CHAPTER 4

Culture independent of actinobacteria associated with *Nostoc commune* Voucher Bornet & Flahault

4.1 Introduction

Actinobacteria represent an extremely diverse group of high GC-containing Gram-positive bacteria belonging to the class *Actinobacteria.* They are widely distributed in terrestrial environments and have long been source of commercially useful enzymes and therapeutically bioactive molecules [Cook and Meyers, 2003], producing over half of the bioactive compounds in the Antibiotic Literature Database [Lazzarini *et al*., 2000]. Over two-thirds of natural product antibiotics are produced by actinobacteria [Strohl, 2003]. However, with the intensive exploitation of terrestrial actinobacteria for many years, the discovery rate of novel bioactive compounds has fallen steadily, with an estimate 95% rediscovery rate of known compounds [Fenical *et al.*, 1999]. There is a need for search and exploitation of actinobacteria from unique environments for novel bioactive compounds [Kurboke, 2011]. There are many reports related to the diversity of actinobacteria associated with many organisms *e.g.* ants [Currie et al. 1999], honey bees [Gilliam, 1997 ; Patil *et al.*, 2010], marine mollusks [Romanenko *et al*., 2008], marine sponges [Dharmaraj and Sumantha, 2009 ;Jiang *et al*., 2008], lichens [González *et al*., 2005], submersed freshwater macrophytes [Wohl and McArthur, 1998], and marine macroalgae [Wiese *et al.*, 2009]. To date, however, there was no report about this organism from freshwater macroalgae.

Macroalgae is one of the most interesting sources waiting for actinobacteria discovery. Previous publication reported microbes that were isolated from the seaweed with potent antibacterial activities [Wiese *et al*., 2009]. *Nostoc commune* is a terrestrial blue-green macroalgae in family *Nostocaceae*, order *Nostocales*, Division Cyanophyta [Komárek *et al.*, 2003] which form jelly clumps on nutrient-poor soils and limestones, where growths may achieve macroscopic structure [Wright *et al.*, 2001]. In Thailand, this alga commonly known as “Hed Lab” is used for consumption by local people in the North-eastern areas. This alga occurs abundant only in rainy season where moist ground induces its growth. It is also used as health food or folk medicines in China and as food in Japan [Ninomiya *et al*., 2011]. *N. commune* is known to be a rich source of bioactive compounds such as antimicrobials, cytotoxic compounds or enzyme inhibitors In this present study, 16S rDNA clone library was constructed to investigate diversity of actinobacteria associated with *Nostoc commune*. The results could be a starting point for an understanding about actinobacterial population associated with this macroalgae.

4.2 Materials and Methods

4.2.1 Sample collection

Nostoc commume Voucher ex Bornet & Flahault was collected in October 2009 from Khemarat District, Ubon Ratchathani Province (16°02'18.35"N 105°13'8.98"E) Northeastern Thailand (Figure 4.1 - 4.2). Algal specimens were collected and transferred into sterile plastic bags and kept at 4 °C until subsequent processing in the laboratory.

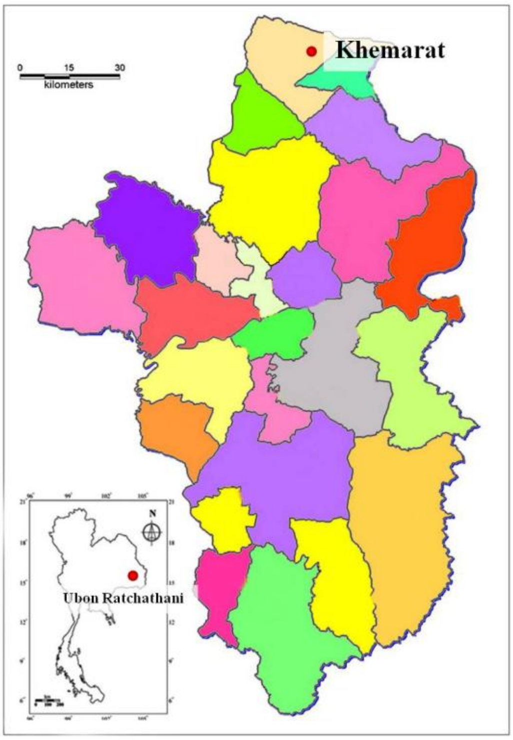


Figure 4.1 Map of sampling site in Khemarat District, Ubon Ratchathani Province

Figure 4.2 *Nostoc commune* in field (1-3) and trichome of *N. commune* (4)

4.2.2 DNA extraction and PCR amplification of partial 16S rRNA gene

Algal specimens were washed in sterile water to remove the loosely attached microorganisms. Direct extraction of DNA from algal sample was carried out using Genomic DNA Mini Kit (Plant) (Geneaid Biotech Ltd.). Genomic DNA was used as a template. Actinobacterial specific primers S-C-Act-0235-a-S-20 (5'-CGC GGCCTATCAGCTTGTTG-3') and S-C-Act-0878-a-A-19 (5'-CCGTACTCCC CAGGCGGGG-3') were used to amplify an approximately 640 bp fragment of the actinobacterial 16S rRNA genes [Stach *et al.*, 2003]. PCR was carried out using a MyCycler™ Thermal Cycler (Bio-RAD) in a 20 µl volume. PCR mixtures contained 2x PCR Master mix Solution (*i*-Taq™) (iNtRON BIOTECHNOLOGY) 10 µl, 1 µl S-C-Act-0235-a-S-20 and 1 µl S-C-Act-0878-a-A-19. Cycling conditions were as follows: initial denaturation at 94°C for 5 min, 35 cycles of 94°C for 30 sec, 55°C for 30 sec, and 72 °C for 30 sec, and final extension of 7 min at 72 °C. In the second PCR, first PCR product was used as template and re-amplified with final volume 200 µl. The products were combined and analysed by electrophoresis in a 1% agarose gel.

