4.1 Genomic DNA Concentration and Purity

Initially, total genomic DNA extracted from soil samples collected at each site was checked for purity and concentration (Table 4.1).

Table 4.1
Genomic DNA concentration of soils collected from Rothera (RO), Viking Valley (VV) and Léonie Island (LE)

OD / Sites	dsDNA (ng/µl)	260/280	260/230
RO	15.0	1.00	2.00
VV	15.0	0.59	2.00
LE	20.0	0.89	0.44

As can be seen from Table 4.1, the OD readings of three samples are not very high. One reason for this might be the low values of A_{260}/A_{280} ratio since the accepted range of this value is 1.8 to 2.0 and none of the samples meet with this criterion. From here an unwanted contamination by either protein or single stranded DNA/RNA might jeopardize the purity of the DNA sample. Furthermore, by comparing the A_{260}/A_{230} values of the three samples, RO and VV appear to be in the desired range (i.e. ≥ 1.5) while LE has a lower value than expected. This may suggest contamination of this sample by organic compounds which could contribute to insufficient DNA purity.

4.2 Primary PCR Reaction

The initial optimization of PCR reaction as well as appropriate thermal cycles for 27F and 1492R primer set was given in Table 3.2, section 3.3.

4.2.1 Template

Total genomic DNA extracted from soil samples at different locations in maritime Antarctica was inspected for bacterial SSU ribosomal gene. The DNA samples to be used as template in PCR amplification needed to be diluted with dH_2O before each round of PCR. This is a crucial step since it helps to dilute out certain impurities present in samples such as humic compounds, decreasing the level of any unwanted components that might interfere with PCR amplification (Volossiouk *et al.*, 1995).

In case of RO, a 1 in 50 dilution of extracted DNA did not generate satisfactory band and a 1 in 20 dilution was required in order to achieve best band quality (Fig. 4.1.a). To indicate the necessary amount of this dilution as template, three different reactions were set up with 1, 2 and 5 µl of 20× diluted DNA in a 50 µl PCR reaction. The best band intensity was observed when 5 µl of template was used (Fig. 4.1.a).

Similarly for VV, different dilutions were tested for optimal results. Since DNA sample from VV site had an OD value similar to RO, primarily a 20× dilution was used as template. However the resulting bands were faint (figure not shown) and a less diluted sample was required. A 10× dilution did not generate satisfactory results either. Eventually, 1-2 µl of the undiluted sample was used as template in a 50 µl PCR reaction which resulted in sufficient band quality (figure not shown).

The absorbance reading of the LE DNA sample was the highest out of three, and therefore 5 µl of the 50× dilution as PCR template generated best results (figure not shown). These optimized values were used to produce a final volume of 100 µl of PCR product per study site sample and was repeated for duplicate reactions accordingly.

