CHAPTER IV

RESULTS AND DISCUSSION

4.1 Communities of ammonia-oxidizing bacteria and archaea in full-scale wastewater treatment plants (WWTPs)

4.1.1 Description of full-scale WWTPs

Sludge sample were taken from 10 full-scale WWTPs, including 4 industrial WWTPs (I1-I4), 4 large municipal WTTPs (LM1-LM4), and 2 small municipal WWTPs (SM1 and SM4). The WTTPs were selected based on the difference in influent wastewater characteristics, system configuration, and system operation (Table 4.1). Four industrial WWTPs observed in this work were categorized into 3 types, depending on ammonia concentrations in influent wastewaters. Plant I2 represented the industrial WWTP with high ammonium concentration, Plants I3 and I4 represent the industrial WWTPs with moderate ammonium concentrations, and Plant I1 represented the industrial WWTP with low ammonium concentration. All industrial WWTPs were operated with activated sludge processes. All municipal WWTPs belong to Bangkok Metropolitan Administration (BMA). All of them were not different in influent characteristics. All municipal WWTPs received low ammonium concentration (5-13 mg-N/l), but different in system configuration and operation. All large and one small municipal WWTPs were operated with activated sludge processes, another small WWTPs was aerated lagoon system. The detail of each plants were shown in Table 4.1

Table 4.1 Description of wastewater treatment plants

WWTP	Treatment process	Flow (m^3/d)	ammonia (mg-N/l)		nitrite (mg-N/l)		nitrate (mg-N/l)		BOD (mg/l)		COD (mg/l)		DO (mg/l) Eff.	MLSS (mg/l)	HRT (hr)	SRT (day)
			Inf.	Eff.	Inf.	Eff.	Inf.	Eff.	Inf.	Eff.	Inf.	Eff.				
I1	AS	4,500	12.90	3.27	0.03	ND	22.56	35.24	NA	NA	NA	NA	NA	NA	1.20	NA
I2	AS	5,000	422.26	29.19	0.04	0.21	3.24	31.05	1,400	9	NA	NA	NA	4,000	24	14
I3	AS	1,300	72.50	3.32	0.04	0.02	3.76	46.87	NA	NA	NA	NA	NA	4,000	117.6	10
I4	AS	6,740	36.05	13.25	ND	0.15	1.43	0.96	192	3	505	26	3.68	5,530	118.5	10
LM1	AS	102,132.61	8.12	4.22	0.02	0.33	0.81	3.95	27.31	7.04	63.1	21.68	6.5	12,067	4	10
LM2	AS	27,634	5.54	5.05	0.03	0.18	0.79	1.80	49.16	12.47	85.63	45.38	5.74	NA	2.17	22
LM3	AS	133,986	5.74	7.30×10^{-3}	ND	0.03	0.78	7.23	27.07	3.21	NA	NA	6.12	3,933	4.27	11.04
LM4	AS	16,596	0.03	0.06	0.08	ND	3.56	12.32	57.42	5.45	106.85	19.42	NA	5,006	63.60	30
SM1	AS	600	13.87	2.35×10^{-3}	0.01	0.02	2.27	18.15	102	2	433	20	NA	6,790	43.20	21
SM4	Aerated lagoon	1,442	5.68	4.53	ND	0.23	0.94	1.33	75	5	185	15	1.58	105	67.24	> 1year

ND, not detected; NA, data not available
AS, activated sludge process; HRT, hydraulic retention time; SRT, solid retention time; MLSS, mixed liquor suspended solid;
[a] The values were the averages of the month in which sludge was collected.
[b] The values were analyzed from one-day grab samples collected on the day close to the day of sludge collection.;

4.1.2 Communities of ammonia-oxidizing bacteria in full-scale wastewater treatment plants

Communities of AOB in samples from full-scale WWTPs were investigated using specific PCR amplification, followed by DGGE, and sequencing of 16S rRNA gene of AOB belonging to beteproteobacteria. All bands recovered from DGGE were cut, reamplified, and run on new gels until they were purified before selecting for sequencing. In total, 14 bands of AOB 16S rRNA gene sequences were tested for sequence similarity using blast program (Table 4.2). All of the analyzed sequences showed 95 -100% identity at nucleotide level to the previous reported sequences in the database.

Table 4.2 Closely related sequences of AOB 16S rRNA gene fragments

Sample	Band	Score	Gap	Percent Identity	Accession No. of closely related sequence	closely related sequence
I1	AOB-I1-1	619	2/383 (0%)	367/383 (95%)	AY123811	*Nitrosomonas* sp. Nm59
I2	AOB-I2-1	667	0/364 (0%)	363/364 (99%)	AL954747	*Nitrosomonas europaea* ATCC 19718
I3	AOB-I3-1	662	0/364 (0%)	362/364 (99%)	AB176858	DGGE A-W-3
	AOB-I3-2	699	0/378 (0%)	378/378 (100%)	FM997803	Clone LEQUIA_R0CTO43
I4	AOB-I4-1	397	2/215 (0%)	212/215 (98%)	AJ297415	Clone GaN50304
LM1	AOB-LM1-1	787	1/443 (0%)	436/443 (98%)	AB222811	DGGE 0NO2c-3
	AOB-LM1-2	806	0/444 (0%)	441/444 (99%)	AJ297415	Clone GaN50304
	AOB-LM1-3	811	0/442 (0%)	441/442 (99%)	AJ297415	Clone GaN50304
LM2	AOB-LM2-1	623	3/357 (0%)	351/357 (98%)	EU224365	Clone 9R-27
LM3	AOB-LM3-1	577	2/349 (0%)	337/349 (96%)	EF016119	*Nitrosomonas oligotropha*
	AOB-LM3-2	401	2/227 (0%)	224/227 (98%)	AJ297415	Clone GaN50304
LM4	AOB-LM4-1	577	2/349 (0%)	337/349 (96%),	AB176858	DGGE A-W-3
SM1	AOB-SM1-1	630	0/347 (0%)	345/347 (99%)	AB176858	DGGE A-W-3
SM4	AOB-SM2-1	462	1/263 (0%)	259/263 (98%)	FM997808	Clone LEQUIA_R0CTO49

Phylogenetic trees were constructed by using three different methods comprising of distance matrix, maximum parsimony, and maximum likelihood. All methods exhibited the same grouping of AOB sequences in the tree (data not shown).

For phylogenetic presented in Figure 4.1, we add our partial 400-bp AOB 16S rRNA sequences using parsimony method into the phylogenetic tree prior constructed by neighbor joining (distance matrix) methods using 1000-bp sequences of all reference AOB species to avoid changing in the tree topology when shorter sequences than 1000-bp were used to constructed the tree. AOB found in each sample were summarized in Table 4.3.

Table 4.3 Summary of AOB found in full-scale WWTPs

AOB Cluster	I1	I2	I3	I4	LM1	LM2	LM3	LM4	SM1	SM4
Nitrosospira cluster										
unknown *Nitrosomonas* cluster			✓							
Nitrosomonas cryototerans cluster										
Nitrosomonas europaea-Nitrosococcus mobilis cluster		✓								
Nitrosomonas communis cluster			✓	✓	✓✓	✓	✓	✓	✓	
Nitrosomonas marina cluster										
Nitrosomonas oligotropha cluster	✓				✓		✓			✓

✓, present (amount of symbols represents numbers of band found)

AOB communities in industrial WWTPs (I1-I4) were more diverse than those in the municipal WWTPs (LM1-LM4 and SM1, SM4). AOB found in industrial WWTPs fell in 4 clusters that are unknown *Nitrosomonas* cluster, *N. europaea-Nc. mobilis* cluster, *N. communis* cluster, and *N. oligotropha* cluster. While AOB found in municipal WWTPs were restricted to only *N. communis* cluster and *N. oligotropha* cluster. The difference of wastewater characteristic might be the key factor causing distinct distribution patterns of AOB communities in both types of WWTPs. In the case of industrial WWTPs, characteristics of wastewater were varied considerably (Table 4.1). BOD and ammonia concentrations were in a range of 192 – 1,400 mg/l and 13 – 420 mg/l respectively. In contrast, influent wastewater of municipal WWTPs were similar in their characteristics. BOD and ammonia concentration were in a narrow ranges between 27 -102 mg/l and 5 – 14 mg/l respectively. It has been reported that ammonia concentrations is the important factor influencing the presence of AOB in different environments (Suwa *et al.*, 1994; Stehr *et al.*, 1995a; Koops and

Pommerening-Roser, 2001. Study on physiological properties of isolated AOB cultures suggested the difference in affinity constant for ammonia among the distinct AOB species. It is believed that this reflects the preference of AOB existing in the habitats (Koops *et al.*, 2003).