4.2.3 Construction of partial 16S rRNA gene clone library

PCR product of approximately 640 bp was excised and recovered using High Pure PCR Product Purification Kit (Roche). Purified PCR products was ligated in to pGEM®-T easy vectors (Promega, USA) and transformed into competent cell (*E. coli* JM 109) following the manufacturer's protocols. LB agar Plates with ampicillin (100 µg/ml) were spread on agar surface with 100 µl of 100mM IPTG and 20 µl of 50 mg/ml X-Gal, then were dried at 37°C for 12 hours before use for transformation clone detection. After spreading the transformed cells on LB agar, these plates were incubated at 37°C for 14-18 hours. The white colonies were subcultured in LB broth which contained 100 µg/ml ampicillin, incubated at 37 °C for 14 – 18 hours. White colonies indicated that *E. coli* transformants containing 16S rRNA gene inserts. These inserts were identified by colony PCR amplification using 2x PCR Master mix Solution (*i*-Taq^{TM}) with specific primer S-C-Act-0235-a-S-20 and S-C-Act-0878-a-A-19. The PCR reactions for 16S rRNA gene inserts were performed in total volume of 20 µl containing ;

Clone DNA template	1.0	µl	(Direct cell suspension)
Sequencing primer:	2.0	µl	(20nM)
(1 µl of Forward and Reward)			
2x PCR Master mix Solution	10	µl	
Deionized water	7.0	µl	

Initial denaturation	**Amplification**			**Final extension**
	Denaturation	**Annealing**	**Extension**	
94°C	94°C	55°C	72°C	72°C
5 min	30 sec	30 sec	30 sec	7 min
1 cycle	30 cycles			1 cycle

Positive PCR products were checked by 1% agarose gel electrophoresis in 1X TAE buffer [40mM Tris-acetate, 1mM EDTA (pH 8.0)]. Positive clones were purified using NucleoSpin® Plasmid (MACHETEY-NAGELGmbH & CO.KG) and sequence analysis carried out at Macrogen (Korea)**.** Pure positive clones were stabbed and streaked technique on LB slant for sequencing, and keep at 4°C for short term preservation (3-6 months).

4.2.4 Phylogenetic analysis

The sequence data were proof read using Chromas, version 1.45 (Technelysium Pty Ltd.) and examined for the formation of chimeras using Bellerophon server (http://comp-bio.anu.edu.au/bellerophon/bellerophon.pl) [Huber *et al.*, 2004]. EzTaxon-e server (http://eztaxon-e.ezbiocloud.net/; [Kim *et al.*, 2012]) was used for species identification. Phylogenetic analysis was carried out using CLUSTAL-W program in MEGA 4.0.2 (Molecular Evolutionary Genetics Analysis, version 4.0.2) [Tamura *et al.*, 2007]. The tree topologies were evaluated by bootstrap analyses based on 1,000 replicates and phylogenetic trees were generated using the neighbor-joining method [Saitou and Nei, 1987]. Only bootstrap values of more than 50% were shown on the phylogenetic trees.

All clone sequences were placed into operational taxonomic units (OTUs) at the genus level (cutoff score >97%). The OTU richness (S_{chao1}) was calculated for each of the sediment samples using the non-parametric estimator Chao index as

$$S_{chao1} = S_{obs} + \frac{a^2}{2b}$$

Where $\mathbf{S_{obs}}$ is the number of species (OTUs) observed in the library, **a** is the number of species observed just once and **b** is the number of species observed twice [Chao, 1987].

Coverage C estimator [Colwell and Coddington, 1994] calculated according to the following equation.

$$\mathbf{C = 1 - (\mathit{ni/N})}$$

Where ***ni*** is the number of species observed just once and ***N*** is the number of total species observed.

Rarefaction analysis, as well as the Shannon-Weiner index for diversity and Chao1 index for total species richness were analyzed using the FASTGROUPII program available at http://biome.sdsu.edu/fastgroup/fg_tools.htm [Yu *et al*., 2006], with a default of 97% similarity in sequence match.

Species diversity indices were estimated using Shannon-Wiener and Simpson index. If species **i** composes proportion ***Pi*** of the total individuals in a clone library of **S** species, the Shannon-Wiener index [Shanon and Weaver, 1949] was calculated as follow.

$$\mathbf{H}' = -\sum_{i=}^{s} Pi \ln Pi$$

4.2.5 Nucleotide sequence accession numbers

All sequences obtained from this study have been deposited in GenBank under accession numbers JQ765702- JQ765746.

4.3 Results and Discussion

The diversity of actinobacteria was assessed by cloning and sequencing of actinobacterial-derived partial 16S rRNA gene. In total, 70 clones were obtained from *N. commune*. The majority of clones (45 clones, 64.3%) were actinobacteria (Table 4.1). However, twenty-five clones contained members of proteobacterial sequences and other Gram positive bacteria (Table 4.2). Although actinobacterial specific primers were used for amplification of 16S rRNA gene, other sequences belong to other bacterial phyla were also recovered in the clone library. Most of them were Proteobacteria. Similar observation was also reported by Khan *et al.* [2012] on the work with actinobacteria associated with 3 marine sponges. Chimeric sequences were checked using Bellerophon server and left out from further analysis.

Table 4.1 Sequence analysis of the culture-independent actinomycete clones from the edible freshwater macroalgae *Nostoc commune*, based on partial 16S rRNA gene sequencing