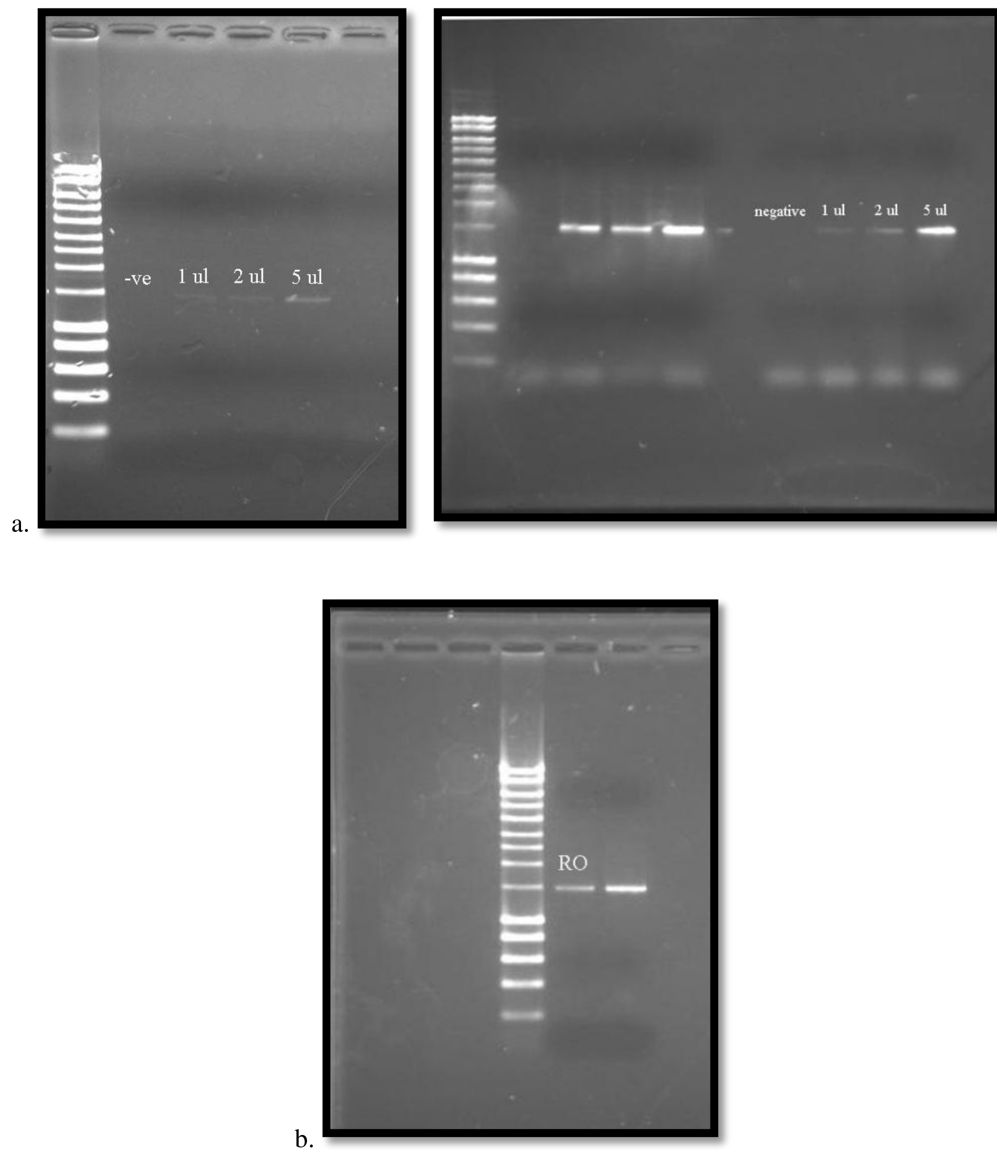


Figure 4.1 Primary PCR amplification of RO site DNA sample **a.** Resulting bands after 50× (left) and 20× dilutions (right). The lanes are marked according to the volume of template used; no band is observed in negative control **b.** Purified DNA. All bands are 1,500 bp in size as indicated by HyperLadder weight marker.

4.3 PCR Purification

The desired PCR products were purified before being cloned into corresponding vectors. The PCR purifications were carried out to remove excess salt ($MgCl_2$) present in PCR reagents and the primer dimers resulting from self-annealing during PCR reactions. These impurities were removed as they may interfere with the ligation step in cloning process.

For each clone library construction, a total of 100 µl DNA was produced by PCR amplification prior to cloning. The products were purified as described (refer to chapter 3, section 3.5) provided that no band was observed in the negative control reaction (Fig. 4.1). Once confirmed by gel electrophoresis, the concentration of the purified samples was measured by spectrophotometry (Table 4.2) in order to specify the amount of DNA for ligation process. The calculated volume needed for ligation for each DNA sample is listed in Table 4.2 (refer to section 3.6.2 for calculations).

Table 4.2
Purified DNA concentration post PCR amplification and calculated amount of DNA required for ligation reaction for each cloning session (duplicates carried out for each site indicated by Roman numerals)

Site	OD (ng/µl)	Volume for ligation (µl)
ROI	17.5	3
ROII	33.7	1.5
VVI	22.5	2.2
VVII	32.9	1.5
LEI	47.9	1
LEII	47.9	1

4.4 Cloning

Clone libraries were constructed concerning bacterial 16S rRNA gene from total community DNA extracted directly from soil from three different sites in Antarctica using pGEM®-T Easy Vector System (Fig. 3.3; see section 3.6). For each cloning reaction, freshly prepared PCR products were used in the TA Cloning® process.

As can be seen in Fig. 3.3, the vector is linearized with a single 3′ T-overhang at both ends. This will greatly improve the ligation efficiency of the insert into the vector by offering a compatible overhang for the PCR product that has an additional 3′ A-overhang. This in turn, is made possible by the basic concept of transferase activity of most DNA polymerases which lack 5′→3′ proofreading ability, such as *Taq* polymerase, adding a single adenosine base to the 3′-ends of double stranded DNA

regardless of the template (Zhou and Gomez-Sanchez, 2000). The single 3′ A-overhang, however, has the tendency to degrade over time due to long period of storage or several freeze-thaw cycles. Therefore it is recommended to prepare the PCR products whenever they are needed to be cloned. The T-A base complementation is stabilized with the help of T4 DNA ligase.