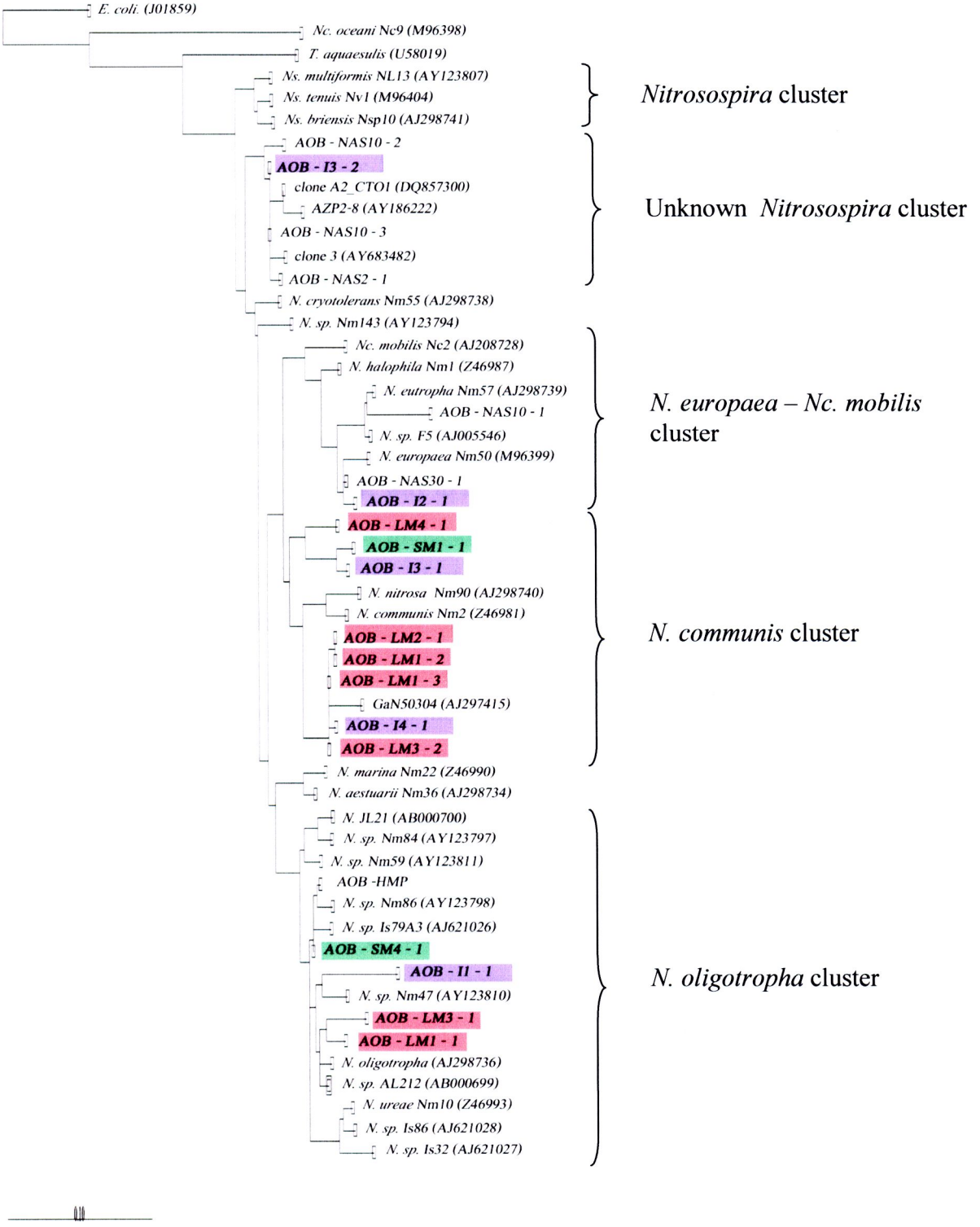

Figure 4.1 Phylogenetic tree showing 16S rRNA genes of AOB belonging to Betaproteobacterial with addition of 400-bp sequences from full-scale WWTPs into the distance tree that was previously constructed based on comparison of 1000-bp sequences of described AOB (Koops *et al.*, 2003)

Ammonium concentration in all 4 industrial WWTPs were varied significantly Plant I2 represented a WWTP receiving high ammonium concentration (400 mg-N/l). Phylogenetic analysis suggested that sequences recovered from plant I2, related closely to AOB in *N. europaea-Nc. mobilis* cluster. All members of *N. europaea-Nc. mobilis* cluster have relatively high affinity constants for ammonia (50 -100 μM). This make AOB in this cluster prefer eutrophic environments such as fertilized soil (Koops *et al.,* 2003). In addition *N. europaea* are common AOB found in WWTPs receiving high ammonium loads (Limpiyakorn *et al.,* 2007). Plants I3 and I4 represented WWTPs receiving mid-low ammonium concentrations (30 – 70 mg-N/l). Bands retrieved from these two plants fell in unknown *Nitrosomonas* cluster and *N. communis* cluster. So far, no isolate culture has been obtained for the unknown *Nitrosomonas* cluster. Therefore the physiological properties of AOB in this cluster have not been revealed. The only one way to make a discussion on the AOB cluster is using information obtained by direct molecular study. Previously, clone A2_CTO1 and clone 3 which are members of this cluster were recovered from activated sludge and aquarium biofilter which are mid-low ammonia concentrations. This implied that this AOB clusters are a group that have moderate affinity to ammonia. *N. communis* cluster have been classified as AOB with moderate affinity constants for ammonia (14 - 43 μM). From this information, they should restrict to moderate eutrophic habitats; however, this group of AOB is occasionally observed in freshwater which is one of oligotrophic environments (Koops *et al.,* 2003). In addition, they have been detected in soil, activated sludge, and biofilm system (Purkhold *et al.,* 2000; Gieseke *et al.,* 2001). Plant I1 represented a WWTP with low ammonium loads (10 mg-N/l), AOB found in this plant was the member of *N. oligotropha* cluster. *N. oligotropha* cluster are low in affinity constants for ammonia (1.9 – 4.2 μM). They frequently found in oligotrophic environments such as freshwater, unfertilized soil, WWTPs with relatively low ammonium loads (Koops and Pommerening-Roser 2001; Limpiyakorn *et al.,* 2005). Only members of *N. communis* cluster and *N.oligotropha* cluster were found in all 7 minicipal WWTPs. All municipal WWTPs received low ammonium strength wastewater (5 – 13 mg-N/l). If ammonium is the major factor, it is not surprising to find only 2 restrict AOB groups in all WWTPs. It has been reported extensively that *N. communis* cluster and *N.oligotropha* cluster were common AOB in

municipal WWTPs (Limpiyakorn *et al.*, 2007; Siripong and Rittmann 2007). Five out of the six plants contained AOB belonging to *N. communis* cluster. SM4 harbored only AOB of *N.oligotropha* cluster. It seemed that *N. communis* cluster might be more important than *N.oligotropha* cluster. From the study of Limpiyakorn 2007 by using real-time PCR to quantify each specific group of AOB in municipal WWTPs in Tokyo, *N.oligotropha* cluster were more abundant than *N. communis* cluster in all plants studied.

Other than ammonium loads or ammonium concentrations in the influent wastewater, system configuration and operation can be the other factors, influencing AOB communities in WWTPs. In this case only municipal WWTPs will be considered as it is possible to avoid the effect of influent characteristics. Almost all municipal WWTPs in this study were activated sludge process with the only exception for plant SM4 that was aerated lagoon system. In general, the SRT of aerated lagoon are relatively longer than the activated sludge process. SRT was firstly considerable as one of a major factor for system configuration and operation. However the results suggested that the AOB communities in all municipal WWTPs are similar. Therefore, system configuration and operation did not influence in this case.

4.1.3 Communities of ammonia-oxidizing archaea in full-scale wastewater treatment plants

Communities of AOA in samples from full-scale WWTPs were analyzed using specific PCR amplification, followed by clone libraries, and sequencing of AOA *amo*A gene fragments. For each library, 10 clones were randomly selected for sequencing. In total 72 clones, analyzed for sequencing, were tested for sequence similarity using blast program (Table 4.4). Results suggested that all analyzed showed 93 – 99% identity at nucleotide level to previously reported AOA *amo*A gene sequences. All analyzed sequences were calculated by DOTUR program to arrange for operational taxonomic units (OTUs) (Scholoss *et al.*, 2005). Any sequences from the same library that showed 100% identity OTUs were assembled as one OTU. All 72 AOA *amo*A sequences were categorized into 38 OTUs. The best quality AOA *amo*A sequence of each OTU was selected to be the representative one. The amounts

of the sequences of each OTU were displayed by the number in parentheses of Table 4.4.

Table 4.4 Closely related sequences of AOA *amo*A gene fragments

Sample	Clone	Score	Percent Identity	Gap	Accession No. of closely related sequence	closely related sequence	Source
I3	AOA-I3-1 (6)	1070	585/588 (99%)	0/588 (0%)	EU590198	Clone BGA-661	Soil
	AOA-I3-2 (1)	1050	579/584 (99%)	1/584 (0%)	EU590198	Clone BGA-661	Soil
	AOA-I3-3 (1)	1046	579/585 (98%)	2/585 (0%)	EU590198	Clone BGA-661	Soil
	AOA-I3-4 (1)	1048	580/586 (98%)	1/586 (0%)	EU590198	Clone BGA-661	Soil
	AOA-I3-5 (1)	1053	579/583 (99%)	1/583 (0%)	EU590198	Clone BGA-661	Soil
I4	AOA-I4-1 (1)	979	553/564 (98%)	2/564 (0%)	EU239976	Clone MamSp.H08	Mammoth Hot Spring sediment
LM1	AOA-LM1-1 (2)	1029	566/570 (99%)	1/570 (0%)	EU651295	Clone SF05-BG30-E01	Estuary sediments
	AOA-LM1-2 (3)	924	543/564 (96%)	1/564 (0%)	DQ278527	Clone DI-20	WWTP operated with low dissolved oxygen levels and long retention times
	AOA-LM1-3 (1)	985	558/570 (97%)	1/570 (0%)	EU239976	Clone MamSp.H08	Mammoth Hot Spring sediment
	AOA-LM1-4 (2)	996	560/570 (98%)	1/570 (0%)	EU239976	Clone MamSp.H08	Mammoth Hot Spring sediment
	AOA-LM1-5 (1)	1003	560/568 (98%)	1/568 (0%)	EU239976	Clone MamSp.H08	Mammoth Hot Spring sediment
	AOA-LM1-6 (1)	981	540/544 (99%)	1/544 (0%)	EU651295	Clone SF05-BG30-E01	Estuary sediments
LM2	AOA-LM2-1 (7)	1064	587/592 (99%)	1/592 (0%)	DQ304863	Clone 2	Trrestrial archaea
	AOA-LM2-2 (1)	1046	581/588 (98%)	2/588 (0%)	DQ304863	Clone 2	Terrestrial archaea
	AOA-LM2-3 (1)	1035	578/586 (98%)	3/586 (0%)	DQ304863	Clone 2	Terrestrial archaea
	AOA-LM2-4 (1)	1042	581/589 (98%)	3/589 (0%)	DQ304863	Clone 2	Terrestrial archaea
LM3	AOA-LM3-1 (2)	963	546/558 (97%)	2/558 (0%)	EU239976	Clone MamSp.H08	Mammoth Hot Spring sediment
	AOA-LM3-2 (1)	977	553/564 (98%)	3/564 (0%)	EU239976	Clone MamSp.H08	Mammoth Hot Spring sediment
	AOA-LM3-3 (1)	826	526/565 (93%)	2/565 (0%)	FJ227760	Clone WBM050405_45P2A1	Sediment
	AOA-LM3-4 (2)	841	530/567 (93%)	1/567 (0%)	FJ227760	Clone WBM050405_45P2A1	Sediment
	AOA-LM3-5 (2)	854	535/571 (93%)	1/571 (0%)	FJ227760	Clone WBM050405_45P2A1	Sediment
	AOA-LM3-6 (1)	990	557/567 (98%)	1/567 (0%)	EU239976	Clone MamSp.H08	Mammoth Hot Spring sediment
	AOA-LM3-7 (1)	965	567/589 (96%)	1/589 (0%)	EU852665	Clone PLANTC AR RSF-I OTU1	Water sampled