Actinomycete clone	Accession number	Most closely related hit	Accession number	Similarity (%)
Clone NT1	JQ765702	*Leucobacter chromiireducens* subsp. *chromiireducens* L-1$^{(T)}$	AJ781046	99.84
Clone NT4	JQ765703	*Yimella lutea* YIM 45900$^{(T)}$	FJ528304	97.19
Clone NT5	JQ765704	*'Pseudonocardia rhizophila'* YIM 67013$^{(T)}$	GU322368	50.55
Clone NT6	JQ765705	*Leucobacter chromiireducens* subsp. *chromiireducens* L-1$^{(T)}$	AJ781046	99.84
Clone NT7	JQ765706	*Pseudonocardia ailaonensis* YIM45505$^{(T)}$	DQ344632	99.84
Clone NT8	JQ765707	*Arthrobacter oryzae* KV-651$^{(T)}$	AB279889	99.22
Clone NT9	JQ765708	*Microbacterium ginsengiterrae* DCY37$^{(T)}$	EU873314	99.38
Clone NT10	JQ765709	*Janibacter hoylei* DSM 21601$^{(T)}$	FR749912	99.53
Clone NT13	JQ765710	*Pseudonocardia ailaonensis* YIM45505$^{(T)}$	DQ344632	99.69
Clone NT15	JQ765711	*Kytococcus sedentarius* DSM 20547$^{(T)}$	CP001686	99.69
Clone NT16	JQ765712	*Amycolatopsis minnesotensis* 32U-2$^{(T)}$	DQ076482	51.25
Clone NT21	JQ765713	*Demetria terragena* HKI 0089$^{(T)}$	Y14152	97.84
Clone NT22	JQ765714	*Arthrobacter soli* SYB2$^{(T)}$	EF660748	100.00
Clone NT24	JQ765715	*Arthrobacter oryzae* KV-651$^{(T)}$	AB279889	99.07
Clone NT25	JQ765716	*Dermacoccus profundi* MT2.2$^{(T)}$	AY894329	99.84
Clone NT26	JQ765717	*Ornithinimicrobium kibberense* K22-20$^{(T)}$	AY636111	98.44
Clone NT27	JQ765718	*Amycolatopsis azurea* IMSNU 20053$^{(T)}$	AJ400709	99.69
Clone NT28	JQ765719	*Pseudonocardia ailaonensis* YIM45505$^{(T)}$	DQ344632	99.53
Clone NT30	JQ765720	*Solirubrobacter* soli Gsoil 355$^{(T)}$	AB245334	99.09
Clone NT31	JQ765721	*Dermacoccus profundi* MT2.2$^{(T)}$	AY894329	99.69
Clone NT32	JQ765722	*Arthrobacter oryzae* KV-651$^{(T)}$	AB279889	98.91
Clone NT33	JQ765723	*Microbacterium ginsengiterrae* DCY37$^{(T)}$	EU873314	99.22
Clone NT37	JQ765724	*Microbacterium aurantiacum* DSM 12506$^{(T)}$	AM182159	47.79
Clone NT38	JQ765725	*Corynebacterium matruchotii* ChDC OS35	AF543287	99.70
Clone NT40	JQ765726	*Pseudonocardia compacta* IMSNU 20111$^{(T)}$	AJ252825	48.44
Clone NT41	JQ765727	*Micrococcus endophyticus* YIM 56238$^{(T)}$	EU005372	100.00
Clone NT43	JQ765728	*Microbacterium oleivorans* DSM 16091$^{(T)}$	AJ698725	99.69
Clone NT45	JQ765729	*Micrococcus endophyticus* YIM 56238$^{(T)}$	EU005372	100.00
Clone NT46	JQ765730	*Pseudonocardia ailaonensis* YIM45505$^{(T)}$	DQ344632	99.69
Clone NT47	JQ765731	*Micrococcus endophyticus* YIM 56238$^{(T)}$	EU005372	99.84
Clone NT48	JQ765732	*Yimella lutea* YIM 45900$^{(T)}$	FJ528304	99.69
Clone NT49	JQ765733	*Actinomyces odontolyticus* CCUG 20536$^{(T)}$	AJ234040	99.69
Clone NT50	JQ765734	*Mycobacterium pallens* czh-8$^{(T)}$	DQ370008	99.22
Clone NT53	JQ765735	*Micromonospora halophytica* DSM 43026$^{(T)}$	X92601	99.53
Clone NT56	JQ765736	*Actinomyces graevenitzii* CCUG 27294$^{(T)}$	AJ540309	99.85
Clone NT57	JQ765737	*Fodinibacter luteus* YIM C003$^{(T)}$	EU878005	99.22
Clone NT59	JQ765738	*Leucobacter iarius* 40$^{(T)}$	AM040493	99.84
Clone NT61	JQ765739	*Mycobacterium parafortuitum* DSM 43528$^{(T)}$	X93183	99.69
Clone NT62	JQ765740	*Microbacterium aurantiacum* DSM 12506$^{(T)}$	AM182159	49.29
Clone NT64	JQ765741	*Arthrobacter oryzae* KV-651$^{(T)}$	AB279889	98.91
Clone NT65	JQ765742	*Micrococcus endophyticus* YIM 56238$^{(T)}$	EU005372	99.84
Clone NT67	JQ765743	*Arthrobacter oryzae* KV-651$^{(T)}$	AB279889	98.91
Clone NT68	JQ765744	*Microbacterium oleivorans* DSM 16091$^{(T)}$	AJ698725	99.69
Clone NT69	JQ765745	*Solirubrobacter ginsenosidimutans* BXN5-15$^{(T)}$	EU332825	92.90
Clone NT70	JQ765746	*Leucobacter chromiireducens* subsp. *chromiireducens* L-1$^{(T)}$	AJ781046	99.69

Table 4.2 Sequence analysis of the culture-independent proteobacteria and other Gram positive bacteria from the edible freshwater macroalgae *Nostoc commune*, based on partial 16S rRNA gene sequencing