In case of successful ligation, more than 100 colonies are expected to form of which 10-40% is blue, according to the manufacturer's protocol. As can be seen from Table 4.3, this was the case in all of duplicate cloning sessions and the white colonies from sample plates were picked randomly for PCR screening and later for RFLP analysis. In case of low number of colonies on sample plates or growth in negative plate as a result of contamination, the cloning for that sample was repeated until satisfactory results were achieved (Fig. 4.2).

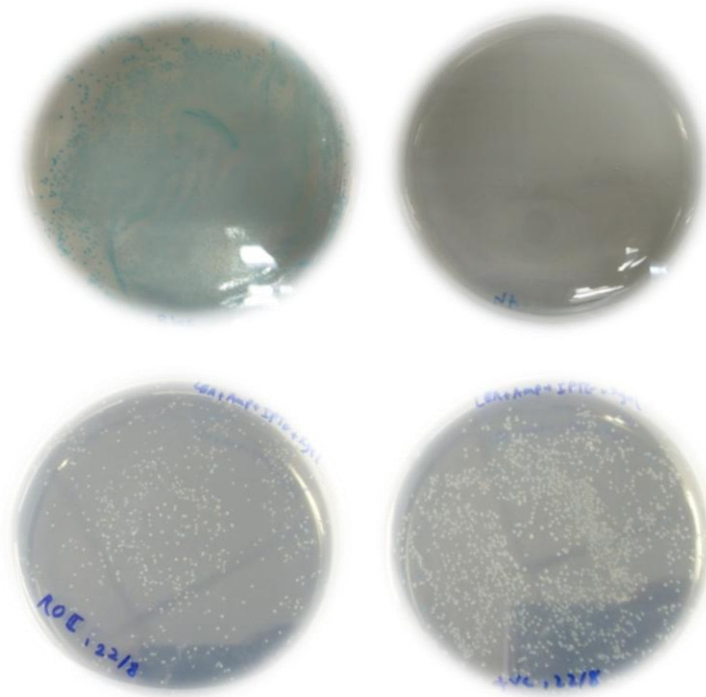
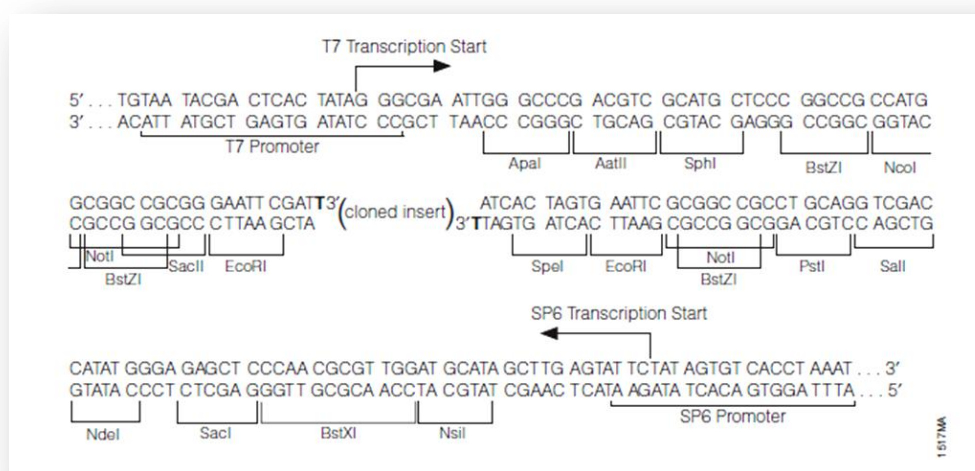


Figure 4.2 Blue/white colony screening of plates of RO cloning session (from left to right, top row transformation and negative control plates, bottom row sample and positive control plates)

Table 4.3
Colony count of duplicate cloning sessions, number of white colonies and blue colonies for both sample and positive control plates

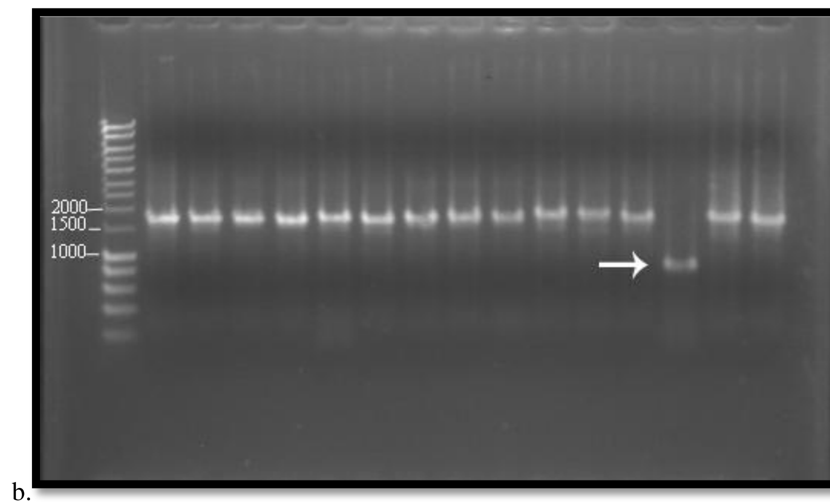
Plates	White		Blue	
	Sample	Positive	Sample	Positive
ROI	167	617	24	83
ROII	258	750+	65	127
VVI	192	688	46	95
VVII	235	780+	94	186
LEI	253	700+	89	159
LEII	203	674	57	140