Sample	Clone	Score	Percent Identity	Gap	Accession No. of closely related sequence	closely related sequence	Source
LM3	AOA-LM3-8 (1)	979	555/567 (97%)	1/567 (0%)	EU239976	Clone MamSp.H08	Mammoth Hot Spring sediment
LM4	AOA-LM4-1 (9)	1013	573/585 (97%)	1/585 (0%)	EU885673	Clone 3063-A-04	Deep-sea sediments
	AOA-LM4-2 (1)	1009	568/579 (98%)	0/579 (0%)	EU885673	Clone 3063-A-04	Deep-sea sediments
SM1	AOA-SM1-1 (9)	981	554/565 (98%)	3/565 (0%)	FJ755701	Clone AOA-10	Sediment
	AOA-SM1-2 (1)	904	546/571 (95%)	15/571 (2%)	FJ755701	Clone AOA-10	Sediment
SM4	AOA-SM4-1 (1)	1057	586/592 (98%)	4/592 (0%)	EU885647	Clone 3057-A-16	Deep-sea sediments
	AOA-SM4-2 (1)	994	557/566 (98%)	1/566 (0%)	EU239976	Clone MamSp.H08	Mammoth Hot Spring sediment
	AOA-SM4-3 (1)	1018	576/588 (97%)	2/588 (0%)	EU885647	Clone 3057-A-16	Deep-sea sediments
	AOA-SM4-4 (1)	771	502/544 (92%)	2/544 (0%)	EU022958	Clone HB_C_0604__C02	Coastal sediments
	AOA-SM4-5 (1)	828	527/566 (93%)	1/566 (0%)	FJ227760	Clone WBM050405_45P2A1	Sediment
	AOA-SM4-6 (1)	924	553/579 (95%)	2/579 (0%)	FJ601561	Clone MS_26B4	Tropical marine estuary sediment
	AOA-SM-7 (1)	948	550/567 (97%)	5/567 (0%)	EF382617	Clone PA6-23	Coral colony
	AOA-SM4-8 (1)	1044	576/581 (99%)	1/581 (0%)	EU590435	Clone SGX-123	Soil
	AOA-SM4-9 (1)	1018	563/569 (98%)	0/569 (0%)	DQ501096	Clone MX_5_JAN_6	Estuarine sediments
	AOA-SM4-10 (1)	922	542/562 (96%)	5/562 (0%)	EF382617	Clone PA6-23	Coral colony

Number in parenthesis indicated amounts of AOA *amo*A clones showing 100% identity

Due to the limited information on AOA phylogenetic taxonomy, phylogenetic trees for AOA was constructed by tree different methods comprising of distance matrix, maximum parsimony, and maximum likelihood to confirm the grouping of AOA analyzed in this study and all major AOA reported in previous studies so far (Figure 4.3, 4.4, 4.5). It must be noted that Figure 4.3, 4.4, 4.5 have been used to confirm AOA clusters only. No information of species was provided in these three trees. Figure 4.2 showed phylogenetic tree constructed based on distance matrix (neighbor joining) with the complete sequence detail that will be used for further discussion. AOA clusters were defined based on OTUs using the DOTUR program (Scholoss *et al.*, 2005). Any AOA sequences, showing >86% identity, were

identified as the same AOA cluster. AOA communities in seed sludge, and enriched NAS were summarized in Table 4.5.

Table 4.5 Summary of AOA communities in full-scale WWTPs

Cluster	I1	I2	I3	I4	LM1	LM2	LM3	LM4	SM1	SM4
B					✓					
E										✓
A					✓✓					
F										✓
C								✓✓		✓✓✓
G			✓✓✓✓✓							
N										✓
D										
I							✓✓✓		✓✓	✓
J						✓✓✓✓				
K				✓	✓✓✓		✓✓✓✓			✓
L							✓			
M										✓✓

✓, present (amount of symbols represents numbers of OTUs)

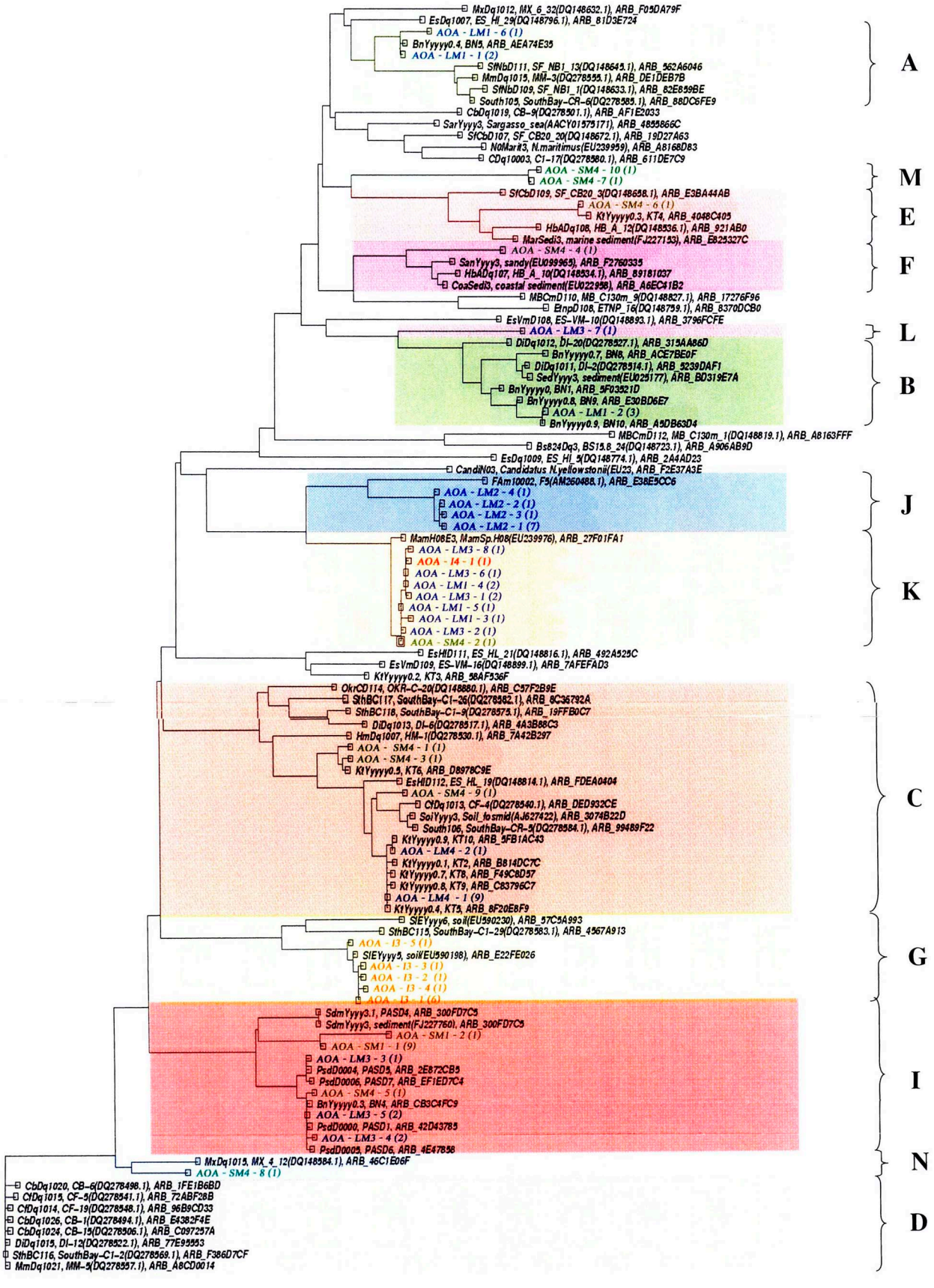

Figure 4.2 Neighbor joining tree of AOA *amo*A sequences from full-scale WWTPs (Details information is provided in this tree and this tree will be used for discussion)

