CHAPTER 5: PHYLOGEOGRAPHY OF VERRUCULINA ENALIA INFERRED FROM THE NUCLEAR ITS (INTERNAL TRANSCRIBED SPACER) SEQUENCE

5.1 Results

5.1.1 Genomic DNA of Verruculina enalia

Genomic DNA of 14 isolates of *V.enalia* from various countries was successfully extracted (Figure 5.1). DNA concentration ranged from 64.5 to 97.2 ug/mL. The OD reading gave A260/A280 ratios ranging from 1.5 to 1.9. The concentrations of the genomic DNA varied due to the difference in fungal biomass used for extraction.

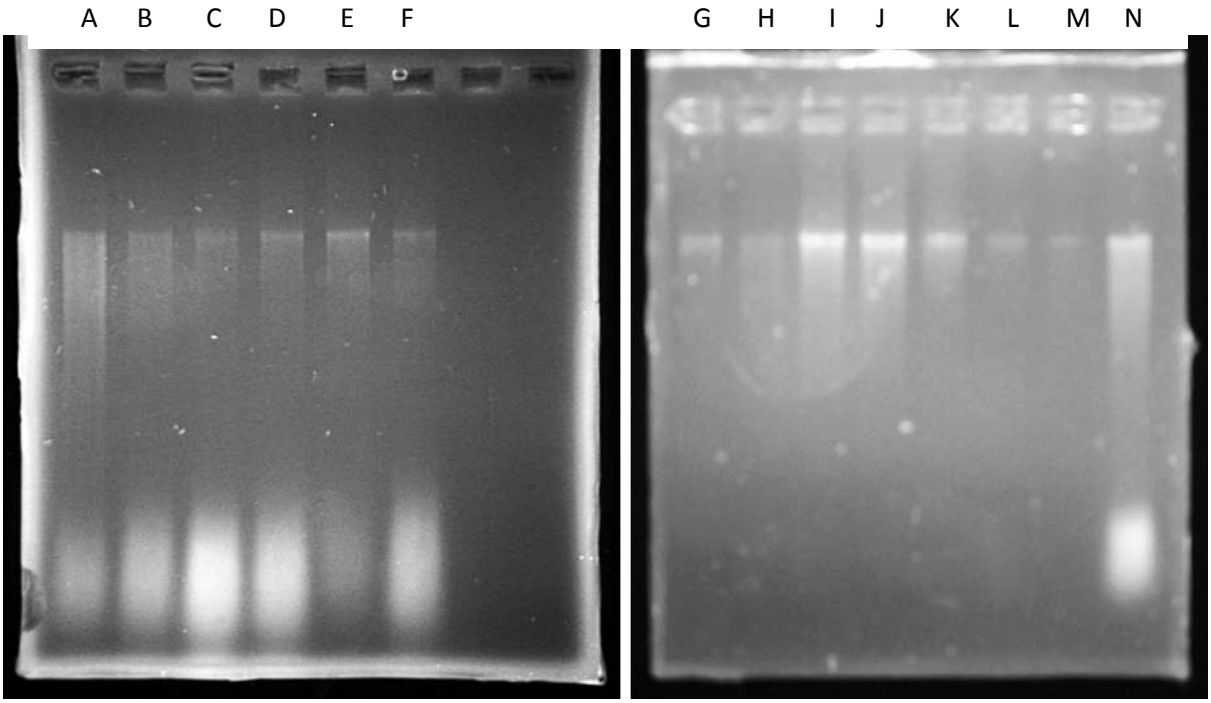

Figure 5.1 Genomic DNA extracted from *V.enalia* isolated from Malaysia, Taiwan, Hong Kong, Philippines and Singapore using the method of Pang *et al.* (2003) and Sakayaroj *et al.* (2005) visualized on 1% (w/v) of TAE agarose gel. A) ISB0201- Tioman Island, Malaysia B) ISB0301- Kukup, Malaysia C) ISB0361- Bagan Lalang, Malaysia D) ISB0362- Bagan Lalang, Malaysia E) ISB3532- Sai Kung, Hong Kong F) ISB3533- Sai Kung, Hong Kong G) ISB2952- Samal, Philippines H) ISB2953- Samal, Philippines I) ISB2954- Samal, Philippines K) ISB5059- Sai Kung, Hong Kong L) ISB1350- Three Fathom Cove, Hong Kong M) ISB0657- Jici Rockyshore, Taiwan N) ISB0658- Jici Rockyshore, Taiwan.

5.1.2 PCR of ITS regions

The ITS1-5.8S-ITS2 region for all 14 isolates was successfully amplified by PCR (Figure 4.3). The size of the entire region ranged from 519 to 522 (Appendix). The small differences in length suggest that the sequences are not rich in length mutations.

