CHAPTER 5

PCR-RFLP STUDY

Introduction

Recently, many techniques in molecular biology have been used to study structure and evolution of genes. One of these techniques, namely Restriction Fragment Length Polymorphisms (RFLPs), is a technique in which organisms may be differentiated by analysis of patterns derived from cleavage of their DNA. If two organisms differ in the distance between sites of cleavage of a particular restriction endonuclease, the length of the fragments produced will be different when the DNA is digested with a restriction enzyme. The fragments are then separated by size using electrophoresis. The similarity of the patterns generated can be used to differentiate taxa from one another (Page and Holmes, 2001).

PCR-RFLP uses a set of primer pairs to amplified regions of the genome via polymerase chain reaction (PCR) and length polymorphisms are detected by digestion of the PCR-products with a set of restriction endonucleases, and followed by electrophoresis of resulting fragments. Presence and absence of fragments resulting from changes in recognition sites are used to identify species or populations. This technique has gained popularity for its easy accessibility and rapidity. Moreover, it detects a larger number of variations in at least 50% of the cpDNA variations being attributable to small insertions and deletions (Gielly and Taberlet, 1994).

Analysis of variations in chloroplast DNA (cpDNA) is a suitable tool for studying molecular evolutionary relationships among and within plant species. This because chloroplast genes are assumed to have slower rates of evolution than nuclear genes (Clegg et al. 1991). Due to its uniparental inheritance, and to the fact that it does not undergo recombination, inheritance of the chloroplast genome is linear over generations, changing only by occasional mutations (Parducci and Szmidt, 1999). It provides opportunities for identifying species-diagnostic markers. In the recent years, PCR-RFLP analysis of chloroplast DNA using universal primers has proved to be a

powerful tool for genetic studies in several plant taxa, particularly at the intraspecific and infraspecific or population levels. Examples included *Prunus avium* L. (Mohanty et al., 2001), *Olea europaea* L (Besnard et al., 2002), and *Brassica oleracea* L. (Panda et al., 2003).

The "*Hoya parasitica* complex" is a highly varied species. According to morphological studies there are likely nine forms of this species growing in Thailand. More information and in-depth investigations are needed to clarify their taxonomic status. The PCR-RFLP technique was used to study the chloroplast DNA variations of "*Hoya parasitica* complex". This may be the first report of molecular studies, especially the PCR-RFLP analysis of wild *Hoya parasitica* s.l. species.

The objectives of this study are: 1) to investigate cpDNA polymorphism among nine forms (morphological and anatomical recognized) in the "*H. parasitica* complex" in Thailand, and 2) to utilize cpDNA variations in determining taxonomic status of the "*H. parasitica* complex".

Materials and Methods

1. Plant Materials

Nine forms of the "*H. parasitica* complex" in Thailand were used for PCR-RFLP analyses. Fifty six samples from 46 localities were collected from various parts of Thailand (Table 5.1 and Figure 5.21). One sample of *Hoya parasitica* var. *citrina* from Malaysia was also included.

2. DNA extraction

Total DNA was extracted from fresh leaves using CTAB (cetyltrimethyl-ammonium bromide) method (Dellaporta, et al., 1983) with minor modification as following.

Approximately 1-1.5 g of the fresh leaves was ground using a mortar and pestle with liquid nitrogen. One g of powdered tissue was placed into a 15-ml tube, then 6 ml of extraction buffer (pre-warmed at 65°C: 200 mM Tris-HCl (pH 8.0); 40 mM EDTA; 2.8 M NaCl; 4% CTAB (w/v), 0.2% ß-mercaptoethanol (v/v) and 2% of PVP (w/v)) was added and incubated at 65°C for about 60 min with occasional gentle swirling and mixing. Afterward, one volume of chloroform/isoamylalcohol (24:1) was added. The mixture was mixed, and then was centrifuged for 5 min at 4 °C and 13,000 rpm. Next, the supernatant was transferred to a new tube and 0.1 volume of sodium acetate (3M) was added and mixed, then 2 volume of cold isopropanol (4°C) was added, and incubated for 30 min at -20 °C. A DNA pellet was precipitated by centrifuging for 5 min at 4 °C at 13,000 rpm. The pellet was briefly washed with 70% ethanol, air-dried and dissolved in 200 µl TE buffer (10 mM Tris-HCl; 1 mM EDTA; pH 8.0). To remove RNA, 10 µl RNase A (10µg/ml) was added and incubated for 30 min at 37 °C. To remove RNase A, one volume phenol/chloroform/isoamylalcohol (25:24:1) was added, mixed and pelleted by centrifuging for 10 min at 4°C at 13,000 rpm. The supernatant was transferred to a new 1.5-ml tube. Then one volume of chloroform was added, mixed for 5 min and centrifuged for 10 min at 13,000 rpm. The supernatant was transferred to a new tube and 0.1 volume of sodium acetate was added, mixed, and 2 volume of cold absolute ethanol (-20 °C) was added and incubated for 30 min at -20 °C. A DNA pellet was obtained by centrifuging for 10 min at 4 °C at 13,000 rpm. The pellet was washed with 70% ethanol, air-dry the pellet for 20 min, and resuspended in 50 µl TE buffer. All DNA were kept at -20 °C for the next step.

Table 5.1 Sampling sites and numbers of the *Hoya parasitica* complex in Thailand.

Site no.[a]	Locality	Specimen	Form[b]
1.	Mueang, Chiang Rai Province	407	V
2.	Wang Bua Ban Waterfall, Chiang Mai Province	297	V
		281, 299	IX
3.	Haew Keaw, Mueang, Chiang Mai Province	289	VI
4.	Doi Khun Tan National Park, Lampang Province	276	IX
5.	Silaphet Waterfall, Pua, Nan Province	380	III
		326	VII
6.	Tat Laung Waterfall, Pua, Nan Province	343	VII
7.	Ban Muang Wang Nhua, Phu Phiang, Nan Province	388	IX
8.	Phasing, Mueang, Nan Province	378, 379	V
9.	Lhinan, Na Noi, Nan Province	392	VII
10.	Pla Ba Waterfall, Phu Ruea, Loei Province	561	IX
11.	Than Thong Waterfall, Sri Chiang Mai, Nong Khai Province	559	IX
12.	Than Ngam Waterfall, Nong Wua So, Udon Thani Province	420	IX
13.	Tat Kham Waterfall, Ban Phaeng, Nakhon Phanom Province	504	IX
14.	Phu Phan National Park, Sakon Nakhon Province	429	IX
15.	Tat Ton Waterfall, Mukdahan Province	484	IX
16.	Huai Sai Yai waterfall, Sirinthon, Ubon Ratchathani Province	480	IX
17.	Phu Chongna Yoi National Park, Ubon Ratchathani Province	526	IX
18.	Sam Long Kiat Waterfall, Khun Han, Si Sa Ket Province	508	IX
19.	Phu Wa Kiew Waterfall, Nakhon Ratchasima Province	548	IX
20.	Pu Kae, Saraburi Province	307	IX
21.	Khao Yai National Park, Nakhon Ratchasima Province	997	I
		375	IX
22.	Pang Sida National Park, Sa Kaeo Province	3	IX
23.	Prachantakham, Prachin Buri Province	315	IX
24.	Ban Pe, Rayong Province	82	VIII
25.	Nam Tok Phriu National Park, Chanthaburi Province	120	VIII
26.	Ko Chang, Trat Province	660	VIII
27.	Thi Lo Su waterfall, Tak Province	570	IX