Actinomycete clone	**Most closely related hit**	**Taxonomy**	**Similarity (%)**
Clone NT2	*Novosphingobium nitrogenifigens* Y88$^{(T)}$	Proteobacteria	79.978
Clone NT3	*Novosphingobium nitrogenifigens* Y88$^{(T)}$	Proteobacteria	79.869
Clone NT11	*Byssovorax cruenta* DSM 14553$^{(T)}$	Proteobacteria	48.559
Clone NT12	*Chelatococcus asaccharovorans* TE2$^{(T)}$	Proteobacteria	81.69
Clone NT14	*Altererythrobacter marensis* MSW-14$^{(T)}$	Proteobacteria	50.055
Clone NT17	*Chondromyces crocatus* Cmc6	Proteobacteria	46.274
Clone NT18	*Erythrobacter nanhaisediminis* T30$^{(T)}$	Proteobacteria	47.74
Clone NT19	*Byssovorax cruenta* DSM 14553$^{(T)}$	Proteobacteria	48.837
Clone NT20	*Chondromyces lanuginosus* Sy t2	Proteobacteria	78.93
Clone NT23	*Methylocystis parvus* OBBP$^{(T)}$	Proteobacteria	79.978
Clone NT29	*Methylobacterium nodulans* ORS 2060$^{(T)}$	Proteobacteria	48.673
Clone NT34	*Sphingomonas rubra* BH3$^{(T)}$	Proteobacteria	79.913
Clone NT35	*Altererythrobacter aestuarii* KYW147$^{(T)}$	Proteobacteria	79.891
Clone NT36	*Chelatococcus daeguensis* K106$^{(T)}$	Proteobacteria	83.425
Clone NT39	Hit sequence has been removed	-	-
Clone NT42	*Geothermobacterium ferrireducens* FW-1a$^{(T)}$	Thermodesulfobacteria	51.309
Clone NT44	*Geobacillus debilis* Tf$^{(T)}$	Firmicutes	48.619
Clone NT51	*Altererythrobacter marensis* MSW-14$^{(T)}$	Proteobacteria	50.768
Clone NT52	*Candidatus Desulforudis audaxviator* MP104C	Proteobacteria	48.528
Clone NT54	*Rubidibacter lacunae* KORDI 51-2$^{(T)}$	Blue-green	76.573
Clone NT55	*Planctomyces limnophilus* DSM 3776$^{(T)}$	Planctomycetes	76.739
Clone NT58	Hit sequence has been removed	-	-
Clone NT60	Hit sequence has been removed	-	-
Clone NT63	*Blastopirellula marina* DSM 3645$^{(T)}$	Planctomycetes	75.166
Clone NT66	*Methylocystis parvus* OBBP$^{(T)}$	Proteobacteria	79.501

All 45 actinobacterial clones were submitted to Eztaxon database for BLAST search to obtain the closest match for preliminary taxonomic assignment (Table 4.3). They were assigned to 28 species, 18 genera and 10 families. Ten clones (22.2%) belonged to the family *Microbacteriaceae* and *Micrococcaceae* followed by 8 clones (17.8%) of *Pseudonocardiaceae*. Members of *Dermacoccaceae* were represented by 6 clones (13.3%) whereas *Intrasporangiaceae* were represented by 3 clones (6.7%). *Solirubrobacteraceae, Actinomycetaceae*, *Micromonosporaceae* and *Mycobacteriaceae* were represented by 2 clones each (4.4%). *Corynebacteriaceae* was repersented by 1 clone (2.2%). *Microbacterium* and *Pseudonocardia* were the most diverse among 18 genera with 4 species. On the other hands, *Yimella, Kytococcus*, *Demetria, Dermacoccus*, *Janibacter*, *Ornithinimicrobium*, *Fodinibacter, Micrococcus, Corynebacterium* and *Micromonospora* have least diversity at species level (Table 4.4 and Figure 4.3).

Table 4.3 Actinomycete clones submitted to Eztaxon database

Actinomycete clone	**bp**	**Most closely related hit**	**Source**	**Accession number**	**Similarity (%)**	**Family**
Clone NT1	643	*Leucobacter chromiireducens* subsp. *chromiireducens* L-1$^{(T)}$	chromium contaminated environment	AJ781046	99.84	Microbacteriaceae
Clone NT4	642	*Yimella lutea* YIM 45900$^{(T)}$	contaminant on an agar plate	FJ528304	97.19	Dermacoccaceae
Clone NT5	643	*'Pseudonocardia rhizophila'* YIM 67013$^{(T)}$	rhizosphere soil	GU322368	50.55	Pseudonocardiaceae
Clone NT6	643	*Leucobacter chromiireducens* subsp. *chromiireducens* L-1$^{(T)}$	chromium contaminated environment	AJ781046	99.84	Microbacteriaceae
Clone NT7	644	*Pseudonocardia ailaonensis* YIM45505$^{(T)}$	soil	DQ344632	99.84	Pseudonocardiaceae
Clone NT8	642	*Arthrobacter oryzae* KV-651$^{(T)}$	soil	AB279889	99.22	Micrococcaceae
Clone NT9	643	*Microbacterium ginsengiterrae* DCY37$^{(T)}$	soil of a ginseng field	EU873314	99.38	Microbacteriaceae
Clone NT10	643	*Janibacter hoylei* DSM 21601$^{(T)}$	air from an altitude of 4041.4 km	FR749912	99.53	Intrasporangiaceae
Clone NT13	644	*Pseudonocardia ailaonensis* YIM45505$^{(T)}$	soil	DQ344632	99.69	Pseudonocardiaceae
Clone NT15	642	*Kytococcus sedentarius* DSM 20547$^{(T)}$	seawater	CP001686	99.69	Dermacoccaceae
Clone NT16	643	*Amycolatopsis minnesotensis* 32U-2$^{(T)}$	prairie soil	DQ076482	51.25	Pseudonocardiaceae
Clone NT21	647	*Demetria terragena* HKI 0089$^{(T)}$	compost soil	Y14152	97.84	Dermacoccaceae
Clone NT22	643	*Arthrobacter soli* SYB2$^{(T)}$	wastewater reservoir sediment	EF660748	100.00	Micrococcaceae
Clone NT24	643	*Arthrobacter oryzae* KV-651$^{(T)}$	soil	AB279889	99.07	Micrococcaceae
Clone NT25	643	*Dermacoccus profundi* MT2.2$^{(T)}$	challenger deep sediment	AY894329	99.84	Dermacoccaceae
Clone NT26	642	*Ornithinimicrobium kibberense* K22-20$^{(T)}$	cold desert	AY636111	98.44	Intrasporangiaceae
Clone NT27	642	*Amycolatopsis azurea* IMSNU 20053$^{(T)}$	-	AJ400709	99.69	Pseudonocardiaceae
Clone NT28	644	*Pseudonocardia ailaonensis* YIM45505$^{(T)}$	soil	DQ344632	99.53	Pseudonocardiaceae;
Clone NT30	663	*Solirubrobacter* soli Gsoil 355$^{(T)}$	soil of a ginseng field	AB245334	99.09	Solirubrobacteraceae
Clone NT31	643	*Dermacoccus profundi* MT2.2$^{(T)}$	challenger deep sediment	AY894329	99.69	Dermacoccaceae
Clone NT32	643	*Arthrobacter oryzae* KV-651$^{(T)}$	soil	AB279889	98.91	Micrococcaceae