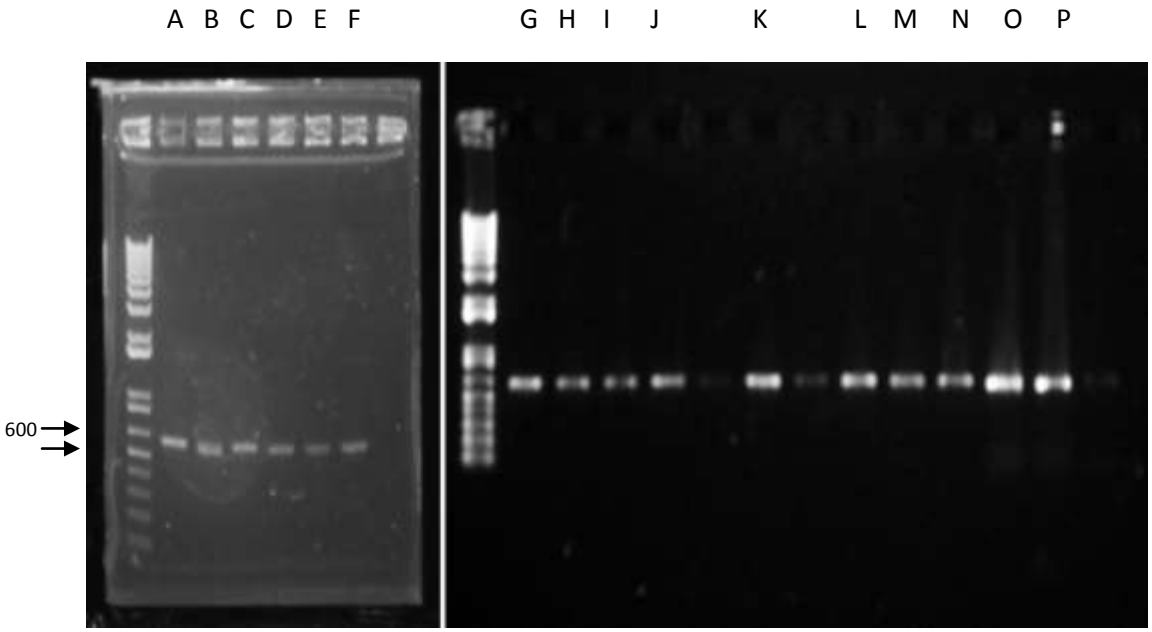

Figure 5.2 PCR amplification of ITS1-5.8S-ITS2 region from V.enalia isolated from Malaysia, Taiwan, Hong Kong, Philippines and Singapore. Representation of isolates showing amplification of the entire ITS1-5.8S-ITS2 region. The PCR products had a molecular weight of 500-550 bp. A) ISB0658 B) ISB0657 C) ISB1350 D) ISB1350 E) ISB5059 F) ISB5059 G) ISB0201 H) ISB0301 I) ISB3532 J) ISB3533 K) ISB5059 L) ISB1350 M) ISB0361 N) ISB0362 O) ISB2952 P) ISB2953

5.1.3 Phylogenetic Analysis of ITS region

5.1.3.1 Identification through BLAST

ITS1-5.8S-ITS2 sequences from all 14 isolates were subjected to BLAST search (Altschul, 1990) comparison in GenBank NCBI to check for the identity of the used isolates. All isolates were confirmed as *Verruculina enalia* based on their ITS sequence.

Table 5.1 Three most significant strains deposited in GenBank associated with *V.enalia* isolates from the study.

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
GQ203796.1	Verruculina enalia strain CBS 304.66 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene, complete sequence; and internal transcribed spacer 2, partial sequence	869	869	93%	0.0	99%
GQ203760.1	Lepidosphaeria nicotiae strain CBS 559.71 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene, complete sequence; and internal transcribed spacer 2, partial sequence	390	390	50%	4e-105	94%
HM123518.1	Fungal sp. ARIZ AZ0829 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence	388	388	50%	2e-104	94%

*All *V.enalia* isolates were associated with the same 3 most significant identity when subjected to BLAST search in NCBI database.

5.1.3.2 Sequence alignment: ITS region characteristic

ITS1-5.8S-ITS2 alignment showing total alignment length of 520 bp, total sites excluding gaps/missing data is 520 bp as well, invariable and variable sites were 344 and 174 respectively and 2 parsimony informative sites.