Using vector-specific primers regarding Sp6 and T7 promoters, the selected colonies were screened for correct insert. The sequence of the promoter as well as the MCS of the vector is shown in Fig. 4.3.a. From there the total size of the amplified region including the 16S rRNA gene is apparent to be around 1,680 bp (1502 bp insert DNA + 78 bp T7+ 101 bp SP6). This is confirmed by agarose gel electrophoresis for all PCR products. The samples showing bands of unwanted sizes or no band at all (in case they persisted after repetition) were discarded (Fig. 4.3.b). The rest was chosen for further RFLP analysis.



a.

Figure 4.3 a. The promoter and MCS of pGEM®-T Easy Vector (Technical Manual for pGEM®-T and pGEM®-T Easy Vector Systems; *retrieved from:* http://www.promega.com/tbs/tm042/tm042.pdf*, on May 30, 2010*)



b.

Figure 4.3 b. Colony PCR of cloned cells from RO cloning session; all bands showing the correct size except one (pointed out with arrow)

4.5 RFLP Analysis

All the PCR products showing the positive insert from clone libraries were screened for similarity by RFLP analysis. For this purpose, two restriction enzymes, *HaeIII* (source organism: *Haemophilus aegypticus*) and *HhaI* (from: *Haemophilus haemolyticus*) were employed. By using a two enzyme system rather than just one, the chances of obtaining undisputed fingerprints increases with better likelihood of each unique pattern corresponding to one certain species. The buffer system C was used with 100% activity for both enzymes (Promega, Madison, WI, USA).

The suggested amount of DNA for best results according to the manufacturer's guide is 1 µg. However, judging by the band quality of PCR products from all amplicon libraries, a volume of 5-10 µl was used for majority of samples whereby adequate band resolution was observed (Fig. 4.4). In case the original PCR product proved to be less concentrated, 15 µl was added to the RE reaction (refer to section 3.8). The resulting bands were inspected by 1.5% (w/v) agarose gel electrophoresis (Fig. 4.4).

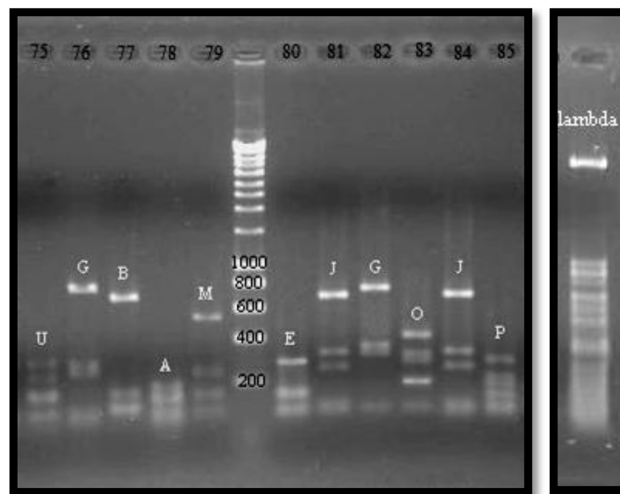


Figure 4.4 An overview of RFLP analysis corresponding to ROI clone library with examples of some of the patterns. Lambda DNA (image to the right) was used as a control for monitoring enzyme digestion.

The main challenge with RFLP fingerprints is to correctly relate and group all the patterns (phylotypes). For the current work, total of 548 clones were screened by restriction fragment length polymorphism. All the documented RE gel pictures were uploaded to the GelComparII software for categorization (refer to section 3.8.2). Each cluster of similar RFLP pattern was considered a phylotype and was further inspected to double-check the proper matching of all individuals lying within that collection. A few samples needed to be manually matched to the existing groups due to either incorrect band configuration by the software or ambiguity.

The clones grouped within 25-35 phylotype patterns per clone library with some of them being rare and site-specific (Table 2S, Appendix B). However, many instances of community-dominant (CD) phylotypes (i.e. those that occurred ≥3 times) were observed in each clone library, especially in clones from RO site sample (Table 4.4). The most occurring phylotype in all three clone libraries belonged to fingerprint type A.