Table 5.1 Continued

Site no.[a]	Locality	Specimen	Form[b]
28.	Sangkhla Buri, Kanchanaburi Province	457	IX
29.	Koeng Kra Wia Waterfall, Kanchanaburi Province	475	IX
30.	Pong Ron, Thong Pha Phum, Kanchanaburi Province	444	IX
31.	Pha Suk Pass,Thong Pha Phum, Kanchanaburi Province	469	I
32.	Ban Thamadua, Thong Pha Phum, Kanchanaburi Province	149	IX
33.	Sai Yok National Park, Kanchanaburi Province	156	IX
34.	Bang Saphan, Prachuap Khiri Khan Province	622	VIII
35.	Namtok Ngao National Park, Ranong Province	34	IX
36.	Ko Wua Ta Lub, Suratthani Province	587	VIII
37.	Mueang, Phangnga Province	685	VIII
38.	Khao Luang National Park, Nakhon Si Thammarat Province	166	I
39.	Noppharat Thara Beach, Krabi Province	642	VIII
40.	Hat Chao Mai National Park, Trang Province	629	VIII
41.	Thung Kai, Trang Province	66	VIII
42.	Sathing Phra, Songkhla Province	214	VIII
43.	Singhanakhon, Songkhla Province	248	VIII
44.	Pak Bang Sakom Beach, Songkhla Province	618	VIII
45.	Tarutao National Park, Satun Province	320	IV
		202, 573	VIII
46.	Sirinthon Waterfall, Waeng, Narathiwat Province	303, 304, 305	II
47.	Bukit Anak takun, Malaysia	998	X

a =Site numbers correspond to those in Fig.1.
b=Nine forms distinguished by morphological and anatomical characters (chapter 3) and one *H. parasitica* var. *citrina* from Malaysia (i.e. form X)

3. PCR amplification

Ten different regions in cpDNA were amplified using universal primers (Table 5.2, Figure 5.1; Grivet et al. 2001). Amplifications were carried out in 30 µl of reaction mixture containing 200 µM dNTPs (Promega), 2 mM $MgCl_2$, 0.5 µM each primers, 1x Taq DNA polymerase buffer, 1 unit of Taq DNA polymerase (Qiagen) and 20-50 ng of total genomic DNA as template. PCR amplifications were carried out in a PTC-100 thermal cycler (MJ Research, Inc.), with a heated lid, using an initial cycle at 94 °C for 4 min, followed by 30 cycles of 45 sec at 94 °C; 45 sec at 45 °C to 62 °C; 2 min to 5 min at 72 °C (annealing temperature and extension time depending on the length of the fragment to be amplified; Table 5.2), and a final extension for 10 min at 72 °C.

Table 5.2 PCR regions of amplification using 10 cpDNA universal primer pairs (Grivet et al., 2001).

No.	Regions	Primers
1.	**AS**	*psaA*: 5'–ACTTCTGGTTCCGGCGAACGAA-3' *trnS*: 5'–AACCACTCGGCCATCTCTCCTA-3'
2.	**C_1C**	*rpoC1*: 5'-GCACAAATTCCRCTTTTTATRGG-3' *trnC*: 5'-CGACACCCRGATTTGAACTGG-3'
3.	**CD**	*trnC*: 5'-CCAGTTCAAATCTGGGTGTC-3' *trnD*: 5'- GGGATTGTAGTTCAATTGGT-3'
4.	**$f_M A$**	*trnf*$_M$: 5'-GAACCCGTGACCTCAAGGTTATG-3' *psaA*: 5'-ATTCGTTCGCCGGAACCAGAAGT-3'
5.	**FV**	*trnF* 5'-CTCGTGTCACCAGTTCAAAT-3' *trnV* 5'-CCGAGAAGGTCTACGGTTCG-3'
6.	**HK**	*trnH*: 5'-ACGGGAATTGAACCCGCGCA-3' *trnK*: 5'- CCGACTAGTTCCGGGTTCGA-3'
7.	**$K_1 K_2$**	*trnK*: 5'–GGGTTGCCCGGGACTCGAAC-3' *trnK*: 5'–CAACGGTAGAGTACTCGGCTTTTA-3'
8.	**K_2Q**	*trnK*: 5'- TAAAAGCCGAGTACTCTACCGTTG-3' *trnQ*: 5'-CTATTCGGAGGTTCGAATCCTTCC-3'
9.	**TC**	*trnT*: 5'-GCCCTTTTAACTCAGTGGTA-3' *psbC*: 5'-GAGCTTGAGAAGCTTCTGGT-3'
10.	**VL**	*trnV*: 5'- CGAACCGTAGACCTTCTCGG-3' *rbcl*: 5'-GCTTTAGTCTCTGTTTGTGG-3'

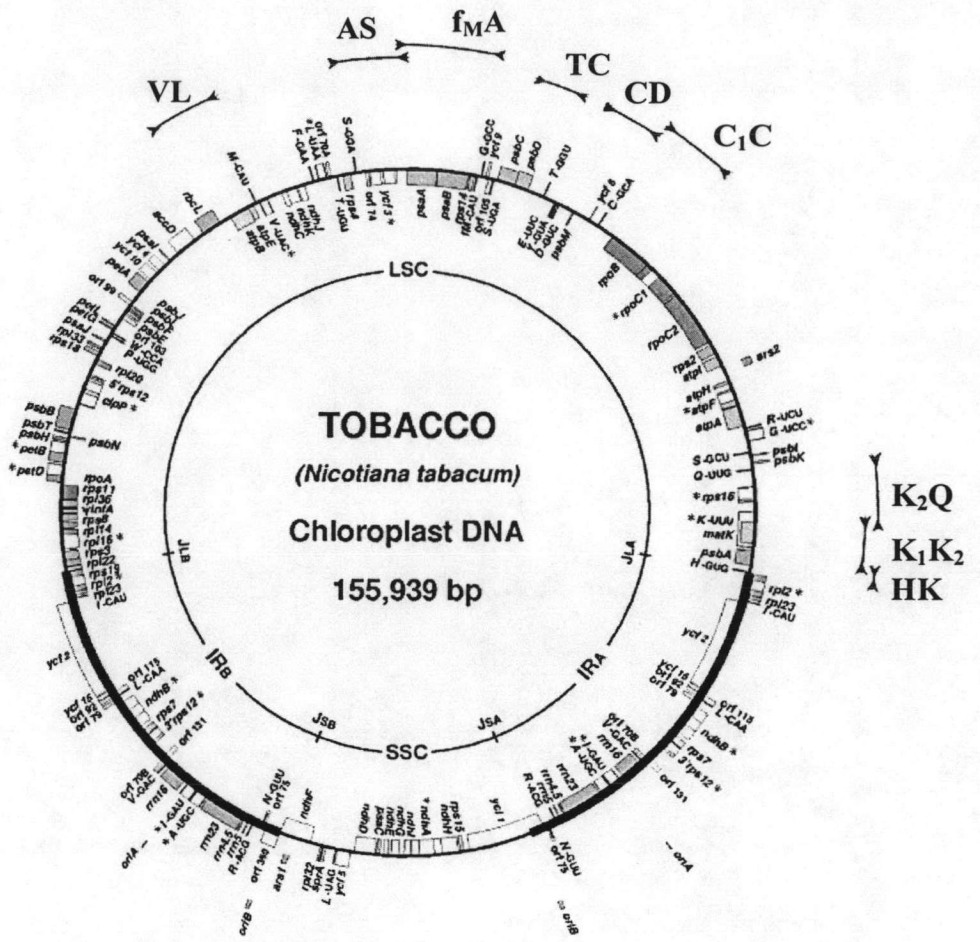

Figure 5.1 Location of the nine regions in this study based on the tobacco cpDNA genome (Grivet et al., 2001).

4. Agarose gel electrophoresis

Agarose gel electrophoresis was performed in order to check the results of DNA extraction and the results of PCR amplification using 0.8% and 1% in TBE buffer (89 mM Tris-HCl, 8.9 mM boric acid and 2.5 mM EDTA pH 8.0), respectively.. Three µl of the loading dye (0.25% bromphenol blue, 40% ficoll 400 and 0.25% xylene cyanol) was mixed with 3 µ1 of each DNA sample, and loaded into each well. Electrophoresis was carried out until bromphenol blue (lowest dye) migrated to approximately 3 cm from the bottom of the gel. The gel was stained with a 2.5 µg/ml ethidium bromide solution for 5 minutes, destained in distilled water for 30 min, and visualized under UV light and photographed. The 1 kb DNA ladder was used as the standard molecular size.