5.1.3.3 ITS phylogenetic tree

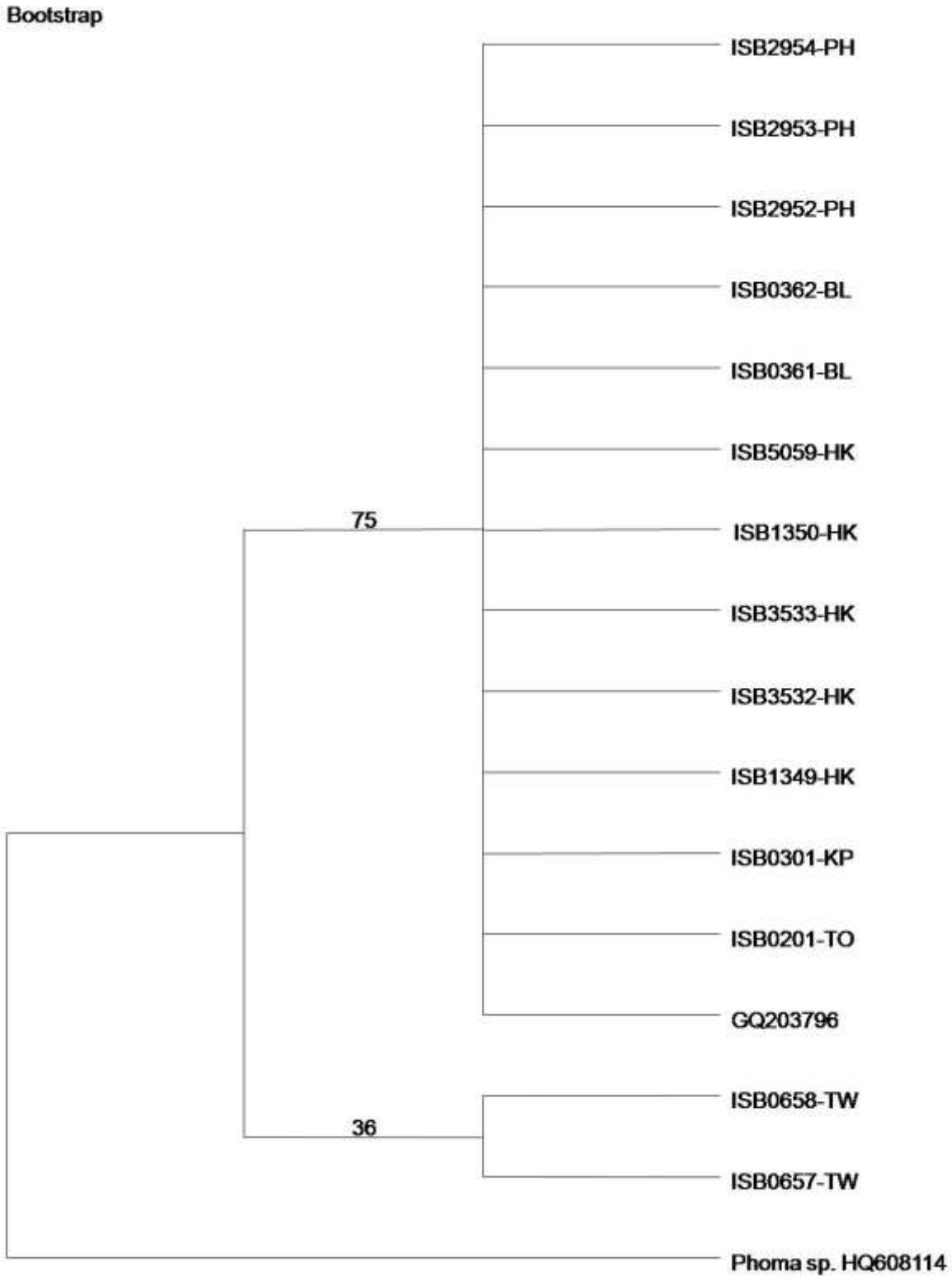

Figure 5.3 A single most parsimonious tree inferred from the ITS1-5.8S-ITS2 sequences data. The tree was produced using branch-swapping algorithm with the Multiple trees option (MULTITREES) disabled (tree length=178 steps, C. I. =1.000, R.I. =1.000). Maximum parsimony tree was produced by the unweighted parsimony analysis where gaps are treated as missing data. Sixteen taxa were used and a *Phoma* sp. was chosen as the outgroup taxa. One tree was resulted with a tree length = 178, a consistency index (C.I.) = 1.000, a retention Index (R.I.) = 1.000 and rescaled

consistency index (R.C.) = 1.0000 Two clades were resulted; the first clade consisted of 13 isolates (ISB2954, ISB2953, ISB2952, ISB0362, ISB0361, ISB5059, ISB1350, ISB3533, ISB3532, ISB1349, ISB0301, ISB0201 and GQ203796) with a moderate bootstrap support and the second clade consisted only 2 isolates (ISB0658 and ISB0657) with a weak bootstrap support. The pairwise matrix (Table 4.2) shows the sequences within both clades are identical, while sequences between the clades differ only for less than 1%.