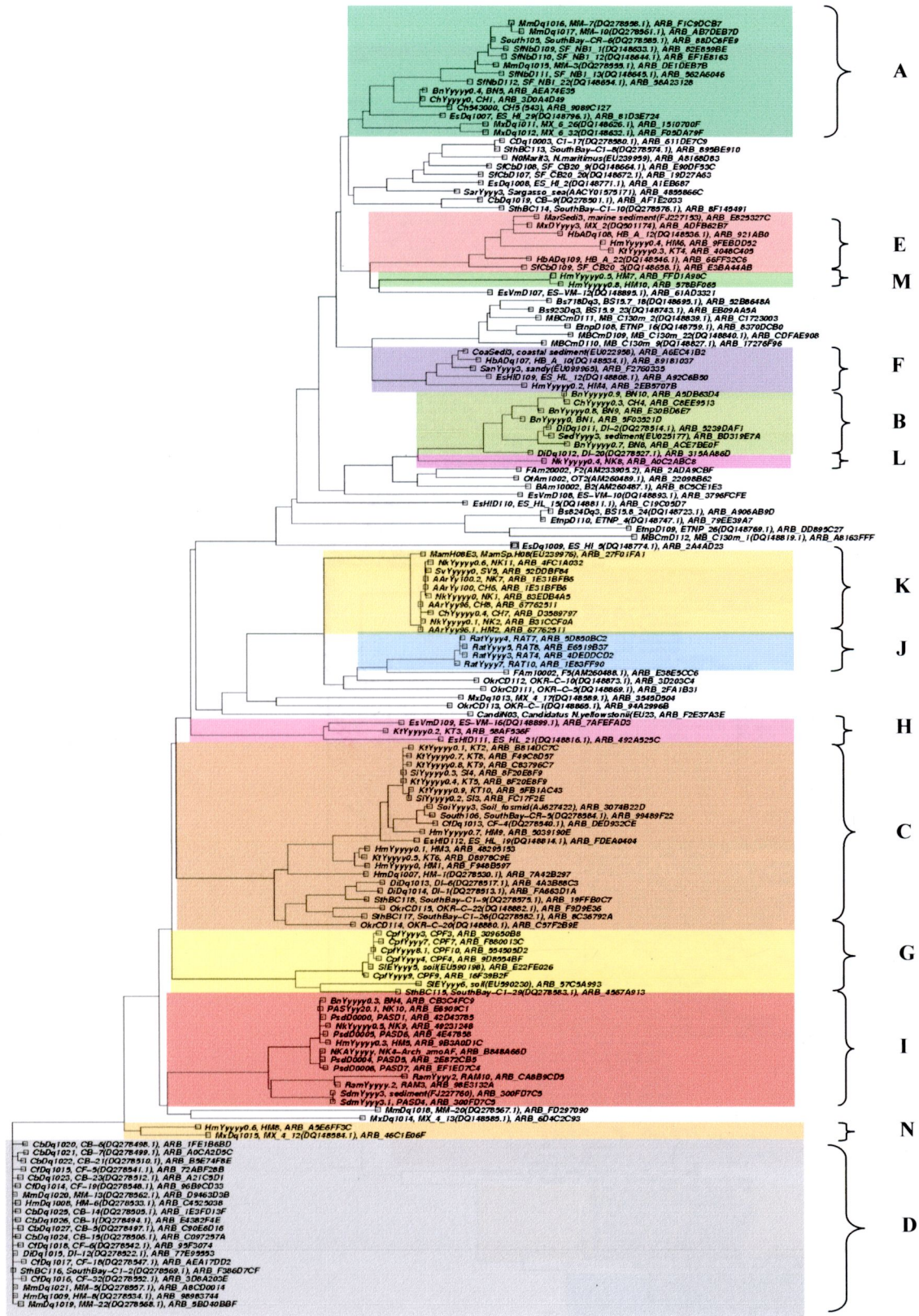

Figure 4.3 Distance matrix tree of AOA *amo*A sequences from full-scale WWTPs. (It must be noted that details information of species is not provided in this tree); Clusters A, B, C, and D were indicated by Park *et al*., 2006, while other than those were found in this study.

Figure 4.4 Maximum parsimony tree of AOA *amo*A sequences from full-scale WWTPs. (It must be noted that details information of species is not provided in this tree) Clusters A, B, C, and D were indicated by Park *et al.*, 2006, while other than those were found in this study.

Figure 4.5 Maximum likelihood tree of AOA *amo*A sequences from full-scale WWTPs. (It must be noted that details information of species is not provided in this tree); Clusters A, B, C, and D were indicated by Park *et al.*, 2006, while other than those were found in this study.

So far, the only one study on AOA communities in WWTPs has been done by Park *et al.*, 2006. Results demonstrated that AOA communities in all of activated sludge of WWTPs were observed using the same technique used in this study. AOA *amo*A sequences, retrieved from activated sludge, fell in 4 clusters (A, B, C, and D) only. Cluster D contained the largest AOA *amo*A sequences, so this cluster was proposed to be the dominant cluster of AOA found in activated sludge. All AOA *amo*A sequences retrieved from full-scale WWTPs examined in this study distributed in 12 clusters (Table 4.5). However, in this study more AOA clusters have been observed. In addition, AOA sequences found were not restricted to the cluster D as well as other clusters indicated by Park *et al.*, 2006. These suggested that it was found more AOA diversity in our WWTP samples. Most clones obtained from this study fell in cluster K. AOA *amo*A clones from WWTPs in this study closely related to those recovered from various habitats including soil, sediment, estuary sediment, marine sediment, deep sea sediment, hot spring sediment, and subsurface thermal spring. Interestingly, the environmental conditions in those sources were far different from those in our WWTPs (such as salinity, temperature, and ammonia concentration). These results suggested that those AOA clusters are not restricted to certain environmental factors. They are more flexible to adapt themselves to survive in more variety of environments.

Communities of AOA in the industrial WWTPs were less diverse than those in the municipal WWTPs. Not all samples of industrial WWTPs showed positive PCR amplification of AOA *amo*A fragments. Only 2 industrial WWTPs for plants I3 and I4, which represented the industrial WWTPs with moderate ammonium loads (40 -70 mg-N/l), contained AOA *amo*A gene fragments. Furthermore, only one AOA cluster was found in each sample (cluster G in plant I3 and cluster K in plant I4). Negative PCR amplification occurred with samples I1 and I2 that represented the industrial WWTPs with low (13 mg-N/l) and high (422 mg-N/l) ammonium loads respectively. The reason that AOA were absent from samples I2 might be because of high ammonium loads for this plant. However, the reason for the case of plant I1 was unclear. In the case of the municipal WWTPs, the influent ammonium concentration comes were in a range of 5 – 13 mg-N/l. AOA *amo*A gene fragments were detected in all samples and they distributed in various clusters (Table 4.5). And in most

samples, more than one AOA clusters was found suggesting more variety of AOA clusters in the municipal WWTPs than in the industrial WWTPs.

Other than influent characteristics, system configuration and operation might be considered as the factors that affected AOA communities. All industrial WWTPs (I3 and I4) and 5 out of 6 municipal WWTPs (LM1, LM2, LM3, LM4, and SM1) were activated sludge processes, whereas another one (SM4) was aerated lagoon system (Table 4.1). For each sample of activated sludge processes, only 1 – 3 clusters were observed, while in the sample of aerated lagoon much more AOA clusters of 6 were found. It was implied that the longer SRT might be a result of more AOA diversity in the WWTPs. It has been reported that 5 out of 9 WWTPs operating with long retention times (>15 days of SRT, >24 h of HRT) contained AOA communities in their system, whereas 4 out of 9 operating with shorter retention times, no AOA was observed (Park *et al.*, 2006). System configuration and operation can be important factors, influencing AOA communities in WWTPs.

4.2 Effect of ammonium concentrations on communities of ammonia-oxidizing bacteria and archaea in enriched nitrifying activated sludge

4.2.1 Enrichment of nitrifying activated sludge by inorganic medium containing different ammonium concentrations of 2, 10, and 30 mM NH_4^+-N (NAS 2, NAS 10, and NAS 30)

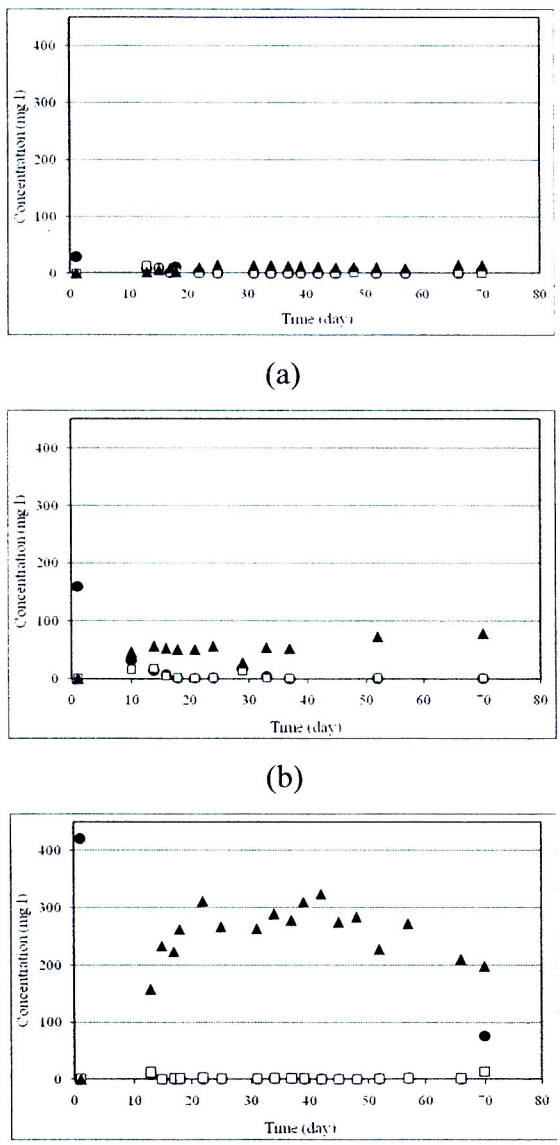

Figure 4.6 Concentrations of ammonium (●), nitrite (□), and nitrate (▲) during enrichment of nitrifying activated sludge by inorganic medium containing different ammonium concentrations (a) NAS 2, (b) NAS 10, and (c) NAS 30

This experiment was conducted to observe effect of ammonium concentrations on communities of AOB and AOA. Three enriched NAS feeding with inorganic medium containing different ammonium concentrations (2, 10, and 30 mM NH_4^+-N) were operated for 80 days. During operation, ammonium, nitrite, and nitrate concentrations were monitored (Figure 4.6). Ammonium concentrations in all three reactors reached the steady-state conditions after certain periods of operation (NAS 2 after 22 days, NAS 10 after 37, and NAS 30 after 15 days of operation). In all cases, ammonium was completely oxidized. Nitrite was detected shortly after starting the operation of all reactors (NAS 2 after 13 days, NAS 10 after 10 days, and NAS 30 after 10 days of operation). Then nitrite gradually decreased nearly zero (NAS 2 after 18 days, NAS 10 after 15 days, and NAS 30 after 18 days of operation). Nitrate was detected in all reactors and temporarily increased until reaching the steady-state condition (NAS 2 after 22 days, NAS 10 after 14 days, and NAS 30 after 18 days of operation). Total nitrogen concentrations in all reactors were slightly lost. It was probably due to denitrification reducing nitrate to nitrogen gas. DO concentration in each reactor was controlled to be above 2 mg/l to ensure absolute aerobic conditions. After 60 days of operation, sludge samples were collected to analyze for communities of AOB and AOA.