Table 4.4
Number of clones from soil clone libraries of three different sites in Antarctica, number of unique and community-dominant (CD) phylotypes, percentage of clones in CD phylotypes and sampling coverage for each clone library

Sample location	No. of clones	No. of phylotype patterns	No. of CD phylotypes	Clones assigned to CD phylotypes (%)	Good's coverage
RO	181	25	20	95	0.99
VV	176	31	22	91	0.98
LE	191	35	20	87	0.97

4.6 Sequencing

A representative of each phylotype was selected for sequence identification. A randomly chosen sample within each phylotype was prepared for sequencing as described in section 3.9. Occasionally a double band would be observed in which case it was repeated with another sample of the same phylotype (Fig. 4.5). All the samples were prepared in the same way and purified before sequencing routine.

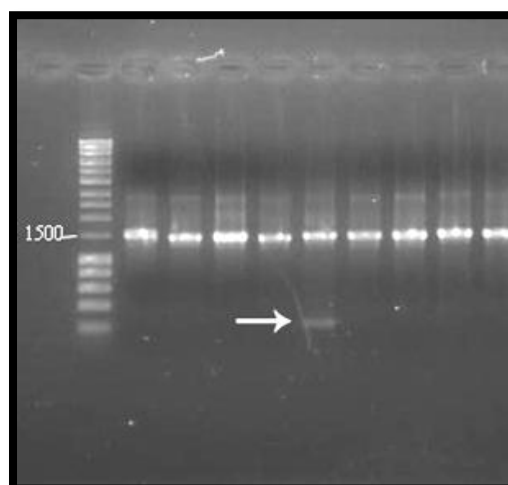


Figure 4.5 PCR amplification of 16S rRNA gene; the sample showing a second band at 300 bp (pointed out by arrow) was discarded.

All the chromatogram files of the sequence results were checked individually by Sequence Scanner version 1.0 (Applied Biosystems, 2005) to ensure maximum quality of the sequences. Most of the results showed reasonable signal peak strength and no mixed signals throughout majority of the sequence length, in which case further sequence analysis was carried out (Fig. 1S, Appendix C).

4.7 Data Analysis

4.7.1 16S rRNA gene sequences

Over 50 DNA sequences were submitted to NCBI website for nucleotide BLAST (BLASTn) search. The sequences showing maximum identity score in a pair-wise alignment were copied along with percentage identity and accession number (Table 2S, Appendix B).

4.7.2 Phylogenetic analysis

Total of 144 sequences including the subject data were studied for homology by constructing a multiple sequence alignment. A final set of about 637 nucleotides was found to be shared unambiguously by all sequences. The online grouping of the trimmed sequences revealed a total of 46 independent groups of OTUs (Table 2S, Appendix B). None of the sequences showed any significant anomaly as suggested by the Pintail tool. Neighbor-Joining (NJ) phylogenetic tree was constructed (refer to section 3.10) to show the phylogenetic affiliations of sample sequences (Fig. 2S, Appendix E). Closely related individuals clustering together were considered belonging to the same phylum after confirmation by the RDP Classifier tool (Table 3S, Appendix D). The observed phyla and their corresponding phylotype relative abundance in each site are presented in Fig. 4.6.