Table 4.3 (continued)

Actinomycete clone	bp	Most closely related hit	Source	Accession number	Similarity (%)	Family
Clone NT33	643	*Microbacterium ginsengiterrae* DCY37$^{(T)}$	soil of a ginseng field	EU873314	99.22	Microbacteriaceae
Clone NT37	641	*Microbacterium aurantiacum* DSM 12506$^{(T)}$	soil and clinical specimens	AM182159	47.79	Microbacteriaceae
Clone NT38	658	*Corynebacterium matruchotii* ChDC OS35	oral isolate	AF543287	99.70	Corynebacteriaceae
Clone NT40	644	*Pseudonocardia compacta* IMSNU 20111$^{(T)}$	-	AJ252825	48.44	Pseudonocardiaceae
Clone NT41	643	*Micrococcus endophyticus* YIM 56238$^{(T)}$	*Aquilaria sinensis* leaves	EU005372	100.00	Micrococcaceae
Clone NT43	642	*Microbacterium oleivorans* DSM 16091$^{(T)}$	-	AJ698725	99.69	Microbacteriaceae
Clone NT45	643	*Micrococcus endophyticus* YIM 56238$^{(T)}$	*Aquilaria sinensis* leaves	EU005372	100.00	Micrococcaceae
Clone NT46	644	*Pseudonocardia ailaonensis* YIM45505$^{(T)}$	soil	DQ344632	99.69	Pseudonocardiaceae
Clone NT47	643	*Micrococcus endophyticus* YIM 56238$^{(T)}$	*Aquilaria sinensis* leaves	EU005372	99.84	Micrococcaceae
Clone NT48	643	*Yimella lutea* YIM 45900$^{(T)}$	contaminant on an agar plate	FJ528304	99.69	Dermacoccaceae
Clone NT49	654	*Actinomyces odontolyticus* CCUG 20536$^{(T)}$	canine and feline clinical specimens	AJ234040	99.69	Actinomycetaceae
Clone NT50	643	*Mycobacterium pallens* czh-8$^{(T)}$	Hawaiian soils	DQ370008	99.22	Mycobacteriaceae
Clone NT53	643	*Micromonospora halophytica* DSM 43026$^{(T)}$	-	X92601	99.53	Micromonosporaceae
Clone NT56	699	*Actinomyces graevenitzii* CCUG 27294$^{(T)}$	human clinical specimens	AJ540309	99.85	Actinomycetaceae
Clone NT57	642	*Fodinibacter luteus* YIM C003$^{(T)}$	salt mine	EU878005	99.22	Intrasporangiaceae
Clone NT59	643	*Leucobacter iarius* 40$^{(T)}$	infective juveniles of the entomopathogenic nematode *Steinernema thermophilum*	AM040493	99.84	Microbacteriaceae
Clone NT61	643	*Mycobacterium parafortuitum* DSM 43528$^{(T)}$	fluoranthene-polluted soil	X93183	99.69	Mycobacteriaceae
Clone NT62	636	*Microbacterium aurantiacum* DSM 12506$^{(T)}$	-	AM182159	49.29	Microbacteriaceae
Clone NT64	643	*Arthrobacter oryzae* KV-651$^{(T)}$	soil	AB279889	98.91	Micrococcaceae
Clone NT65	643	*Micrococcus endophyticus* YIM 56238$^{(T)}$	*Aquilaria sinensis* leaves	EU005372	99.84	Micrococcaceae
Clone NT67	643	*Arthrobacter oryzae* KV-651$^{(T)}$	soil	AB279889	98.91	Micrococcaceae
Clone NT68	642	*Microbacterium oleivorans* DSM 16091$^{(T)}$	-	AJ698725	99.69	Microbacteriaceae
Clone NT69	662	*Solirubrobacter ginsenosidimutans* BXN5-15$^{(T)}$	soil of a ginseng field	EU332825	92.90	Solirubrobacteraceae
Clone NT70	643	*Leucobacter chromiireducens* subsp. *chromiireducens* L-1$^{(T)}$	chromium contaminated environment	AJ781046	99.69	Microbacteriaceae

Table 4.4 Taxonomy and number of actinomycete clones from the edible freshwater macroalgae *Nostoc commun*

Family	**Genus**	**Species**	**Number of clone**
Actinomycetaceae	*Actinomyces*	*Actinomyces odontolyticus* CCUG 20536$^{(T)}$	1
		Actinomyces graevenitzii CCUG 27294$^{(T)}$	1
Corynebacteriaceae	*Corynebacterium*	*Corynebacterium matruchotii* ChDC OS35	1
Dermacoccaceae	*Yimella*	*Yimella lutea* YIM 45900$^{(T)}$	2
	Kytococcus	*Kytococcus sedentarius* DSM 20547$^{(T)}$	1
	Demetria	*Demetria terragena* HKI 0089$^{(T)}$	1
	Dermacoccus	*Dermacoccus profundi* MT2.2$^{(T)}$	2
Intrasporangiaceae	*Janibacter*	*Janibacter hoylei* DSM 21601$^{(T)}$	1
	Ornithinimicrobium	*Ornithinimicrobium kibberense* K22-20$^{(T)}$	1
	Fodinibacter	*Fodinibacter luteus* YIM C003$^{(T)}$	1
Microbacteriaceae	*Leucobacter*	*Leucobacter chromiireducens* subsp. *chromiireducens* L-1$^{(T)}$	3
		Leucobacter iarius 40$^{(T)}$	1
	Microbacterium	*Microbacterium ginsengiterrae* DCY37$^{(T)}$	2
		Microbacterium aurantiacum DSM 12506$^{(T)}$	2
		Microbacterium oleivorans DSM 16091$^{(T)}$	2
Micrococcaceae	*Arthrobacter*	*Arthrobacter oryzae* KV-651$^{(T)}$	5
		Arthrobacter soli SYB2$^{(T)}$	1
	Micrococcus	*Micrococcus endophyticus* YIM 56238$^{(T)}$	4
Micromonosporaceae	*Micromonospora*	*Micromonospora halophytica* DSM 43026$^{(T)}$	1
Mycobacteriaceae	*Mycobacterium*	*Mycobacterium pallens* czh-8$^{(T)}$	1
		Mycobacterium parafortuitum DSM 43528$^{(T)}$	1
Pseudonocardiaceae	*Pseudonocardia*	*Pseudonocardia rhizophila* YIM 67013$^{(T)}$	1
		Pseudonocardia ailaonensis YIM45505$^{(T)}$	4
		Pseudonocardia compacta IMSNU 20111$^{(T)}$	1
	Amycolatopsis	*Amycolatopsis minnesotensis* 32U-2$^{(T)}$	1
		Amycolatopsis azurea IMSNU 20053$^{(T)}$	1
Solirubrobacteraceae	*Solirubrobacter*	*Solirubrobacter* soli Gsoil 355$^{(T)}$	1
		Solirubrobacter ginsenosidimutans BXN5-15$^{(T)}$	1
Total 10	**18**	**28**	**45**