5. Restriction enzyme digestion

Fourteen restriction enzymes (Table 5.3) were used for digesting PCR products. Digestion was carried out in a 15-µl volume containing approximately 50 ng of PCR product. The reaction mixture was incubated at 37 to 65 °C and left overnight with 3-5 units of enzyme in 1X enzyme buffers. The fragments were separated on 1.5-2.5% agarose gel at 50 V for about 5 hours, stained with ethidium bromide, visualized under UV light and photographed. The 1 kb DNA ladder and 100 bp DNA ladder were used as the standard molecular size.

Table 5. 3 List of restriction enzymes used for analyses of cpDNA variation of *Hoya parasitica* complex in Thailand.

No.	Restriction enzymes	Recognition site	Incubated Temp. °C
1.	*Alu* I	AG/CT	37
2.	*Ase* I	AT/TAAT	37
3.	*Bam*H I	G/GATCC	37
4.	*Dpn* I	GA/TC	37
5.	*Eco*R I	G/AATTC	37
6.	*Eco*R V	GAT/ATC	37
7.	*Hae* III	GG/CC	37
8.	*Hin*d III	A/AGCTT	37
9.	*Hinf* I	G/ANTC	37
10.	*Msp* I	C/CGG	37
11.	*Nde* I	CA/TATG	37
12.	*Rsa* I	GT/AC	37
13.	*Taq* I	T/CGA	65
14.	*Xba* I	T/CTAGA	37

6. Data analysis

The PCR-RFLP bands were analyzed to estimate the genetic variations/relationships among the populations and forms studied. The PCR-RFLP bands were interpreted as dominant markers and were scored as characters either as 1 (present) or 0 (absent). A pair-wise similarity matrix was calculated using Dice's coefficient (Dice, 1945). The similarity matrix was employed to construct a dendrogram by the unweighted pair group method with arithmetical averages (UPGMA), using the SAHN-clustering and TREE programs from the NTSYS-pc, version 2.10m (Rohlf, 2000).

Results

1. Total cpDNA extraction

Total cpDNA was extracted from a fresh leaf of each specimen. The fragment of total DNA is larger than 10 kb in length, slightly sheared DNA were observed (Figure 5.2).

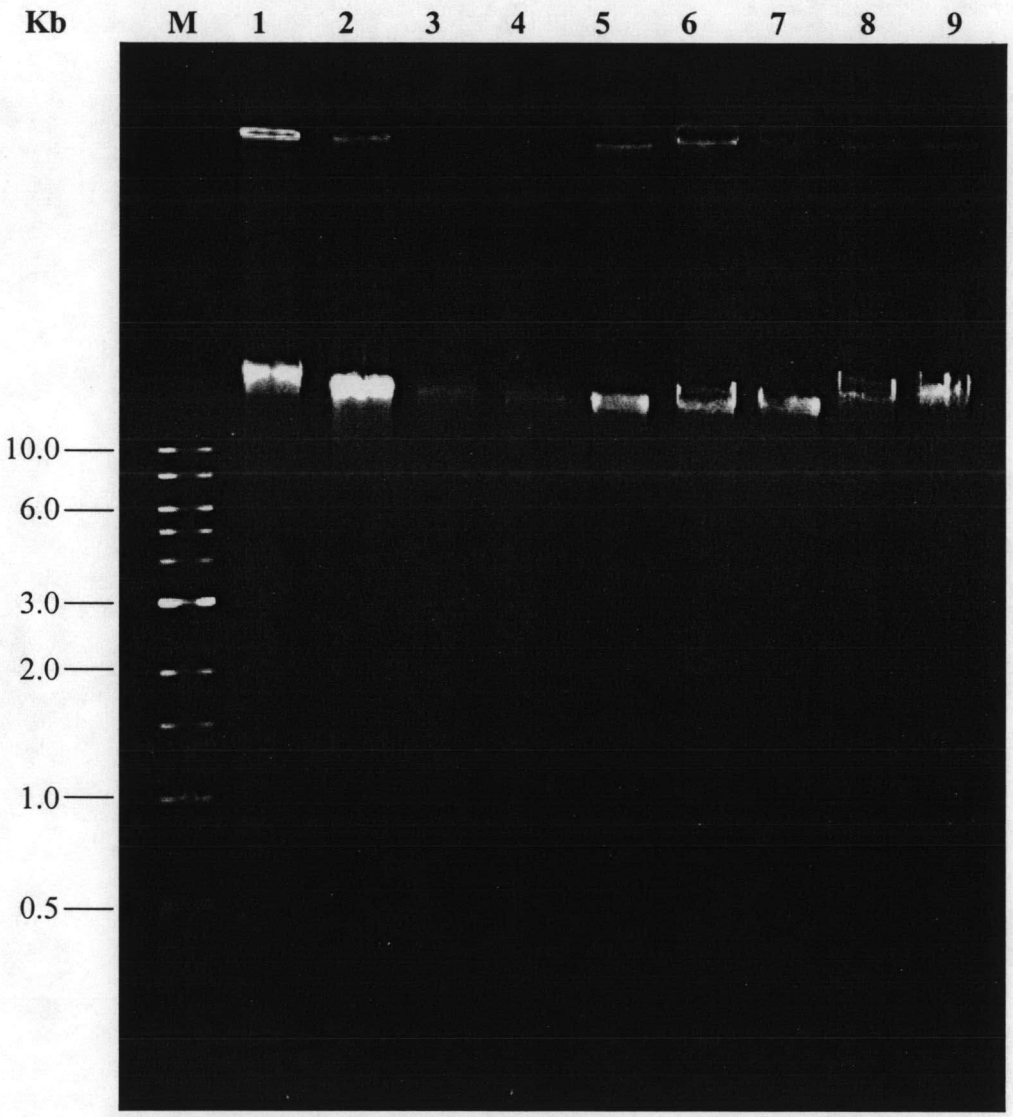

Figure 5.2 Sample gel showing fragment size of total DNA extracted from fresh leaves of *Hoya parasitica* complex. Lane M: 1 kb ladder DNA marker, Lane 1-9: Total cpDNA from nine *H. parasitica* individuals.

2. PCR amplification

PCR technique was used to amplify the selected regions of cpDNA. The chloroplast DNA analysis in this study was based on the ten intergenic-spacers (HK, $K_1 K_2$, K_2Q, C_1C, CD, TC, $f_M A$, AS, FV and VL).

The approximated size of PCR products of these regions are shown in Table 5.4, and Figures 5.3-5.11. No difference in length of PCR products was detected in most regions, except only in CD region. PCR product from specimen number 997 was larger than the others. It was 3420 bp while the others are 2860 bp. The proportion of cpDNA surveyed was about 30 kb, or 20% of cpDNA genome (assuming to be ~150 kb).

Table 5.4 PCR conditions, size of the amplified fragments, and degree of amplification using 10 cpDNA universal primer pairs in *Hoya parasitica* complex in Thailand.

Abberv. of cpDNA primer[a]	PCR conditions		Size (bp)	Degree of amplification[b]
	Annealing Temperature °C)	Extension time (min)		
AS	55	4.5	3215	+
C_1C	47.5	5	4490	+
CD	55	4.5	2860, 3420	+
$f_M A$	47.5	5	5440	+
FV	50	4. 5	NA	NA
HK	62	2.5	1695	++
$K_1 K_2$	53.5	3	2480	++
K_2Q	45	3	3240	+
TC	50	4	3030	++
VL	55	4.3	3730	++

[a] Abbreviations are in Grivet et al. (2001)
[b] NA, no amplification; + faint amplification ; ++, good amplification