Table 5.2 Pairwise matrix.

		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
1	Phoma sp. HQ6081																
2	ISB0658	0.34178															
3	ISB2954	0.34378	0.00385														
4	ISB2953	0.34378	0.00385	0.00000													
5	ISB2952	0.34378	0.00385	0.00000	0.00000												
6	ISB0362	0.34378	0.00385	0.00000	0.00000	0.00000											
7	ISB0361	0.34378	0.00385	0.00000	0.00000	0.00000	0.00000										
8	ISB5059	0.34378	0.00385	0.00000	0.00000	0.00000	0.00000	0.00000									
9	ISB1350	0.34378	0.00385	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000								
10	ISB3533	0.34378	0.00385	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000							
11	ISB3532	0.34378	0.00385	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000						
12	ISB1349	0.34378	0.00385	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000					
13	ISB0301	0.34378	0.00385	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000				
14	ISB0201	0.34378	0.00385	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000			
15	ISB0657	0.34178	0.00000	0.00385	0.00385	0.00385	0.00385	0.00385	0.00385	0.00385	0.00385	0.00385	0.00385	0.00385	0.00385		
16	GQ203796	0.32029	0.00626	0.00214	0.00214	0.00214	0.00214	0.00214	0.00214	0.00214	0.00214	0.00214	0.00214	0.00214	0.00214	0.00626	

5.2 Discussion

An integrated study of ecology and historical biogeography.

A species would not be able to live outside its ecological tolerance range and therefore, its biogeography cannot contradict the nature of its ecology (Ridley, 2004). However, historical factors may also determine where it is living and where it is not within its ecological tolerance range (Ridley *et al.*, 2003). An integrated approach of these two studies needs to be done as exchanges in these two fields are very limited. While the historical studies has become narrowly focused on using phylogenies to discover the history of geological connections among regions, the ecologists often ignore the historical biogeography, even when its input can be crucial (Wiens and Donoghue, 2004).

There are two major groups of marine fungi: the pan-temperate and the pan-tropical, but there is little evidence of species being restricted to a certain countries or continents (Jones, 1993). In the regions between the tropics and subtropics, the composition of the mycobiota is dependent on water temperature, rather than air temperature. Tropical currents or colder currents often cross boundaries and influence the mycobiota therein. Where intermediate sea temperatures occur, there is often a mixture of temperate and tropical fungi (Jones, 1993).

Some marine fungi are cosmopolitan which can be found in temperate as well as tropical region (Jones, 1993) while the intertidal mangrove species have a subtropical distribution (Hyde and Lee, 1995). In most cases, the occurrence of a fungus in a particular habitat is related to water temperatures and the type of substrata. The effect of the latter is particularly striking as the fungi occurring on submerged wood in the open sea usually differ from those on intertidal mangrove wood. In turn, these fungi differ from those occurring on leaves or algae (Hyde, 1988).

Mangrove fungi constitute the second largest ecological group of marine fungi which are widely distributed in Old and New world mangroves (Atlantic, Indian and Pacific Ocean) (Jones, 2011a). Mangroves evolved from terrestrial or fresh water plant species rather than marine plants where these land plants regularly becomes adapted to brackish water. It is believed that the breakup of continental land masses provided conditions, favorable for the development of mangroves in the fringe areas (Chapman, 1976).

The earliest mangrove species is believed to be originated in the Indo-Malayan region or also known as the Indo-West Pacific (IWP) region, where the mangrove species richness is at the highest (Ellison *et al.*, 1999). Due to its unique floating propagules and seeds, these early mangrove species spread westward, borne by ocean currents, to Caribbean and western Atlantic between 66 million years ago (Ellison *et al.*, 1999).

The analysis on *Verruculina enalia.*

Verruculina enalia is known to have a high occurrence on mangrove woods. It is particularly common on lignocellulosic substrata of mangrove branches and twigs (Schoch *et al.*, 2006; Suetrong *et al.*, 2009). *Verruculina enalia* was referred to the Testudinaceae based on molecular sequence (Schoch *et al.*, 2006). However, analysis done by Mugambi and Huhndorf (2009) placed it in the Platystomaceae with a weak support.