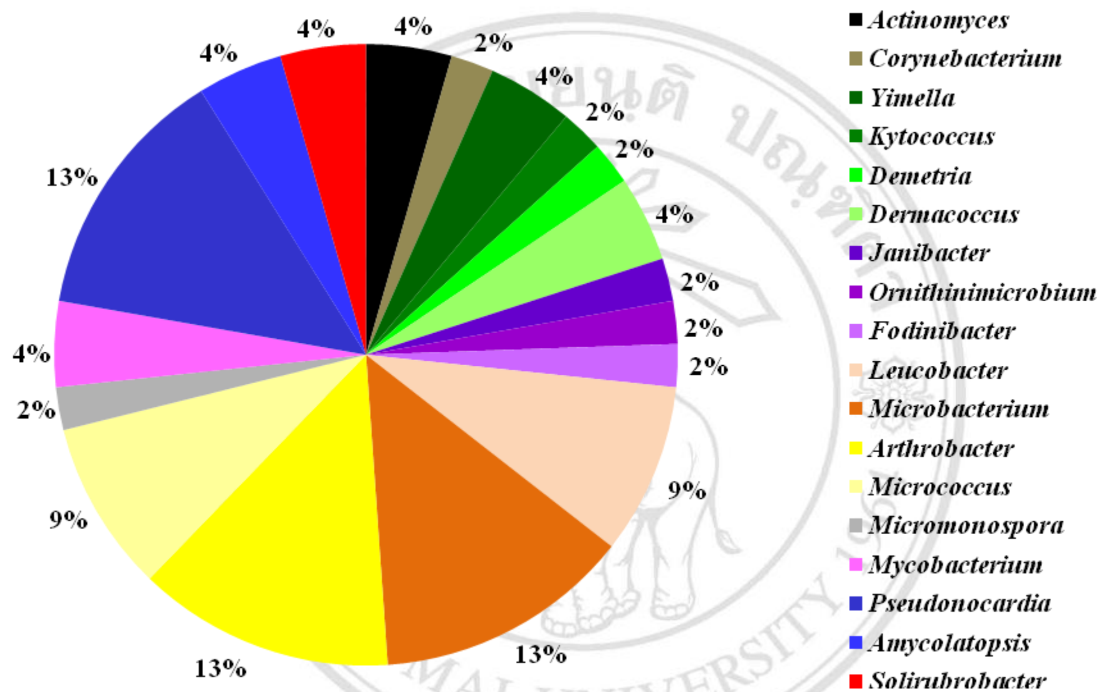


Figure 4.3 Occurrence of actinomycete genera from the edible freshwater macroalgae *Nostoc commune*

black color	= Actinomycetaceae	orange tone color	= Microbacteriaceae
brown color	= Corynebacteriaceae	the red color	= Solirubrobacteraceae
green tone color	= Dermacoccaceae	blue tone color	= Pseudonocardiaceae
purple tone color	= Intrasporangiaceae	the pink color	= Mycobacteriaceae
yellow tone color	= Micrococcaceae	the grey color	= Micromonosporaceae

Phylogenetic analysis of all clone sequences was shown in Figure 4.4. It is evident from the tree that taxonomic placement of these actinobacterial sequences is in agreement with the BLAST result except for Clone NT5, Clone NT16, Clone NT37, Clone NT40 and Clone NT62. These 5 clones showed very low similarity (48-51%) with any known actinobacterial sequences hence formed a separated cluster as seen in Figure 4.4b. These sequences could represent a novel taxa at higher rank above genus level. The common criteria for proposing novel genera of bacteria is <93% identity of previously published 16S rDNA sequences in the GenBank and < 97% for proposing novel species [Rohwer *et al.*, 2002]. Our results suggested some of *Nostoc commune* associated actinobacteria are novel at genus and species level. For example, clone NT69 shared only 92.9% similarity with *Solirubrobacter ginsenosidimutans* BXN5-15^{T} which is likely to be a new genus. Similarly, clone NT4 showed 97.2% similarity with *Yimella lutea* YIM 45900^{T}, an indication of a potential novel species.