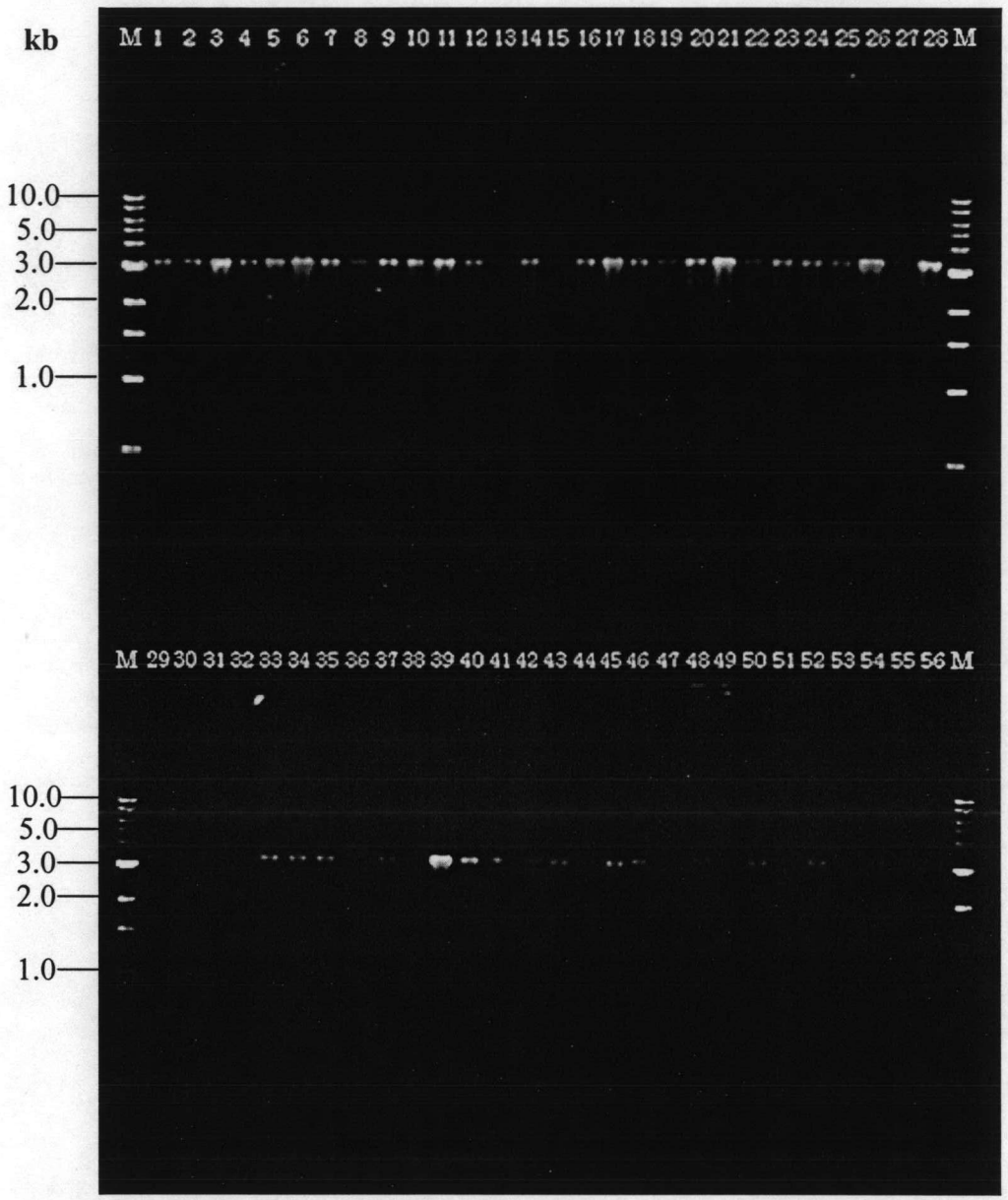

Figure 5.3 PCR amplified of AS region of cpDNA of "*Hoya parasitica* complex". Lane M: 1 kb ladder, Lane 1-56: PCR amplified products from 56 *H. parasitica* complex individuals.

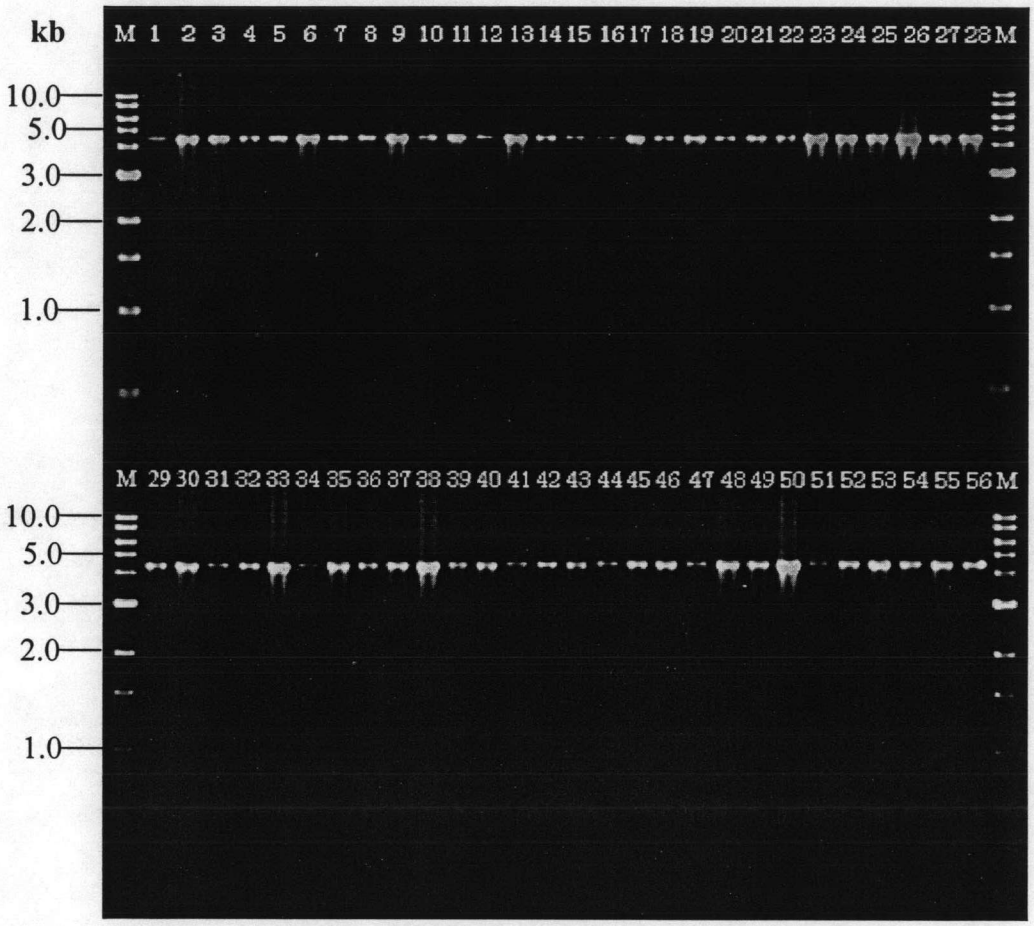

Figure 5.4 PCR amplified of C_1C region of cpDNA of "*Hoya parasitica* complex". Lane M: 1 kb ladder, Lane 1-56: PCR amplified products from 56 *H. parasitica* complex individuals.