4.2.2 Communities of ammonia-oxidizing bacteria in seed sludge and enriched NAS

Communities of AOB in samples (seed sludge, NAS 2, NAS 10, and NAS 30) were investigated by using specific PCR amplification, followed by DGGE, and sequencing of 16S rRNA gene of AOB belonging to beteproteobacteria . All bands recovered from DGGE were cut, reamplified, and run on new gels until they were purified before selecting for sequencing. In total 8 bands, analyzed for sequencing, were tested for sequence similarity using blast program (Table 4.6). Results suggested that all analyzed sequences showed 96 -99% identity at nucleotide level to the previous reported sequences in the database.

Table 4.6 Closely related sequences of AOB 16S rRNA gene fragments

Sample	Band	Score	Percent Identity	Gap	Accession No. of closely related sequence	closely related sequence
Seed sludge	AOB-S-1	787	436/443 (98%)	1/443 (0%)	AB222811	DGGE 0NO2c-3
	AOB-S-2	806	441/444 (99%)	0/444 (0%)	AJ297415	Clone GaN50304
	AOB-S-3	811	441/442 (99%)	0/442 (0%)	AJ297415	Clone GaN50304
NAS 2	AOB - NAS2-1	712	403/411 (98%)	3/411 (0%)	AM295532	Clone Nm 271104_1
NAS 10	AOB – NAS10-1	813	452/457 (98%)	3/457 (0%)	AY123795	*Nitrosomonas eutropha*
	AOB - NAS10-2	739	431/446 (96%)	6/446 (1%)	AM295532	Clone Nm 271104_1
	AOB - NAS10-3	728	405/410 (98%)	1/410 (0%)	AM295532	Clone Nm 271104_1
NAS 30	AOB – NAS30-1	830	455/458 (99%)	1/458 (0%)	AL954747	*Nitrosomonas europaea* ATCC 19718

Phylogenetic trees were constructed by using three different methods comprising of distance matrix, maximum parsimony, and maximum likelihood. All methods exhibited the same grouping of AOB sequences in the tree (data not shown). For phylogenetic presented in Figure 4.7, we add our partial 400-bp AOB 16S rRNA sequences using parsimony method into the phylogenetic tree prior constructed by neighbor joining (distance matrix) methods using 1000-bp sequences of all reference AOB species to avoid changing in the tree topology when shorter sequences than 1000-bp were used to constructed the tree. AOB found in each sample were summarized in Table 4.7.

Table 4.7 Summary of AOB found in seed sludge and enriched NAS

AOB Cluster	Seed sludge	NAS 2	NAS 10	NAS 30
Nitrosospira cluster				
unknown *Nitrosomonas* cluster			✓✓	
Nitrosomonas cryototerans cluster				
Nitrosomonas europaea-Nitrosococcus mobilis cluster			✓	✓
Nitrosomonas communis cluster	✓✓			
Nitrosomonas marina cluster				
Nitrosomonas oligotropha cluster	✓	✓		

✓, present (amount of symbols represents numbers of band found)

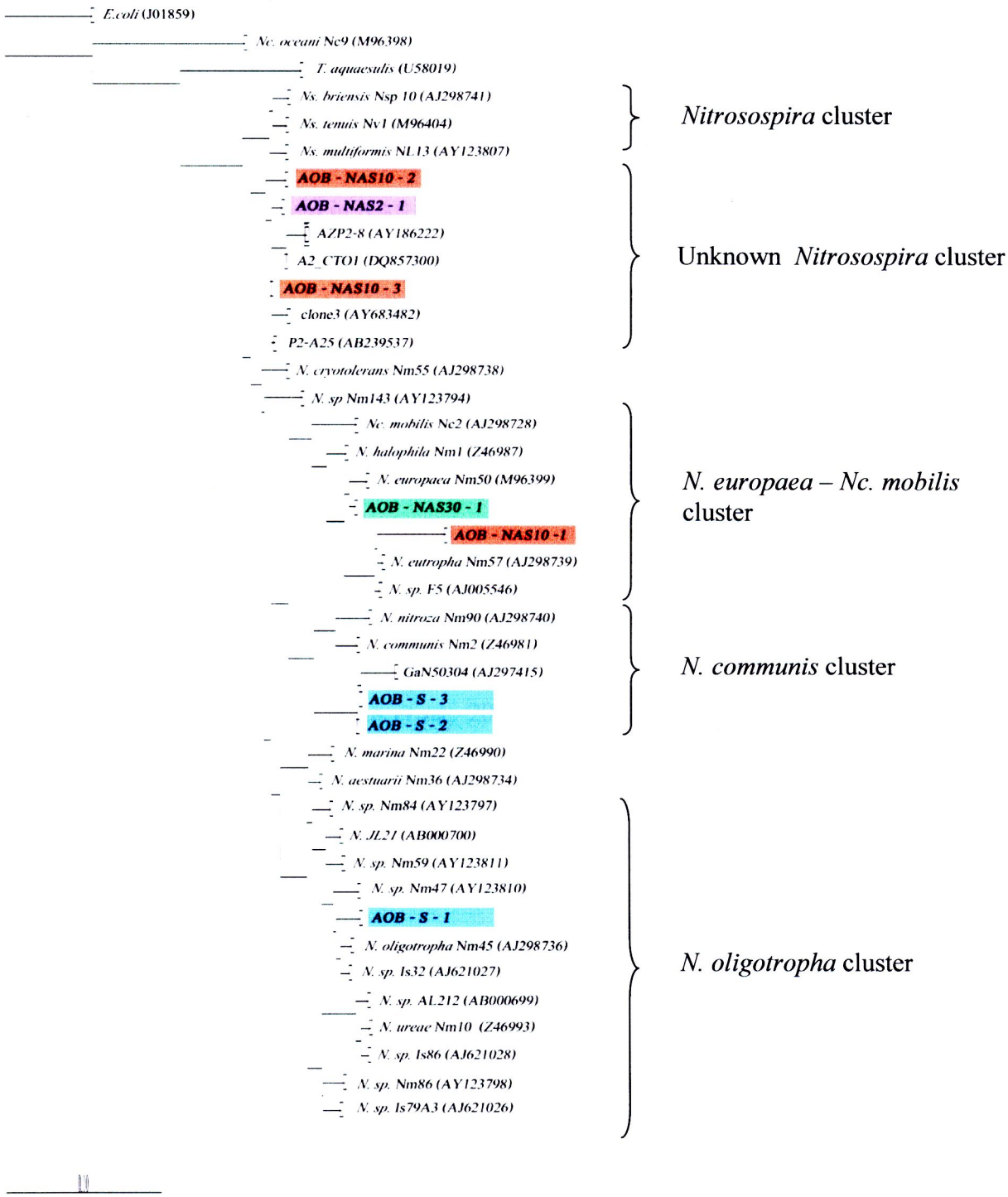

Figure 4.7 Phylogenetic tree showing 16S rRNA genes of AOB belonging to Betaproteobacterial with addition of 400-bp sequences from seed sludge and enriched NAS into the distance tree that was previously constructed based on comparison of 1000-bp sequences of described AOB (Koops *et al.*, 2003)

Sludge taken from a municipal WWTP used as a seed for all reactors contained members of *N. communis* cluster and *N. oligotropha* cluster. After it was enriched under various ammonia concentrations, members of *N. communis* cluster disappeared from all enriched NASs. In NAS 2, only AOB closely related to *N. oligotropha* cluster were found. Sequence types of unknown *Nitrosomonas* cluster and *N. europaea* cluster were recovered from NAS 10. Only AOB related to *N. europaea* cluster were observed in NAS 30.

Results revealed the shift in AOB communities from a seed sludge to enriched NAS, and the communities of AOB in each NAS varied significantly. AOB in a seed sludge related closely to DGGE 0NO2c-3 and clone GaN50304 which were recovered from a sequencing batch biofilm reactor (Gieseke *et al.*, 2001) and laboratory-scale continuous-flow reactor (Limpiyakorn *et al.*, 2006). And fell in the clusters *N. communis* and *N. oligotropha* cluster respectively. Although members of *N. communis* cluster were reported for their moderate affinity to free ammonia (K_s = 14 to 43 μM; Koops *et al.*, 2003), they were often recovered from wastewater treatment systems receiving low ammonium loads (Gieseke *et al.*, 2001, Koops *et al.*, 2003, Limpiyakorn *et al.*, 2005). *N. oligotropha* cluster are well known to be common AOB in municipal WWTPs (Limpiyakorn *et al.*, 2005) as they exhibited high affinity to ammonia (K_s = 1.9 to 4.2 μM; Koops *et al.*, 2003), meaning that they prefer low ammonia habitats. It is not surprised to find these two AOB cluster in the seed sludge, as the seed sludge was taken from a municipal WWTP receiving low ammonium load.