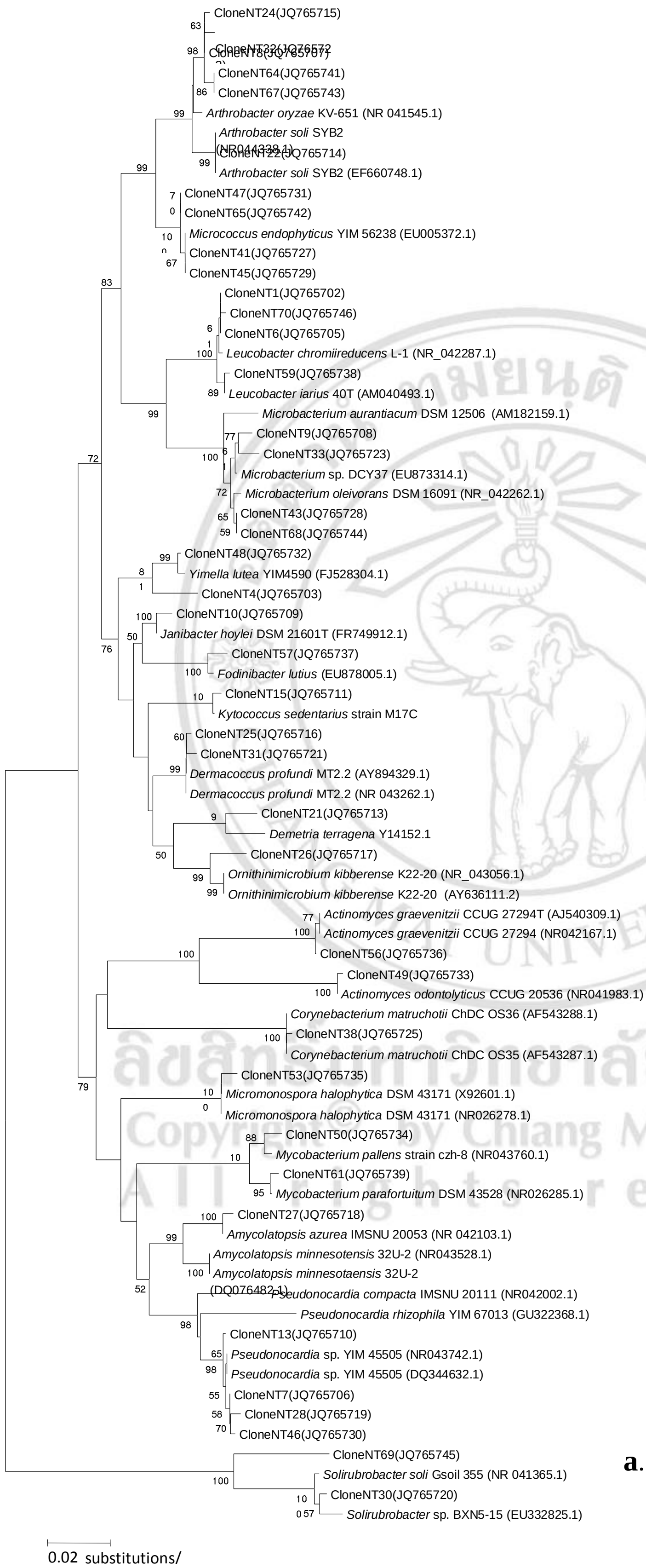
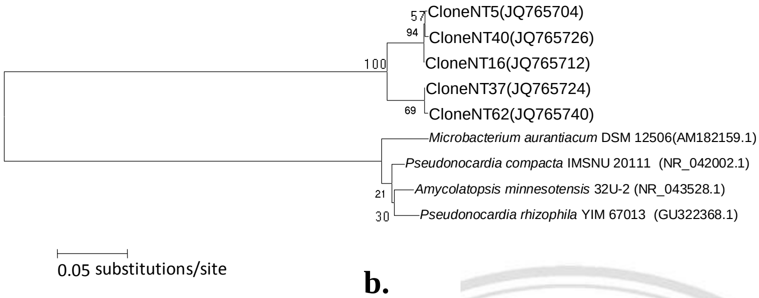


Figure 4.4 Phylogenetic tree of 16S rRNA clone sequences from *Nostoc commune*
(a) Phylogenetic tree of actinobacterial clones with high BLAST similarity
(b) Phylogenetic tree of actinobacterial clones with low BLAST similarity.
The numbers are the percentages indicating the labels of boot strap support, based on a neighbor joining analysis of 1,000 resampled data sets



b.

Figure 4.4 (continued)

Actinobacteria are major source of secondary metabolites for applications. Rare actinobacterial taxa, in particular are producers of unique compounds of high activity and low toxicity [Berdy, 2005; Tiwari and Gupta, 2012]. Data from our clone library also revealed candidates of potential strains for bioactive compounds production. These include clone NT27 which was related to *Amycolatopsis azurea*. Novel antifungal antibiotics Octacosamicins A and B were produced by *A. azurea* MG398-hF9 [Dobashi *et al.,* 1988]. Similary, clone NT53 was found to be closely related to *Micromonospora halophytica* DSM43026^T. This bacteria was a producer of antibiotic lincomycin [Bibikova *et al.*, 1989]. In addition, few clones hold promise as candidates for environmental remediation. Clone NT50 and NT67 showed closed phylogenetic relationship with polycyclic aromatic hydrocarbon degrading *Mycobacterium pallens* [Hennessee *et al*., 2009] and crude oil degrading *Microbacterium oleivorans* [Schippers *et al.*, 2005], respectively.

Since this algae is being consumed by some local people in Thailand, it is noteworthy to mention that some of the clones were affiliated with potential pathogenic strains. Though they are well known antibiotic producers, some actinobacteria are causative agents of human diseases. Clone NT49 and NT56 were affiliated with *Actinomyces odontolyticus* and *A. graevenitzii*, respectively. *Actinomyces* spp. are the most common commensal anaerobic actinobacteria in human oral cavity with 6 species are considered pathogenic in humans includin *A. graevenitzii* and *A. odontolyticus*.

A. graevenitzii, originally isolated from human clinical specimen, was reported to be a cause of bacteremia in patient with alcoholic liver cirrhosis [Hwang *et al*., 2011] and multiple pulmonary abscesses [Nagaoka *et al*., 2012]. Similarly, *A. odontolyticus* has been reported as an opportunistic pathogen causing systemic infections [Mohan *et al*., 2009], bacteremia [Povazan *et al*., 2012] and thoracoactinomycosis [Thomas *et al*., 2007]. Clone NT38 shared high similarity with *Corynebacterium matruchotii*. This organism has been isolated from dental plaque and involved in periodontitis [Amel *et al*., 2010]. Although no gastrointestinal pathogen related clones were found, it is recommended not to consume raw algae for safety precaution.