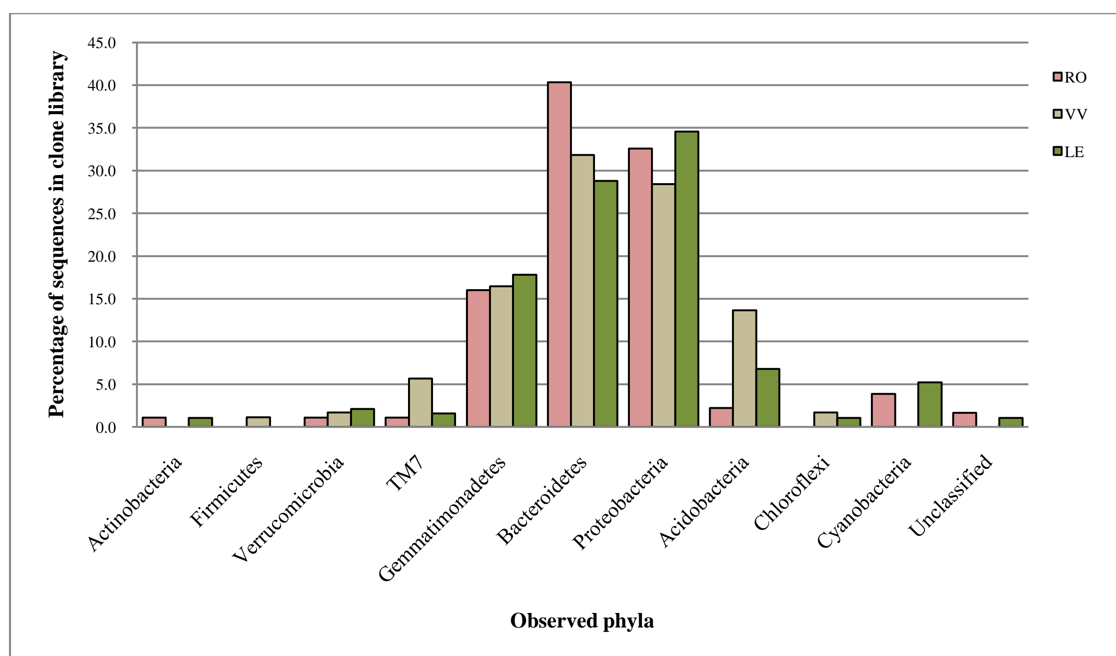


Figure 4.6 Bar charts of clone library composition at phylum level for Rothera (RO), Viking Valley (VV), and Léonie Island (LE) samples.

The dominant phylum in all three studied locations was *Bacteroidetes,* with a total of 13 phylotypes, followed by *Proteobacteria* with 11 phylotypes (8 assigned to *Betaproteobacteria*, 2 to *Alphaproteobacteria* and 1 to the *Gammaproteobacteria*). Seven phylotypes were grouped within the *Acidobacteria*, 3 in each of the *Verrucomicrobia*, *Cyanobacteria*, and the *TM7*, 2 within the *Actinobacteria*, and 1 in the *Gemmatimonadetes*, *Chloroflexi*, *Firmicutes*, and *Unclassified Bacteria* each. In general, most of the phyla were observed in all three sites except for *Actinobacteria*, *Cyanobacteria* and *Unclassified Bacteria* which were missing from Viking Valley. In turn, *Firmicutes* phylum was exclusive to VV site whereas *Chloroflexi* was present in both Viking Valley and Léonie Island but not in Rothera (Fig. 4.6).

Overall, no significant difference between the classification obtained from RDP Classifier and the NJ method of all the cloned 16S rDNA sequences was observed. Clones belonging to phylotype A, which showed close relationship to *Gemmatimonas*,

assembled about 16-18% of all clone libraries, making them the single most dominant taxa undisputedly.

The *Bacteroidetes* dominant in all three soil samples were closely related to the genera *Terrimonas* (phylotypes F, G and AQ), *Ferruginibacter* (W and Y), *Segetibacter* (AJ), *Niabella* (K) or *Lacibacter* (S) of family Chitinophagaceae. In addition, Phylotypes D and E showed close affiliation to genus *Pedobacter* belonging to the family *Sphingobacteriaceae*. Phylotype B showed 64% similarity to genus *Haliscomenobacter* in the family *Saprospiraceae*, and was prevalent in Rothera Point and Viking Valley soil but absent from the Léonie Island. For a complete list of phylogenetic classification of all phylotypes obtained from RDP Classifier, refer to Table 3S in Appendix D.

Not all phylotypes were able to show considerable similarity to finest taxonomic levels (in this case, the finest taxonomic resolution was down to genera level). A few were unable to be assigned to any known phylum; e.g. Phylotype I, which showed 29% similarity to the *Proteobacteria* group in RDP Classifier, formed an independent branch under *Unclassified Bacteria* in the NJ tree showing moderate affiliation to *Fibrobacter* and distant relation to *Cytophagales*/green sulfur bacterium. Another unclassified phylotype, phylotype AI showed 43% homology to *Chloroflexi* group and was clustered closely with uncultured bacterium clone UMABcl83 and to a lesser extent to uncultured Chloroflexi bacterium clone T5P2B_22.

4.7.3 Statistical analysis

4.7.3.1 Observed number of phylotypes

For a basic estimation of phylotype richness, the observed number of phylotypes in each clone library was considered as a representative of the original assemblage. The criterion for phylotype distinction was set by the online program FastGroupII to consider 97% sequence similarity as one operational taxonomic unit (OTU). The list of observed phylotypes from duplicate clone libraries regarding each study site was inspected for similarity and since no significant difference was observed, the results from each set was pooled together to form one final set for each site.

From the total of 46 observed phylotypes, 15 were shared by all three sites, 3 were exclusive to RO and VV, 5 were observed only in RO and LE, 7 shared by VV and LE, 2 specific to RO site, 6 occurring only in VV and 8 exclusive to LE. Clone library corresponding to RO site revealed the least phylotype richness with a total number of 25 phylotypes whereas VV and LE contained 31 and 35 phylotypes, respectively (Table 2S, Appendix B).