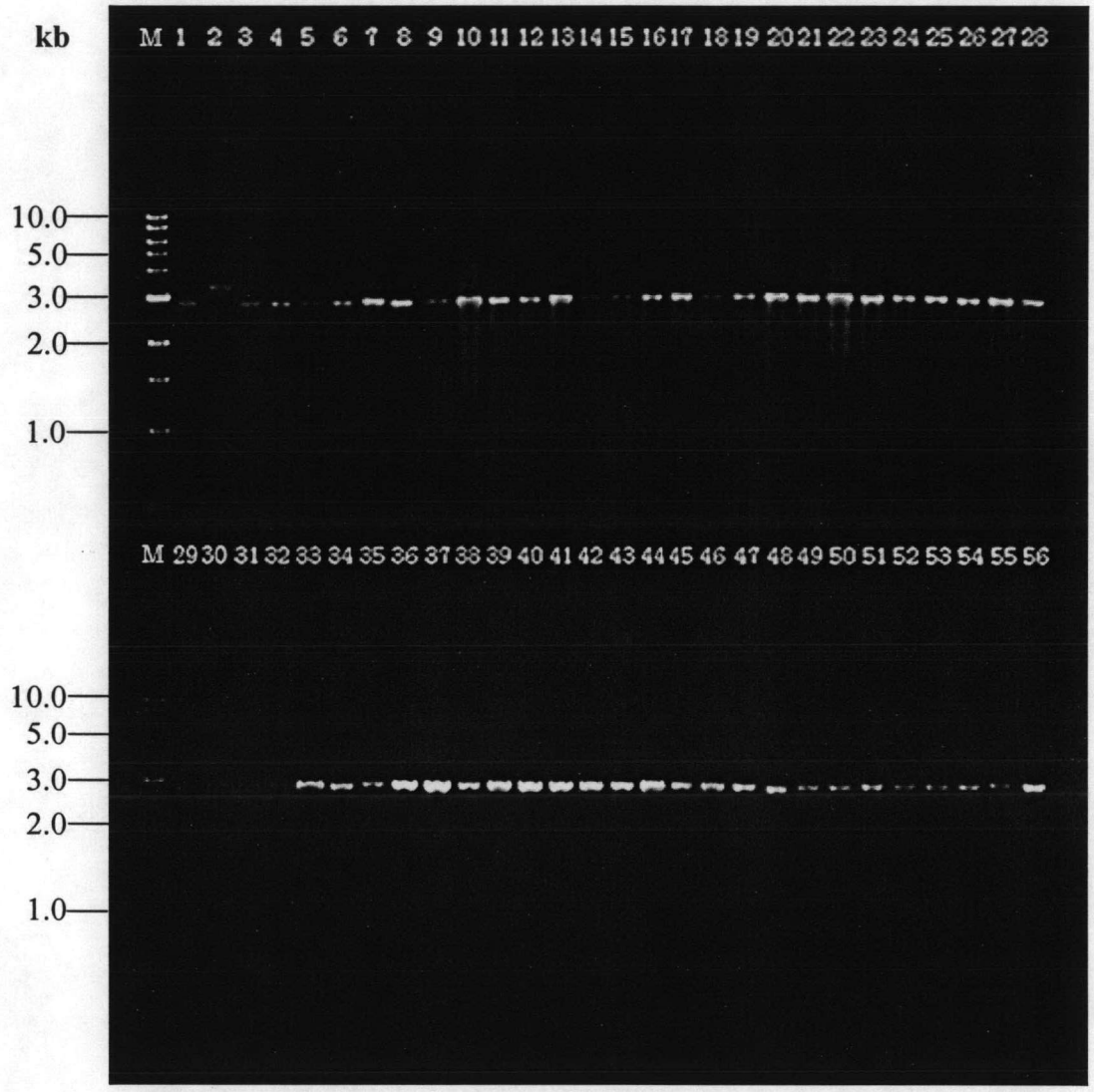

Figure 5.5 PCR amplified of CD region of cpDNA of "*Hoya parasitica* complex". Lane M: 1 kb ladder, Lane 1-56: PCR amplified products from 56 *H. parasitica* complex individuals.

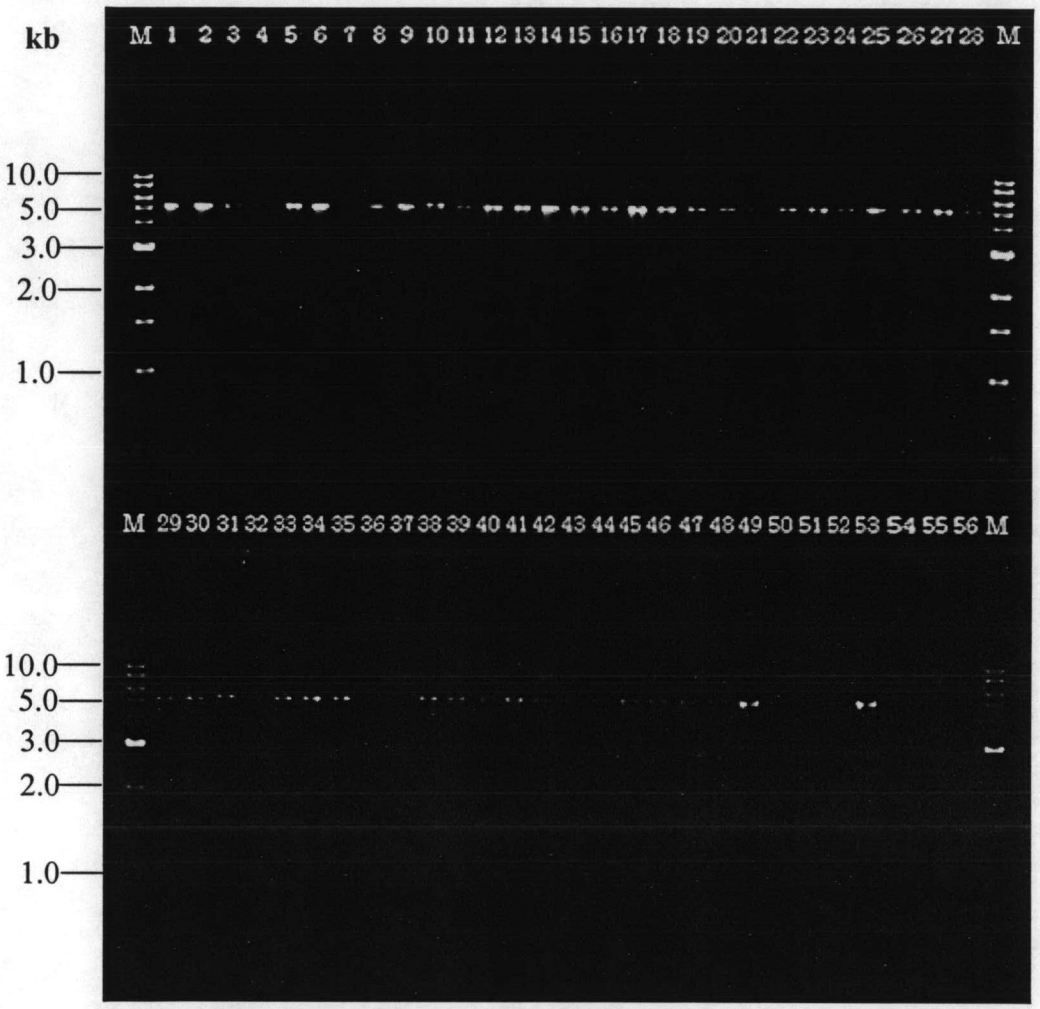

Figure 5.6 PCR amplified of f_MA region of cpDNA of "*Hoya parasitica* complex". Lane M: 1 kb ladder, Lane 1-56: PCR amplified products from 56 *H. parasitica* complex individuals.