It is almost possible to predict the relationship between actinobacteria and the algae from metagenomic analysis data alone. Culture representatives are needed to define their biochemical and physiological properties. We opined that the relationship between actinobacteria and *N. commune* might be similar to that of plant endophytic actinobacteria as this algae is a terrestrial macroalgae which grow on soil. Actinobacteria may obtain nutrients from the algae and in turn promote algal growth by production of antibiotics and phytohormones. Nevertheless, bacteria were reported to affect growth and development of green marine macroalga, *Ulva linza* [Marshall *et al.*, 2006]. The growth and thallus morphogenesis of another green marine *Ulva mutabilis* were also reported to require regulatory factors secreted from its associated bacteria [Spoerner *et al*., 2012]. Toxic marine dinoflagellate, *Gymnodinium catenatum* requires growth-stimulatory marine bacteria for postgermination survival and growth [Bolch and Subramanian, 2011]. The algal samples also showed no sign of disease or any abnormality in appearance hence suggested positive relationship between actinobacteria and algae.

There are very few reports on actinobacteria diversity associated with algae. Some of actinobacteria found in our clone library have previously been isolated in pure cultures. Members of the genera *Corynebacterium, Microbacterium* and *Micrococcus* have been isolated from edible macroalga (seaweed), *Palmaria palmata* [Moore *et al*., 2002].

Arthrobacter and *Agromyces* were also found to present on the surface of brown algae *Desmarestia viridis* [Beleneva and Zhukova, 2006]. Brown algae, *Laminaria saccharina* was found to harbor *Amycolatopsis*, *Arthrobacter* and *Micrococcus* [Wiese *et al*., 2009]. *Frigobacterium* sp. was isolated from marine alga *Ulva linza* [Marshall *et al*., 2006]. However, no actinobacteria was found in association with filaments of marine cyanobacteria, *Nodularia spumigena* [Salomon *et al*., 2003]. To date there are 8 new validly described species of alga-derived actinobacteria from 8 genera including 4 novel genera [Goecke *et al*., 2013] .

Rarefaction analyses of the clone library was done to determine the unique actinobacterial clones as proportion of the estimated total diversity within the library. The rarefaction curve was clearly not reached the saturation point which indicated that more sampling of clone library probably recovered additional diversity (Figure 4.5). Our analysis revealed that the 16S rRNA clone library of the actinobacteria of *N. commune* was diverse with a Shannon-Wiener index of 3.25 and Chao1 of 187.0 ribotypes. The Shannon-Wiener and Chao1 indexes showed that there is species richness (different number of species within the clone library) and low species equitability (how even are the numbers of actinomycete species present in the clone library) in *Nostoc commune.* In the present study, the clone library was diverse with 10 actinobacterial families and the evenness of the clone library was considering high. In addition, the coverage value was 71.11% indicating that this library was not covered all of the diversity presented in the sample. Nevertheless, the diversity of clone library obtained in the present study was more diverse than those previously reported from the mucus of the coral *Acropora digitifera* with Shannon-Wiener index of 2.3586 and Chao1 of 41.16 ribotypes [Nithyanand *et al*., 2010] and 2 Red sea sponges: *Hyrtios erectus* (Shannon-Wiener index of 3.1927 and Chao1 of 94.6667 ribotypes) and *Amphimedon* sp. (Shannon-Wiener index = 2.1587 and Chao1 = 19.0 ribotypes) [Radwan *et al*., 2010].

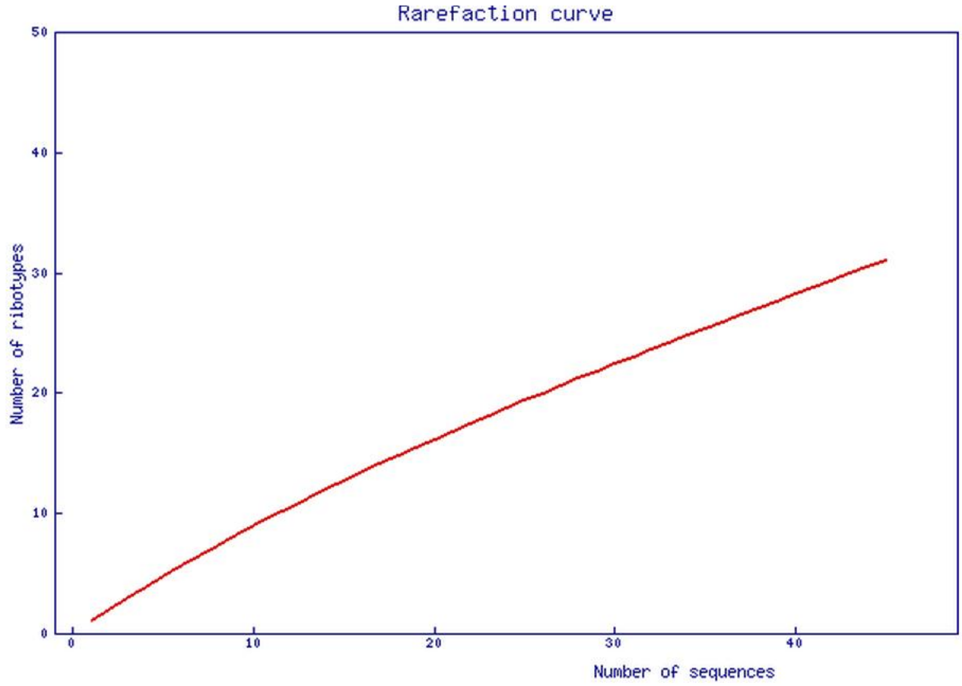


Figure 4.5 Rarefaction curve for the clone library of *Nostoc commune*

This study is the first report on diversity of actinobacteria associated with freshwater macroalgae, *Nostoc commune* Voucher ex Bornet&Flahault. Our results showed that diverse groups of actinobacteria were associated with this macroalgae and some of them were likely to be new taxa. At least 18 genera of actinobacteria were presented in *N. commune*. The high diversity of actinobacteria observed could be the potential source of novel taxa for bioprospecting. The data obtained from this study provides background information which extends our understanding on actinobacterial diversity in freshwater macroalgae.