PCR-DGGE (denaturing gradient gel electrophoresis) analysis of DNA extracted were used in this study it has been used on various substrata to document fungal communities using fungal specific primers (May *et al.*, 2001; Nikolcheva *et al.*, 2005; Duong *et al.*, 2006; Seena *et al.*, 2008)

ITS region were chose as it is variable among the morphologically distinct fungal species according to several studies done by Gardes *et al.*, 1991; Gardes and Bruns, 1991; Baura *et al.*, 1992; Chen *et al.*, 1992; Lee and Taylor, 1992. Other than that, mycologists are working towards DNA barcoding where ITS region is used almost universally and was selected as the standard DNA barcode region. In some groups, ITS is not unique for individual species and need to be combined with a second gene (Rossman, 2007).

The analysis of geographical distribution data on mangrove fungi found that every oceans supported different number of species (Schmit and Shearer, 2004) but the numbers are more likely reflect the intensity and frequency of sampling (Jones and Puglisi, 2006; Alias and Jones, 2008).

Identification of isolates through BLAST shows that all isolates are related with *Verruculina enalia* strain CBS 304.66 and two other sequence deposited in GenBack which are the *Lepidosphaeria nicotiae* culture-collection CBS: 559.71 and Fungal sp. ARIZ AZ0829. This confirms the identity of all isolates used in the study as *V. enalia*.

The alignment length in this study is 520 bp and the number of parsimony informative sites is 2. This number is very low which leaded to unresolved parsimony tree. Phoma sp.HQ608114 remain as the outgroup. All isolates grouped together including one isolate from Liberia (west of Africa) deposited in GenBank with an accession number GQ203796 forming 2 clades: one as an unresolved polytomy and one with 2 isolates from Jici Rockshore, Taiwan (ISB0658-TW, ISB0657-TW) (Figure 4.4).

Dispersal of marine fungi.

Hyphae of marine fungi developed in lignocelluloses which can possibly transported over a long distance on driftwood or inside planks of wooden boats in the early days. In the old days, the ships were made of wooden where the fungi can well grow on them.

While for current situation, the hyphae could be transported by the bilge of ships and ballast water in nowadays ships.

It is impossible for a delicate ascospore to serve transoceanic dispersal, let alone its propagules that need an optimum condition for it to emerge (Kohlmeyer and Kohlmeyer, 1979). However, fungi may also disperse through the wood and not by spore itself. Some birds can transfer wood from one place to another.

After about 10 years later, the adaptation and the mechanisms to salinity has been reviewed from the physiological studies of one targeted species *Dendryphiella salina*, a marine hypomycetes (Clipson and Jennings, 1992; Stanley *et al.*, 1995). The cell membrane of fungi are able to maintained the osmotic gradient and this is crucial for growth as turgor becomes the driving force for apical growth and expansion (Money, 1997; Hooley *et al.*, 2003). This ability showed that the ascospores of marine fungi can survived in aquatic environment which means that it is possible for them to disperse through the oceans. The findings of the study have contradicted the statement by previous researcher who said that transoceanic dispersal are impossible to happen.

There is a weak relation unto whether the human dispersal back in early days does affect the dispersal of marine mangrove fungi. However, the fact that human settlement those days were located along the shoreline including the mangrove ecosystems are somewhat related with the process of dispersion. Sir Charles Darwin, 1859 in his book, The Origin of Species also suggested that organisms might float accidently on rafts of vegetation. He also thinks that the land bridges between continents might have been the dispersal routes as our land masses were actually small groups that form a supercontinents million years ago when over the time and with the climate change, it broke apart. However, he is not convinced with either.

From the results gathered, isolates from Malaysia, Hong Kong, Philippines and Singapore are similar in terms of its sequences while isolates from Jici Rocky shore,

Taiwan are significantly different from the other. It could be due to the nature of its habitat which is different compare to the others. The rest of the isolates are isolated from twigs and branches collected from mangrove ecosystem. Very likely, *V. enalia* has not evolved long enough to accumulate mutations. More isolates from wider geographical locations are required to explain the current distribution of *V. enalia*.

The molecular analysis outcome of this study is not enough in order to discuss further on the role of human interference, the mangrove habitat and the vicariance dispersal from the changes of the ancient continent that happens overtime. More biogeographical isolates, targeted species and genes are needed in order to get the DNA patterns.

4.5 Conclusion

DNA sequences of the ITS regions of the nuclear rRNA gene for 13 isolates are identical and the 2 isolates from Jici, Taiwan formed a separate clade but the sequences between the two clades differ for less than 1%. However, more isolates from wider geographical locations are required to explain the phylogeographic structure of *Verruculina enalia*.