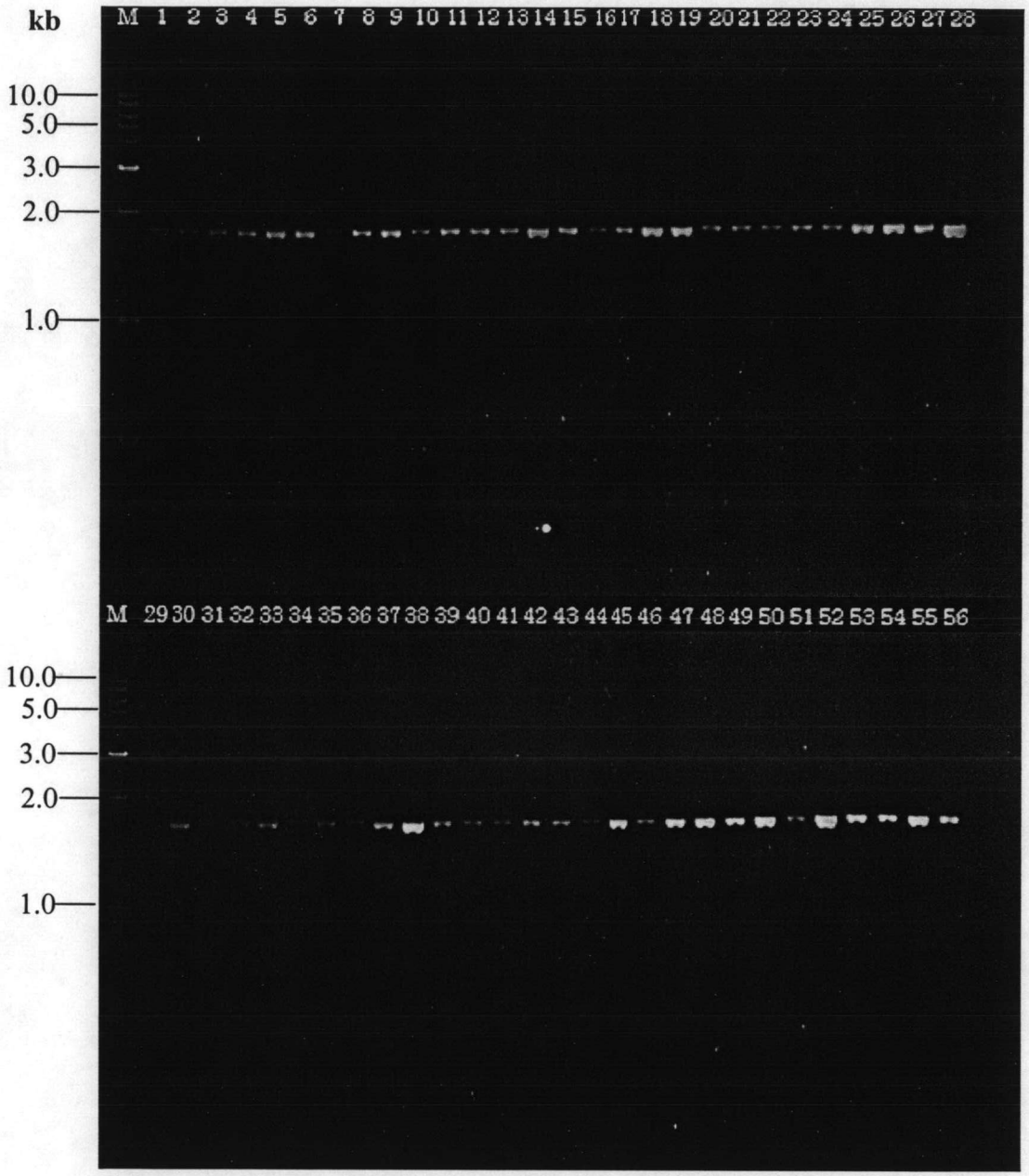

Figure 5.7 PCR amplified of HK region of cpDNA of "*Hoya parasitica* complex". Lane M: 1 kb ladder, Lane 1-56: PCR amplified products from 56 *H. parasitica* complex individuals.

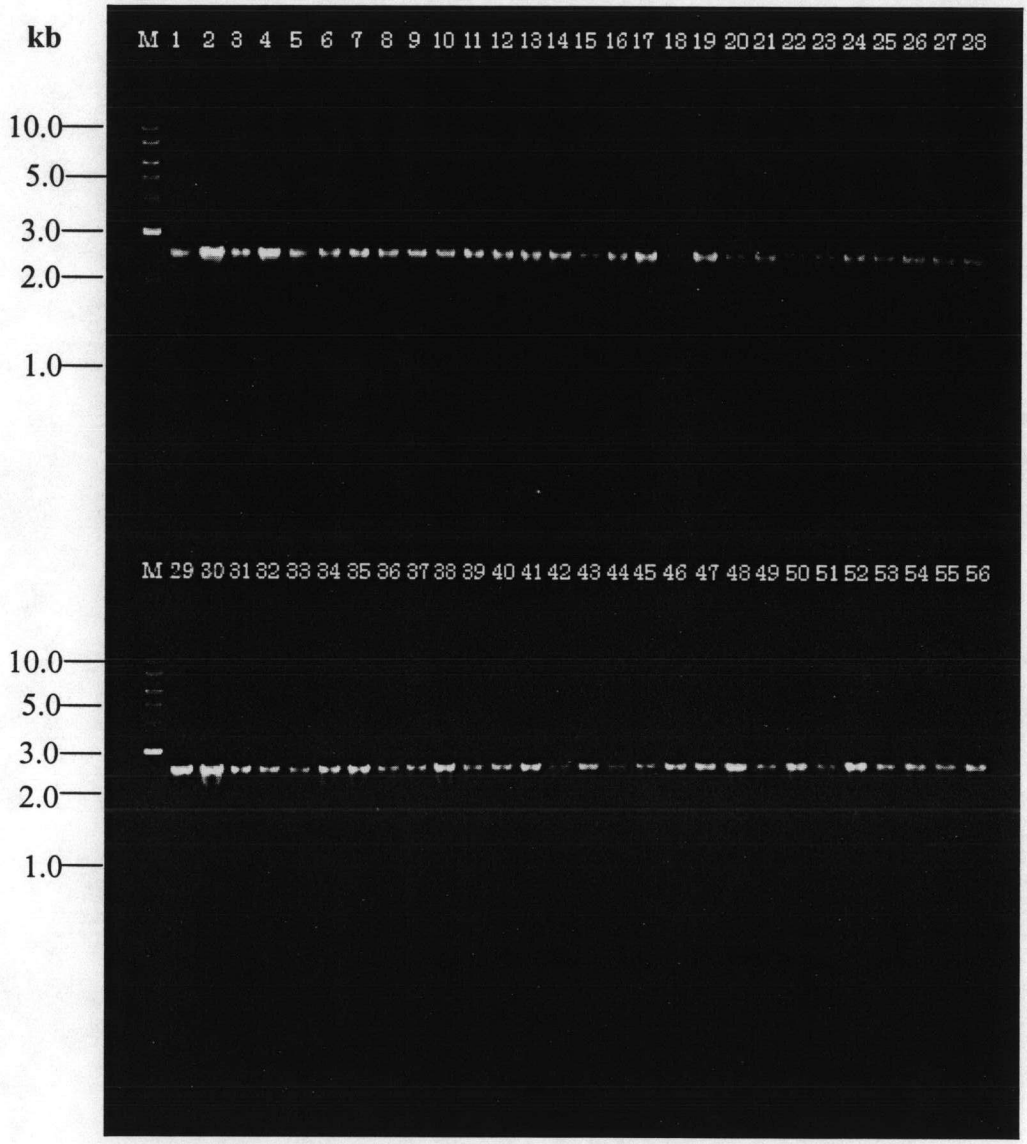

Figure 5.8 PCR amplified of K_1K_2 region of cpDNA of "*Hoya parasitica* complex". Lane M: 1 kb ladder, Lane 1-56: PCR amplified products from 56 *H. parasitica* complex individuals.