4.7.3.2 Biodiversity indices

The observed phylotypes and their abundances were used to calculate diversity indices (Table 4.5). The Shannon diversity index (*H'*) calculated for the clone libraries revealed the highest level of diversity in Léonie Island (3.14) which was comparable to that of Viking Valley (3.09). Rothera Point showed the lowest value (2.93) of the three sites, indicating a less diverse bacterial community compared to VV and LE. The calculated value for Fisher's alpha (estimated number of unseen phylotypes) was also found to be highest in LE (12.57), followed by VV (10.91) and RO (7.87). However, Simpson's diversity (*D*), equitability (*J'*), and Berger-Parker evenness indices were

highest in RO clone library, suggesting a more even distribution of phylotypes across the source environment as opposed to that of VV and LE sites.

Table 4.5
Diversity indices calculated for soil clone libraries

Sites	Taxa *S*	Individuals	Shannon *H'*	Simpson *D*	Fisher's α	Berger-Parker	Equitability *J'*
RO1	25	181	2.93	0.068	7.87	6.24	0.91
VV1	31	176	3.09	0.063	10.91	6.07	0.898
LE3	35	191	3.14	0.064	12.57	5.62	0.883

To indicate the level of overlap or number of species shared by two communities, Sorensen's similarity index was applied. The clone libraries were compared two by two and the least similarity was observed between RO and VV (0.643), whereas RO and LE as well as VV and LE comparison revealed the same value (0.667). Altogether, the values suggest a high level of overlap (more than 60%) between three sites.

4.7.3.3 Accumulation curves and richness estimators

Relative abundance distribution of phylotypes obtained from clone libraries was plotted versus abundance categories in a log abundance plot (Colwell, 2009) to visualize the phylotype distribution throughout different sites (Fig. 4.7). The distribution corresponding to RO and VV site seem to reach a lognormal (bell-shaped) statistical distribution. This type of distribution is common for large, well-inventoried natural communities as described by the author. This may suggest a near complete sampling for these two sites while the LE site phylotype distribution is not lognormal implying that, even though all three sites have been equally sampled, more sampling is required for LE site due to higher biodiversity.

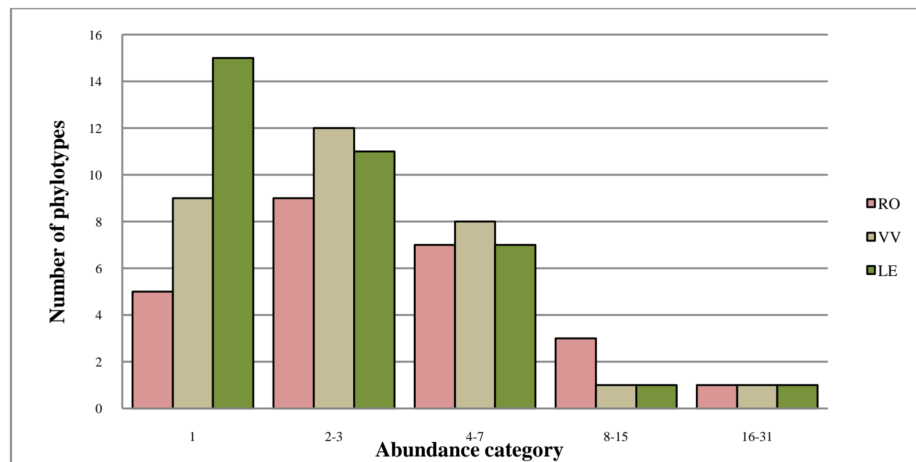


Figure 4.7 Log abundance plots of observed phylotypes considering their relative abundance in each clone library

Another way of assessing sampling effort and whether or not it has been done exhaustively is by plotting number of phylotypes observed versus number of clones identified in an accumulation curve (aka collector's curve) (Kemp & Aller, 2004). Using the EstimateS software and the data from each clone library (duplicates combined), the S_{obs} *(Mao Tau)* against the number of clones characterized was plotted for each data set (Fig. 4.8.a). Additionally, Cole Rarefaction curve corresponding to each data set was plotted against sampling effort for that clone library which was found to be identical to the accumulation curve for respective samples (data not shown) and thus analyzed in the same manner.

