB can catalyze oxidation of hydroxylamine differentially, producing nitrite under aerobic conditions and N_2O and NO under microaerophilic conditions enabling them to thrive under the said conditions [30]. Due to these diverse catalytic activities of both the enzymes involved in the same pathway, different selective pressures could have acted upon them resulting in differences in their evolutionary rates. Evolutionary history of HAO reported by Klotz et al. [27] also supports that *amo*A and *hao* genes may have different evolutionary rates. Despite having differences in the evolutionary rates of *amo*A and *hao* genes their Pearson's correlation coefficient (r-0.949, *p*-value < 0.001) suggested co-evolution of the two genes. Pearson's correlation coefficients for 16S rRNA with *amo*A and *hao* genes were r = 0.806 and 0.757 respectively with *p*-value < 0.001 indicating

significantly lesser correlation of the two genes with 16S rRNA gene compared to the correlation observed amongst the functional genes. The results are in agreement with Li and Rodrigo [24] who showed that functionally related genes co-evolution, while studying 216 genes from 10 prokaryotic species.

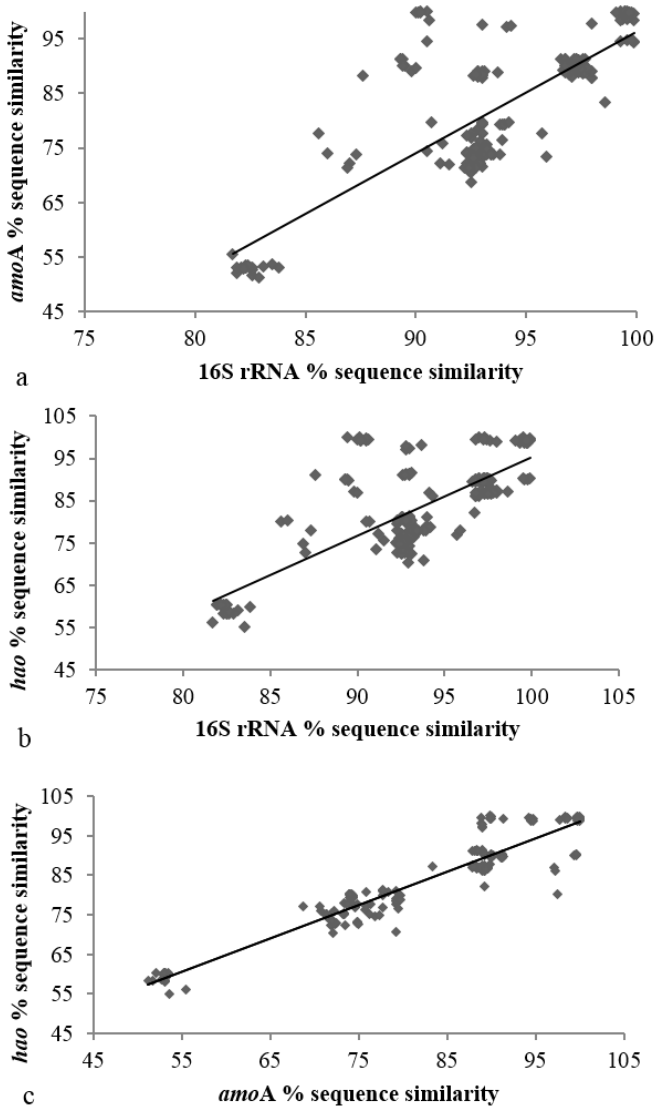


Figure 2: Correlation plots of a) *amo*A and 16S rRNA gene % sequence similarity b) *hao* and 16S rRNA gene % sequence similarity and c) *amo*A and *hao* gene % sequence similarity

Codon based evolutionary relationship between *amo*A and *hao* genes analyzed using MEGA 4 software showed that out of the total mutations in *amo*A and *hao* genes, synonymous mutations in both the genes were interestingly 29.6% and 29.5% respectively and similarly nonsynonymous mutations were 70.4% and 70.5% respectively. This again indicates high correlation between *amo*A and *hao* genes and confirms the co-evolution of *amo*A and *hao* genes. Rates of synonymous (d_S) and nonsynonymous substitutions (d_N) calculated according to Nei and Gojobori [37] using the MEGA 4 software (Table 7) showed higher d_N than d_S in both *amo*A and *hao* genes. Ratio of d_N:d_S

were found to be 1.92 and 1.56 respectively for *amo*A and *hao* genes, indicative of advantageous selection for new mutants than neutral variants and is suggestive of adaptive or positive Darwinian selection in both the genes.

Table 4 Estimate of the mean codon-based evolutionary diversity for the entire population

	*amo*A	*hao*
No. of Synonymous mutations	116.71 (29.6%)	202.41 (29.5%)
No. of Nonsynonymous mutations	277.33 (70.4%)	483.56 (70.5%)
Total mutations	394.043 (100%)	685.04 (100%)
Rate of nonsynonymous mutations (D_N)	0.212	0.180
Rate of synonymous mutations (D_s)	0.110	0.115
D_N/D_S	1.92	1.56

4. CONCLUSION

Phylogenetic trees constructed using the three genes *amo*A, *hao* and 16S rRNA showed statistically significant similar topologies. Grouping of AOB was found to be better using *hao* gene fragment. Diversity index calculated for AOB using the three genes were found to be similar. Sequence variation analysis, of the marker genes of AOB, showed bias towards transitions over transversions. Higher sequence divergence in *amo*A and *hao* gene fragments was observed compared to 16S rRNA gene and hence the marker genes have higher evolutionary rates compared to 16S rRNA gene. Synonymous and nonsynonymous substitutions were exceedingly similar in *amo*A and *hao* gene fragments. *hao* based AOB phylogeny was highly consistent with *amo*A and 16S rRNA gene based phylogenies with better separation of *Nitrosomonas* sp. Furthermore, co-evolution of *amo*A and *hao* genes suggests that *hao* can also be used at par with *amo*A as an alternative phylogenetic marker in studying the diversity and evolution of AOB.

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CONFLICT OF INTEREST

The author declares that there is no financial interest or any conflict of interest.

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