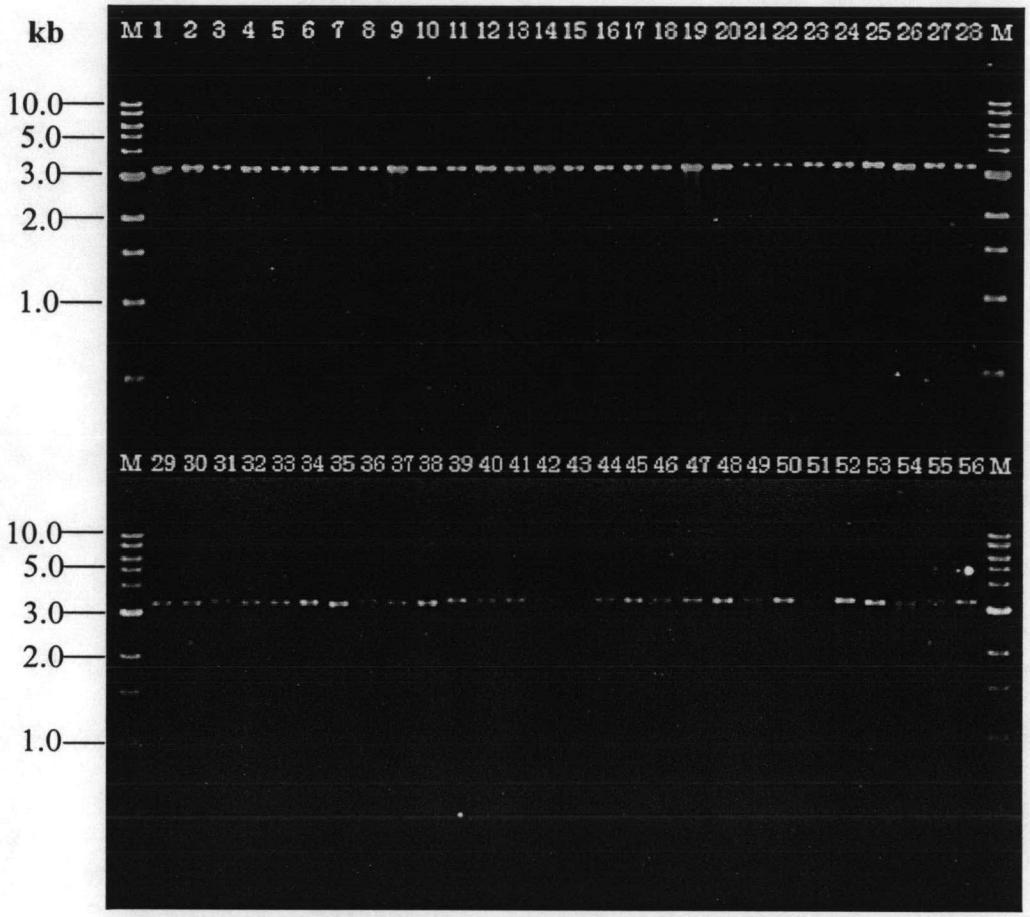

Figure 5.9 PCR amplified of K_2Q region of cpDNA of "*Hoya parasitica* complex". Lane M: 1 kb ladder, Lane 1-56: PCR amplified products from 56 *H. parasitica* complex individuals.

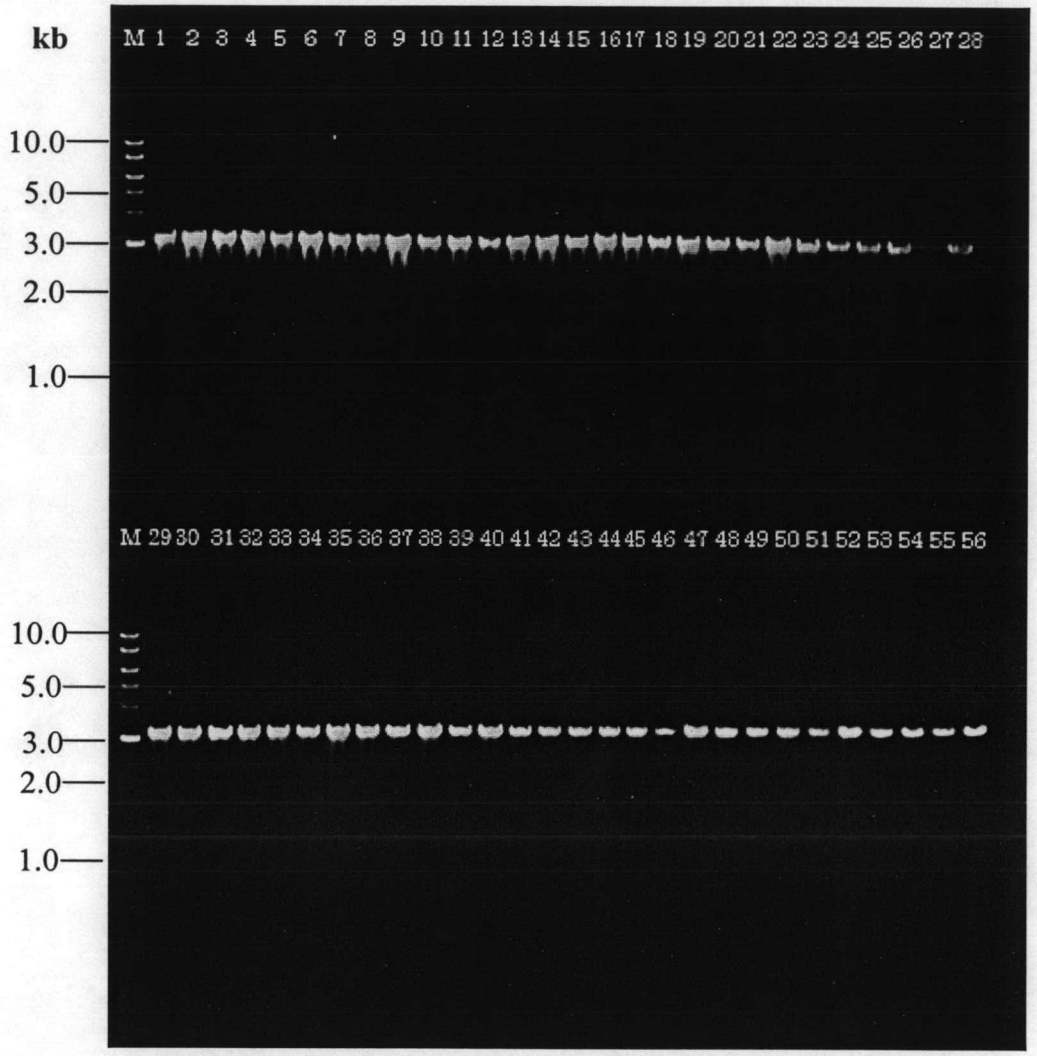

Figure 5.10 PCR amplified of TC region of cpDNA of "*Hoya parasitica* complex". Lane M: 1 kb ladder, Lane 1-56: PCR amplified products from 56 *H. parasitica* complex individuals.

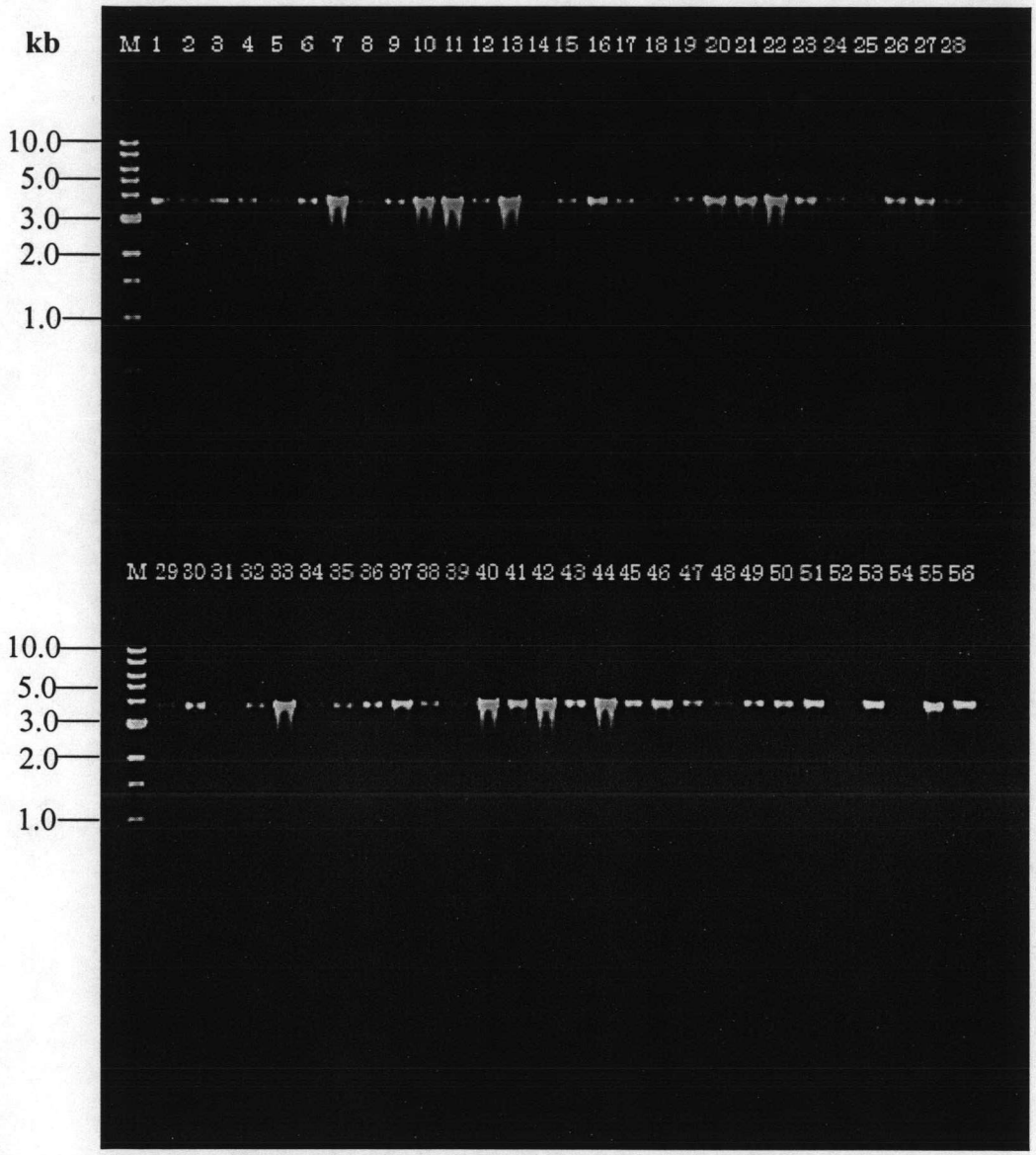

Figure 5.11 PCR amplified of VL region of cpDNA of "*Hoya parasitica* complex". Lane M: 1 kb ladder, Lane 1-56: PCR amplified products from 56 *H. parasitica* complex individuals.