By looking at the curves separately, it is apparent that the one corresponding to RO site is reaching an asymptote first. The next curvilinear plot belongs to VV samples while LE accumulation curve is the last to slowly approach an asymptote. Though when considering the 95% confidence intervals (data not shown), VV and LE curves overlap almost entirely, suggesting that the observed difference between the phylotype richness of these two sites at current sampling effort was due to chance. On the other hand, since the curve corresponding to LE site is not completely asymptotic yet, the phylotype

richness in the source environment is underestimated and richness estimation must not be overinterpreted (Kemp and Aller, 2004).

With that in mind, it is perhaps safe to assume that sampling effort for RO site has been done exhaustively and few additional phylotypes will be recovered by extra sampling beyond the current library size. On the other hand, more sampling might reveal more unique phylotypes in either of VV and LE sites with better differentiation between the two environments (Kemp and Aller, 2004).

In addition, phylotype richness was estimated using *Chao1* formula (Chao, 1984) in EstimateS software program and a rarefaction curve was plotted (Fig. 4.8.b). As can be seen, the curves corresponding to VV and RO clone libraries are reaching a flat line while LE rarefaction curve is not yet steady. This might be due to slight undersampling of LE site as was suggested by the accumulation curve for this clone library. The phylotype richness as calculated by *Chao1* estimator was found to be 25.1 for RO site, 31.8 for VV site and 36.3 for LE site.

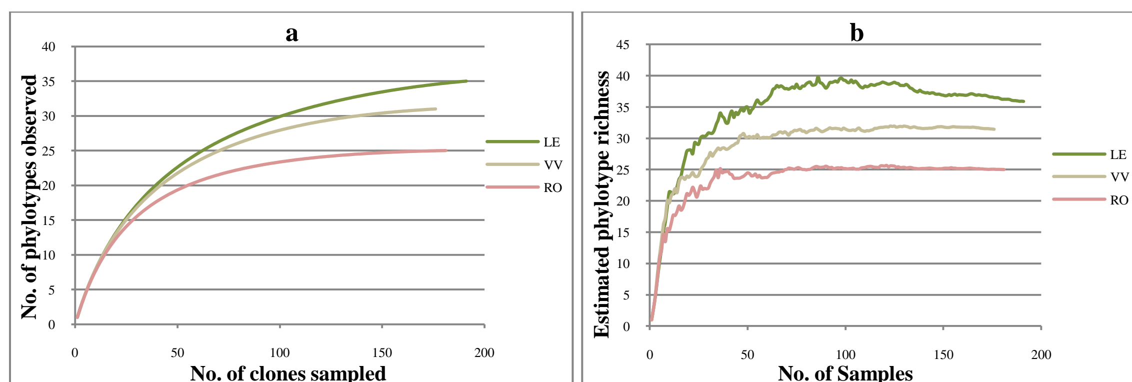


Figure 4.8 a. Phylotype accumulation curves (collector's curves) for different clone libraries from study sites **b.** phylotype richness corresponding to each clone library estimated by *Chao1* unbiased formula

It is still hard at this point to confidently differentiate between the sample sites as the calculated values seem to be very close to each other. The non-metric multidimensional scaling (nMDS) comparison showed no similarity between the three sites as they were distantly oriented on the scale (Fig. 4.9). A non-metric MDS based on the available phylum groups did not reveal any similarity at phylum level either (data not shown), ruling out the doubt of near identical results.

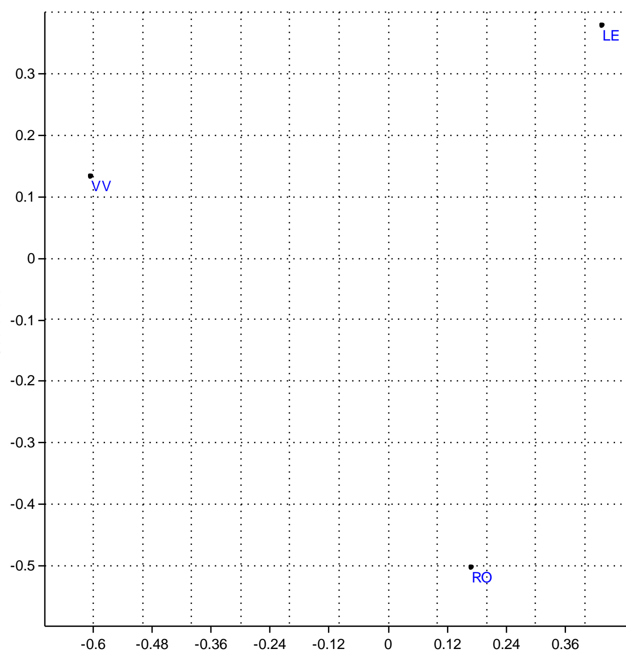


Figure 4.9 Phylotype level non-metric multidimensional scaling (nMDS) of clone library data from three different sites in Antarctica