Band analyzed for NAS 2 closely related to the clone Nm 271104_1 that was recovered from marine aquaculture (Foesel *et al.*, 2007), and fell in *N. oligotropha* cluster. This cluster was AOB with high affinity to ammonia, being the range of a few μM. Member of *N. oligotropha* cluster are absolute majority strains originating from oligotropic freshwaters and generally the dominant AOB representatives in natural freshwater environments. Moreover, *N.oligotropha* are the most common AOB found in WWTPs with low ammonium loads (Limpiyakorn *et al.*, 2005, 2006b).

Bands analyzed from NAS 10 were found to be related to Clone Nm 271104_1, that was obtained from marine aquaculture (Foesel *et al.*, 2007), and fell in unknown *Nitrosomonas* cluster. While, another band of this sample was closely

related to *N. eutropha* C91 (Purkhold *et al.*, 2003), and fell in *N. europaea* cluster. Surprisingly, groups of AOB exhibiting low and high affinity to ammonia was found in this NAS that received the moderate ammonium load.

Band from NAS 30 was affiliated to *N. europaea* ATCC19718 that fell in *N. europaea* cluster. These AOB are low in affinity to ammonia ($K_s > 30$ μM; Koops *et al.*, 2003). They are often found in wastewater with high ammonium loads, eutrophic freshwaters, or fertilized soil (Koops *et al.*, 2003).

AOB can be divided into two groups which are AOB with high and low affinity to free ammonia. AOB with high affinity to ammonia could be retrieved from NAS 2 and NAS 10. Whereas AOB with low affinity to ammonia could be recovered from NAS 10 and NAS 30. AOB communities in NAS 10 were the mixture of AOB of high and low affinity to ammonia. This may be because of the moderate ammonium load supplied to the NAS 10. This demonstrated that ammonium load is the important factor, selecting the communities of AOB in the enriched NAS and the selection is based on the physiological properties (ammonia affinity) reported for the isolated AOB cultures in the previous studies.

4.2.3 Communities of ammonia-oxidizing archaea in seed sludge and enriched NAS

Communities of AOA in samples (seed sludge, NAS 2, NAS 10, and NAS 30) were analyzed using specific PCR amplification, followed by clone libraries, and sequencing of *amo*A gene of AOA. For each library, 10-30 clones were randomly selected for sequencing. In total 88 clones, analyzed for sequencing, were tested for sequence similarity using blast program (Table 4.8). Results suggested that all analyzed showed 88 – 98% identity at nucleotide level to previously reported AOA *amo*A gene sequences. All analyzed sequences were calculated by DOTUR program to arrange for operational taxonomic units (OTUs) (Scholoss *et al.*, 2005). Any sequences from the same library that showed 100% identity OTUs were assembled as one OTU. All 88 AOA *amo*A sequences were categorized into 30 OTUs. The amounts of the sequences of each OTU were displayed by the number in parentheses of Table 4.8.

Table 4.8 Closely related sequences of AOA *amo*A gene fragments

Sample	Clone	Score	Percent Identity	Gap	Accession No. of closely related sequence	closely related sequence
Seed Sludge	AOA-S-1 (5)	957	541/552 (98%)	2/552 (0%)	EU239976	Clone MamSp.H08
	AOA-S-2 (1)	946	539/552 (97%)	2/552 (0%)	EU239976	Clone MamSp.H08
	AOA-S-3 (1)	953	548/563 (97%)	4/563 (0%)	EU239976	Clone MamSp.H08
	AOA-S-4 (1)	833	527/564 (93%)	3/564 (0%)	FJ227760	Clone WBM050405_45P2A1
	AOA-S-5 (1)	693	473/537 (88%)	2/537 (0%)	FJ227760	Clone WBM050405_45P2A1
	AOA-S-6 (1)	819	518/555 (93%)	1/555 (0%)	FJ227760	Clone WBM050405_45P2A1
NAS 2	AOA-NAS2-1 (5)	976	552/563 (98%)	3/563 (0%)	EU239976	Clone MamSp.H08
	AOA-NAS2-2 (9)	981	553/563 (98%)	3/563 (0%)	EU239976	Clone MamSp.H08
	AOA-NAS2-3 (2)	977	553/564 (98%)	3/564 (0%)	EU239976	Clone MamSp.H08
	AOA-NAS2-4 (1)	955	549/564 (97%)	4/564 (0%)	EU239976	Clone MamSp.H08
	AOA-NAS2-5 (1)	941	546/563 (96%)	5/563 (0%)	EU239976	Clone MamSp.H08
	AOA-NAS2-6 (1)	815	537/583 (92%)	7/583 (1%)	FJ227153	Clone 3_15
	AOA-NAS2-7 (1)	992	557/566 (98%)	3/566 (0%)	EU022958	Clone HB_C_0604__C02
	AOA-NAS2-8 (1)	673	497/561 (88%)	10/561 (1%)	EU239976	Clone MamSp.H08
NAS 10	AOA-NAS10-1 (14)	979	553/564 (98%)	2/564 (0%)	EU239976	Clone MamSp.H08
	AOA-NAS10-2 (6)	983	554/564 (98%)	3/564 (0%)	EU239976	Clone MamSp.H08
	AOA-NAS10-3 (1)	955	549/564 (97%)	3/564 (0%)	EU239976	Clone MamSp.H08
	AOA-NAS10-4 (1)	966	551/564 (97%)	3/564 (0%)	EU239976	Clone MamSp.H08
	AOA-NAS10-5 (1)	966	557/573 (97%)	3/573 (0%)	EU239976	Clone MamSp.H08
	AOA-NAS10-6 (1)	963	556/573 (97%)	2/573 (0%)	EU590230	Clone BGA-781
	AOA-NAS10-7 (1)	896	539/564 (95%)	7/564 (1%)	DQ501174	Clone MX_2_OCT_29
	AOA-NAS10-8 (1)	965	566/587 (96%)	4/587 (0%)	EU025177	Clone S18-A-16
NAS 30	AOA-NAS30-1 (19)	990	559/570 (98%)	1/570 (0%)	EU239976	Clone MamSp.H08
	AOA-NAS30-2 (5)	992	558/569 (98%)	1/569 (0%)	EU239976	Clone MamSp.H08
	AOA-NAS30-3 (1)	970	552/565 (97%)	2/565 (0%)	EU239976	Clone MamSp.H08
	AOA-NAS30-4 (1)	972	553/565 (97%)	5/565 (0%)	EU239976	Clone MamSp.H08
	AOA-NAS30-5 (1)	963	551/565 (97%)	3/565 (0%)	EU239976	Clone MamSp.H08
	AOA-NAS30-6 (1)	970	551/563 (97%)	3/563 (0%)	EU239976	Clone MamSp.H08
	AOA-NAS30-7 (1)	987	560/572 (97%)	3/572 (0%)	EU239976	Clone MamSp.H08
	AOA-NAS30-8 (1)	961	551/566 (97%)	2/566 (0%)	EU239976	Clone MamSp.H08

Number in parenthesis indicated amounts of AOA *amo*A sequences showing 100% identity

Due to the limited information on AOA phylogenetic taxonomy, phylogenetic trees for AOA was constructed by tree different methods comprising of distance matrix, maximum parsimony, and maximum likelihood to confirm the grouping of AOA analyzed in this study and all major AOA reported in previous studies so far (Figure 4.9, 4.10, 4.11). It must be noted that Figure 4.9, 4.10, 4.11 have been used to confirm AOA clusters only. No information of species was provided in these three trees. Figure 4.8 showed phylogenetic tree constructed based on distance matrix (neighbor joining) with the complete sequence detail that will be used for further discussion. AOA clusters were defined based on OTUs using the DOTUR program (Scholoss *et al.*, 2005). Any AOA sequences, showing >86% identity, were identified as the same AOA cluster. AOA communities in seed sludge, and enriched NAS were summarized in Table 4.9.

Table 4.9 Summary of AOA communities in seed sludge and enriched NAS

Cluster	Seed sludge	NAS 2	NAS 10	NAS 30
B			✓	
E		✓	✓	
F		✓		
G			✓	
I	✓✓✓			
K	✓✓✓✓✓✓✓	✓✓✓✓✓✓✓✓✓ ✓✓✓✓✓✓✓✓✓ ✓✓✓	✓✓✓✓✓✓✓✓✓ ✓✓✓✓✓✓✓✓✓ ✓✓✓✓✓	✓✓✓✓✓✓✓✓✓ ✓✓✓✓✓✓✓✓✓ ✓✓✓✓✓✓✓✓✓ ✓✓✓

✓, present (amount of symbols represents numbers of clones)

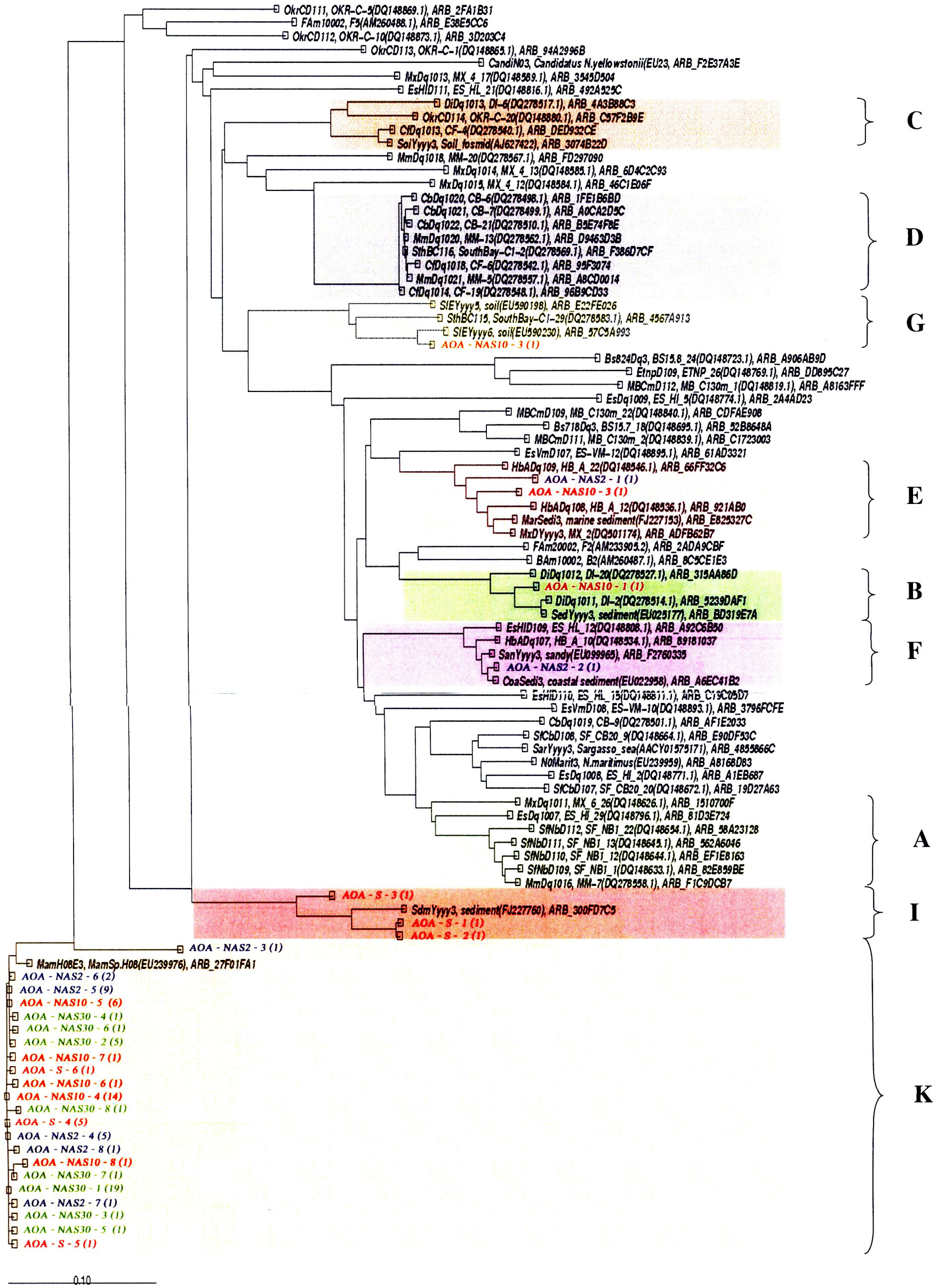

Figure 4.8 Neighbor joining tree of AOA *amo*A sequences from seed sludge and enriched NAS (Details information is provided in this tree and this tree will be used for discussion)

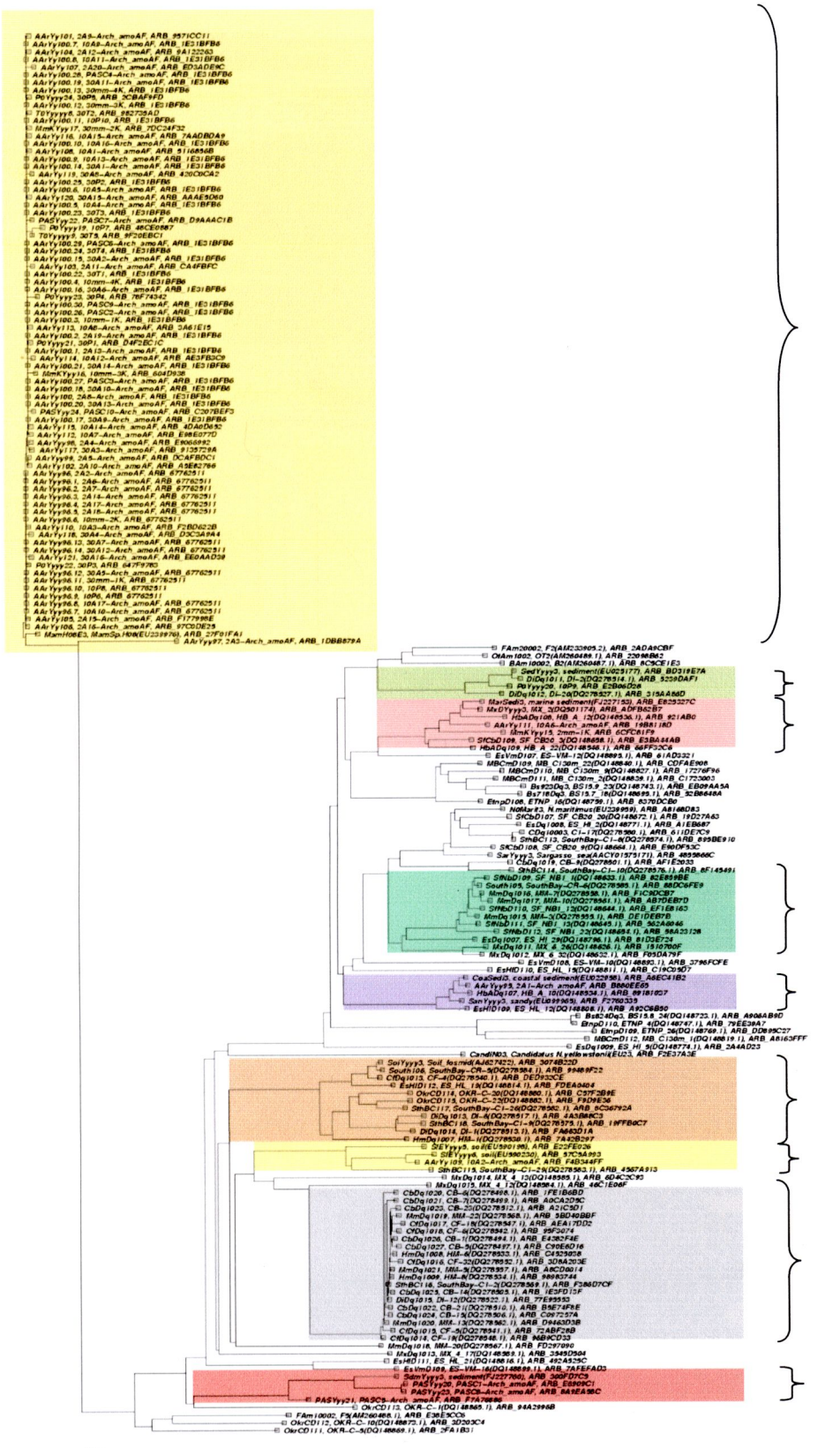

Figure 4.9 Distance matrix tree of AOA *amo*A sequences from seed sludge and enriched NAS. (It must be noted that details information of species is not provided in this tree); Clusters A, B, C, and D were indicated by Park *et al.*, 2006, while other than those were found in this study.

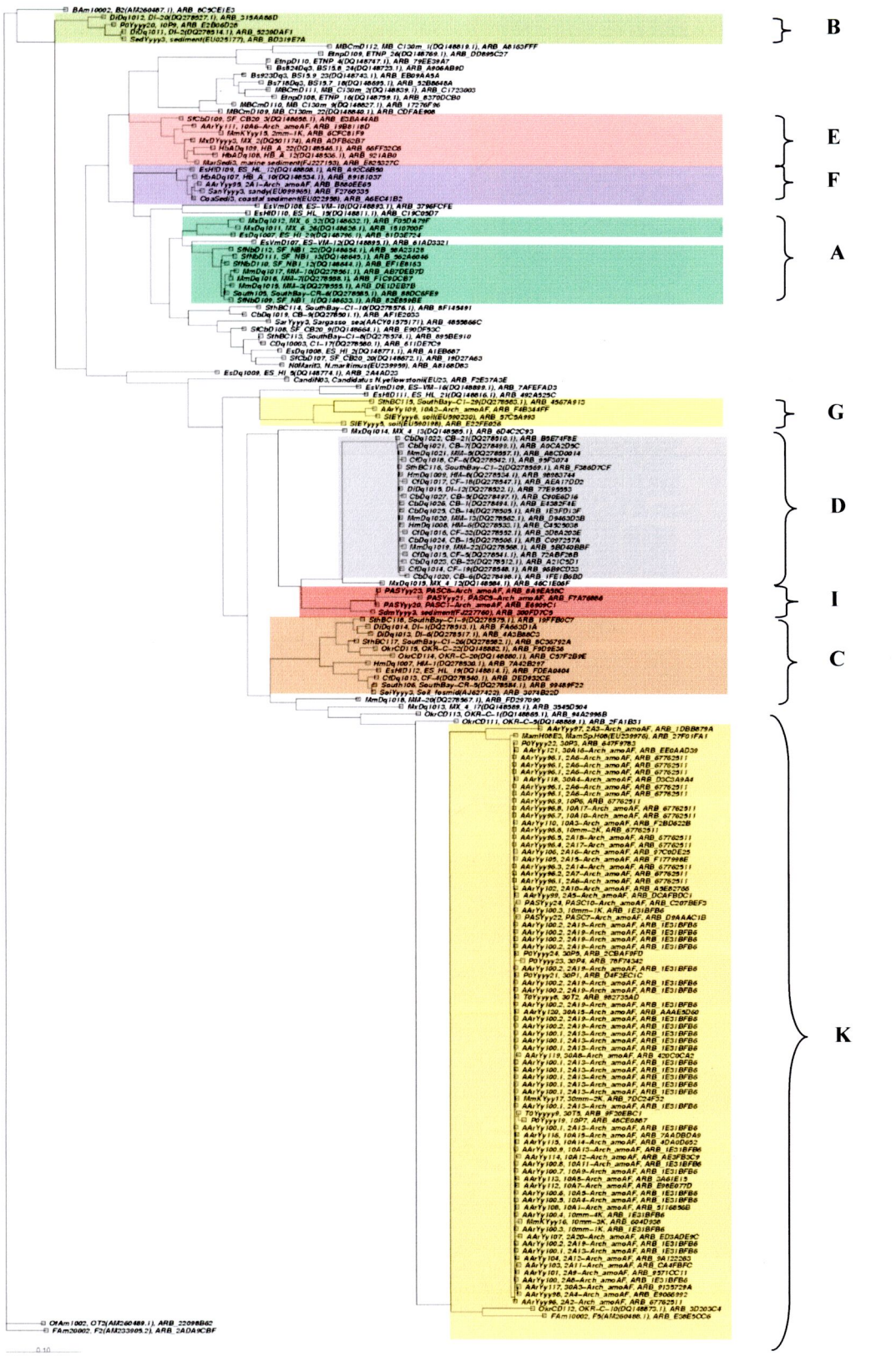

Figure 4.10 Maximum parsimony tree of AOA *amo*A sequences from seed sludge and enriched NAS. (It must be noted that details information of species is not provided in this tree); Clusters A, B, C, and D were indicated by Park *et al*., 2006, while other than those were found in this study.

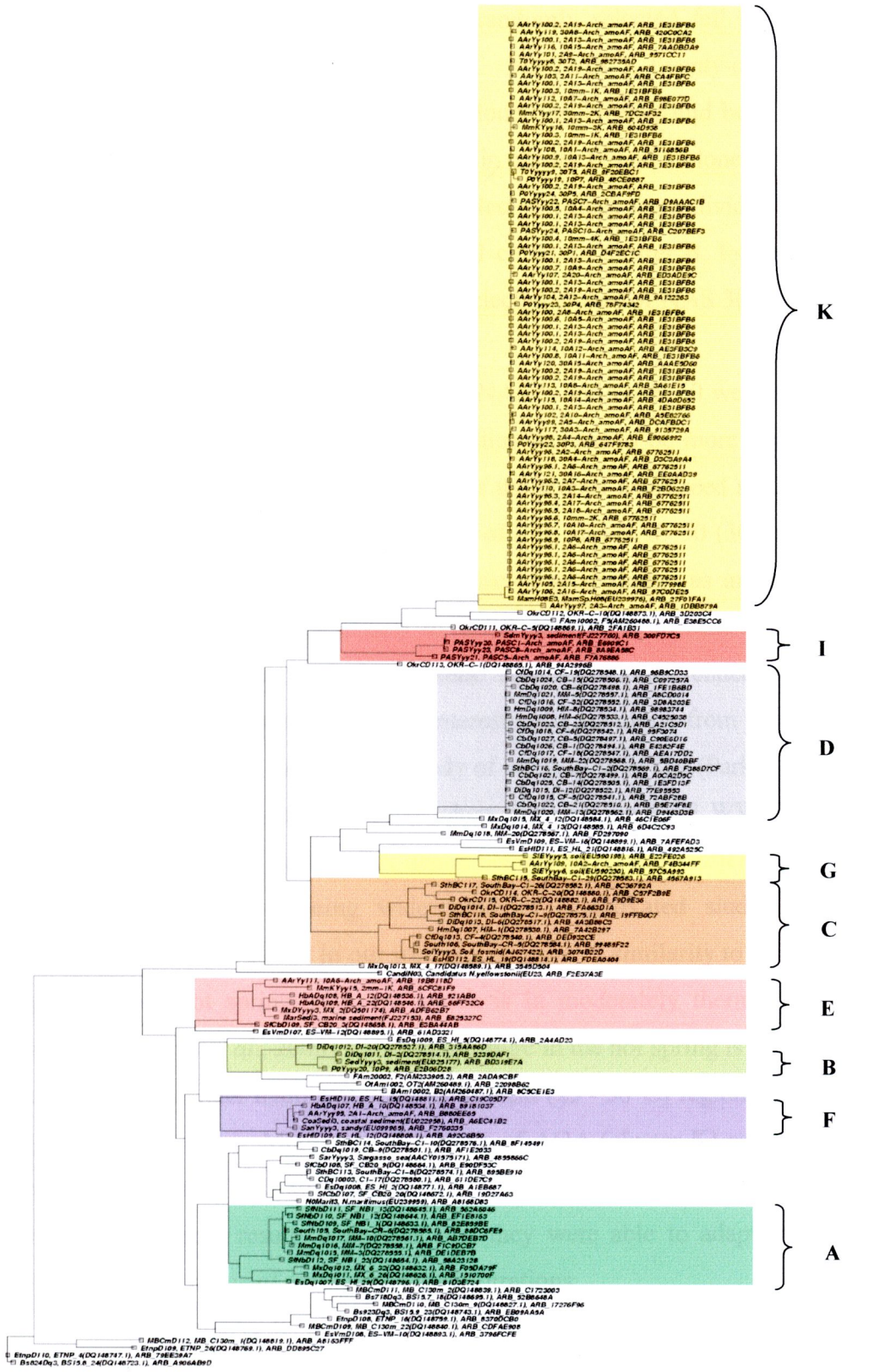

Figure 4.11 Maximum likelihood tree of AOA *amo*A sequences from seed sludge and enriched NAS. (It must be noted that details information of species is not provided in this tree); Clusters A, B, C, and D were indicated by Park *et al.*, 2006, while other than those were found in this study.

For seed sludge sample, ten clones randomly selected fell in 2 clusters: 7 clones in cluster K and 3 clones in cluster I. For NAS 2, twenty-one clones were randomly selected for sequencing. All 21 cloned analyzed could be divided into 3 AOA clusters: 19 clones in cluster K, 1 clone in cluster E, and 1 clone in cluster F. In the case of NAS 10, twenty-six randomly selected clones were divided into 4 AOA clusters which were 23 clones for cluster K, 1 clone for cluster B, 1 clone for cluster E, and 1 clone for cluster G. All randomly selected clones of NAS 30 were members of cluster K only.

It seemed that AOA communities in NAS 2 and NAS 10 were more diverse than NAS 30. Unlike AOB, AOA communities seemed to be more stable by being less influence by ammonium loads. Most of the clones from seed sludge (7 out of 10), NAS 2 (19 out of 21), NAS 10 (23 out of 26), and NAS 30 (30 out of 30) fell only in the same AOA cluster K. In the case of seed sludge as mentioned above, seven out of ten clones related closely to those obtained from Mammoth hot spring sediment (cluster K) (Torre *et al.*, 2008) while another 3 clones related to AOA found in sediment (cluster I) (unpublished). Interestingly, no clone from the seed sludge related closely to those in the only one study of AOA in WWTPs (Park *et al.*, 2006) in spite the seed sludge in our study was taken from a municipal WWTPs (activated sludge). In the study of Park, AOA *amo*A sequences of the cluster D (Figure 4.8) were the common AOA being widespread in 5 activated sludge bioreactors. Surprisingly, the major clones in our seed sludge showed similarity to those recovered from Mammoth hot spring sediment which is in moderately thermal (42 - 50°C) environment (Torre *et al.*, 2008). The temperature in the hot spring is elevated than in a municipal wastewater treatment plant in tropical (25 - 30°C) region where our seed sludge was taken from. Moreover, this group of AOA (cluster K) could survive and probably dominate in all ammonium load conditions (NAS 2, NAS 10, and NAS 30) in this study. These results suggested that they were able to adapt themselves to survive in a broad range of ammonium concentrations.

Other that cluster K, clones from NAS 2 related to the clones retrieved from coastal marine sediment (cluster E) (unpublished) and coastal sediment (cluster F) (Santoro *et al.*, 2008). In addition, three clones from NAS 10 were closely related to those obtained from estuarine sediment (cluster E) (Beman *et al.*, 2006), sediment

(cluster B) (Dang *et al.*, 2008), and soil (cluster G) (unpublished). Those closely related to the clones from NAS 2 and NAS 10 were from saline environments, while all enriched NASs in our study were operated without salt. These results implied that some AOA clusters were flexible to exist in both salt and non salt environments. Unlike AOA, distinct AOB species are very restrict to salt tolerance. In non salt environment *N.europaea*, *N. nitrosa*, and *N. ureae* are found, while in the salt environments *N. marina*, *N. aestuarii*, and *Nc. oceani* are found. It was questionable that whether salt is the factor influencing communities of AOA.

This experiment revealed that AOB communities obviously shifted from seed sludge to each enriched NAS, communities of AOB in each enriched NAS varied particularly. Ammonium load was confirmed to be the major factor selecting communities of AOB. AOB with high affinity to ammonia presented in NAS 2, AOB with low affinity to ammonia presented in NAS 30, and both strains can survive in NAS 10. These results corresponded to physiological properties reported in previous study on isolated AOB cultures. In the case of AOA, only isolated AOA culture (*N. maritimus*) has been obtained. Therefore, information on physiological properties of AOA is very limited. Thus this is the first study in the world that indirectly study physiological properties of AOA by using molecular tools. In contrast to AOB, AOA communities were more stable under ammonium load variation. Almost all AOA *amo*A sequences from all enriched NASs fell in the same cluster (cluster K). It was emerged the question that ammonia is the sole energy source for AOA whether it is important enough to affect their communities. However, it must be noted that the enriched NAS in this study is an ordinary reactors aimed to enriched mainly AOB. The inorganic medium used designed for AOB; consequently, no additional vitamin and trace elements being essential for AOA was supplied (Konneke *et al.*, 2005). Therefore AOA found in this study might be the common one survived under these conditions only.