3. PCR-RFLP analysis

Each of the 9 amplified fragments (HK, K_1 K_2, K_2Q, C_1C, CD, TC, f_M A, AS and VL) was digested by fourteen restriction enzymes (*Alu I, Ase* I, *Bam*H I, *Dpn* I, *Eco*R I, *Eco*R V, *Hae* III, *Hind* III, *Hinf* I, *Msp* I, *Nde* I, *Rsa* I, *Taq* I, *Xba* I), generating 126 primer-enzyme combinations (Table 5.5).

Table 5.5 The results of digestions of 9 amplified cpDNA regions with 14 restriction enzymes.

Enzymes	Amplified cpDNA [1, 2]								
	K_2Q	C_1C	AS	f_MA,	HK	K_1K_2	TC	VL	CD
*Alu*I	5	9	6	9	6	4	6	9	4
*Ase*I	4	3	2	3	3	**4**	4	2	**3**
*Bam*HI	4	3	-	4	-	-	-	-	-
*Dpn*I	-	-	2	-	-	-	-	-	2
*Eco*RI	2	4	3	-	-	**2**	-	2	4
*Eco*RV	-	3	-	-	2	2	-	-	-
*Hae*III	3	5	4	11	3	4	6	7	-
*Hind*III	-	2	5	-	-	-	-	-	-
*Hin*fI	**8**	13	8	8	4	8	8	6	-
*Msp*I	2	**8**	3	10	-	5	8	6	5
*Nde*I	-	2	4	4	-	-	2	-	-
*Rsa*I	4	5	6	11	3	4	5	**10**	4
*Taq*I	**7**	7	10	7	-	5	**8**	**10**	5
*Xba*I	-	4	-	-	3	-	-	-	6

[1] -, no recognition site in these amplified DNA/ enzyme combinations
[2] The number show number of the recognition sites and the **bold** letter denote that these are polymorphic patterns in these amplified DNA/ enzyme combinations

Nine combinations (C_1C- *Msp* I, CD- *Ase* I, K_1K_2- *Ase* I, K_1K_2- *Eco*R I, K_2Q- *Hinf* I, K_2Q- *Taq* I, TC- *Taq* I, VL- *Rsa* I, VL- *Taq* I) exhibited polymorphic patterns (Table 5.6 and Figures 5.12-5.20). The fragment amplified by the primer pairs HK, f_MA, and AS showed the monomorphic patterns with the fourteen restriction enzymes.

Table 5.6 Numbers and appoximated size of restriction fragments and number of polymorphic patterns recognized by PCR-RFLP analysis.

Region	Restriction enzymes	No. of patterns	No. of bands	Approximated fragment size (bp)	Specimens
C_1C	*Msp* I	2	8	1100, 800, 730, 600, 490, 300, 280, 190	548
			9	1100, 800, 730, 600, 350, 300, 280, 190, 140	all specimens, except 548
CD	*Ase* I	3	3	1800, 880, 180	469, 407, 289, 297, 281, 299, 276, 380, 326, 343, 388, 378, 379, 392, 0, 559, 420, 315, 457, 444, 149, 303, 304, 305
			4	1800, 720, 180,160	166, 504, 429, 484, 480, 526, 508, 548, 375, 307,3, 82, 120, 660, 570, 475, 156, 622, 34, 587, 685, 642, 629, 66, 248, 214, 618, 202, 573, 320, 998
			4	1800, 880, 560, 180	997
K_1K_2	*Ase* I	2	4	900, 720, 700, 160	997
			5	800, 720, 700, 160, 100	all specimens, except 997
	*Eco*R I	2	2	2050, 430	469, 997, 166, 407, 289, 297, 281, 299, 276, 380, 326, 343, 388, 378, 379, 392, 0, 559, 420, 504, 429, 484, 480, 508, 548, 315, 660, 457, 444, 149, 66, 248, 214, 618, 202, 573, 303, 304, 305, 998
			3	1630, 430, 420	526, 375, 307, 3, 82, 120, 570, 475, 156, 622, 34, 587, 685, 642, 629, 320
K_2Q	*Hinf* I	2	9	520, 410, 350, 290, 280, 230, 170, 140, 110, ? (none visually detected band)	469, 997, 166, 407, 289, 297, 281, 299, 276, 380, 326, 343, 388, 378, 379, 392, 0, 559, 420, 504, 429, 484, 480, 508, 548, 315, 660, 457, 444, 149, 66, 248, 214, 618, 202, 573, 303, 304, 305, 998
			8	520, 510, 410, 350, 290, 170, 140, 110, ? (none visually detected band)	526, 375, 307, 3, 82, 120, 570, 475, 156, 622, 34, 587, 685, 642, 629, 320

Table 5.6 (Continued)

Region	Restriction enzymes	No. of patterns	No. of bands	Approximated fragment size (bp)	Specimens
K_2Q	*Taq* I	2	7	900, 450, 420, 330, 280, 210, 130, ? (none visually detected band)	469, 407, 289, 297, 281, 299, 276, 380, 326, 343, 388, 378, 379, 392, 0, 559, 420, 504, 548, 457, 444, 149, 303, 304, 305
			6	900, 450, 420, 330, 210, 130, ? (none visually detected band)	997, 166, 429, 484, 480, 526, 508, 375, 307, 3, 315, 82, 120, 660, 570, 475, 156, 622, 34, 587, 685, 642, 629, 66, 248, 214, 618, 202, 573, 320, 998
TC	*Taq* I	2	7	1280, 480, 420, 360, 200, 160, 140	166
			8	900, 480, 420, 380, 360, 200, 160, 140	all specimens, except 166
VL	*Rsa* I	3	11	800, 450, 400, 330, 315, 300, 210, 190, 160, 110, 90, ? (none visually detected band)	997, 484, 307, 120, 570, 475, 156, 622, 34, 587, 320
			11	800, 450, 400, 330, 325, 300, 210, 190, 160, 110, 90, ? (none visually detected band)	469, 166, 407, 289, 297, 281, 299, 276, 380, 326, 343, 388, 378, 379, 392, 0, 559, 420, 504, 429, 526, 508, 548, 375, 3, 315, 82, 660, 457, 444, 149, 685, 642, 629, 66, 248, 214, 618, 202, 573, 303, 304, 305, 998
			10	800, 450, 400, 330, 325, 300, 210, 190, 110, 90, ? (none visually detected band)	480
	Taq I	3	11	620, 540, 420, 390, 340, 320, 270, 210, 185, 120, 90, ? (none visually detected band)	997, 484, 307, 120, 570, 475, 156, 622, 34, 587, 320
			11	620, 540, 420, 390, 340, 320, 270, 200, 195, 120, 90, ? (none visually detected band)	469, 166, 407, 289, 297, 281, 299, 276, 380, 326, 343, 388, 378, 379, 392, 0, 559, 420, 504, 429, 480, 526, 508, 548, 375, 3, 315, 82, 660, 457, 444, 149, 685, 642, 629, 66, 248, 214, 618, 202, 573, 303, 304, 305, 998
			10	930, 620, 420, 340, 320, 270, 200, 195, 120, 90, ? (none visually detected band)	420

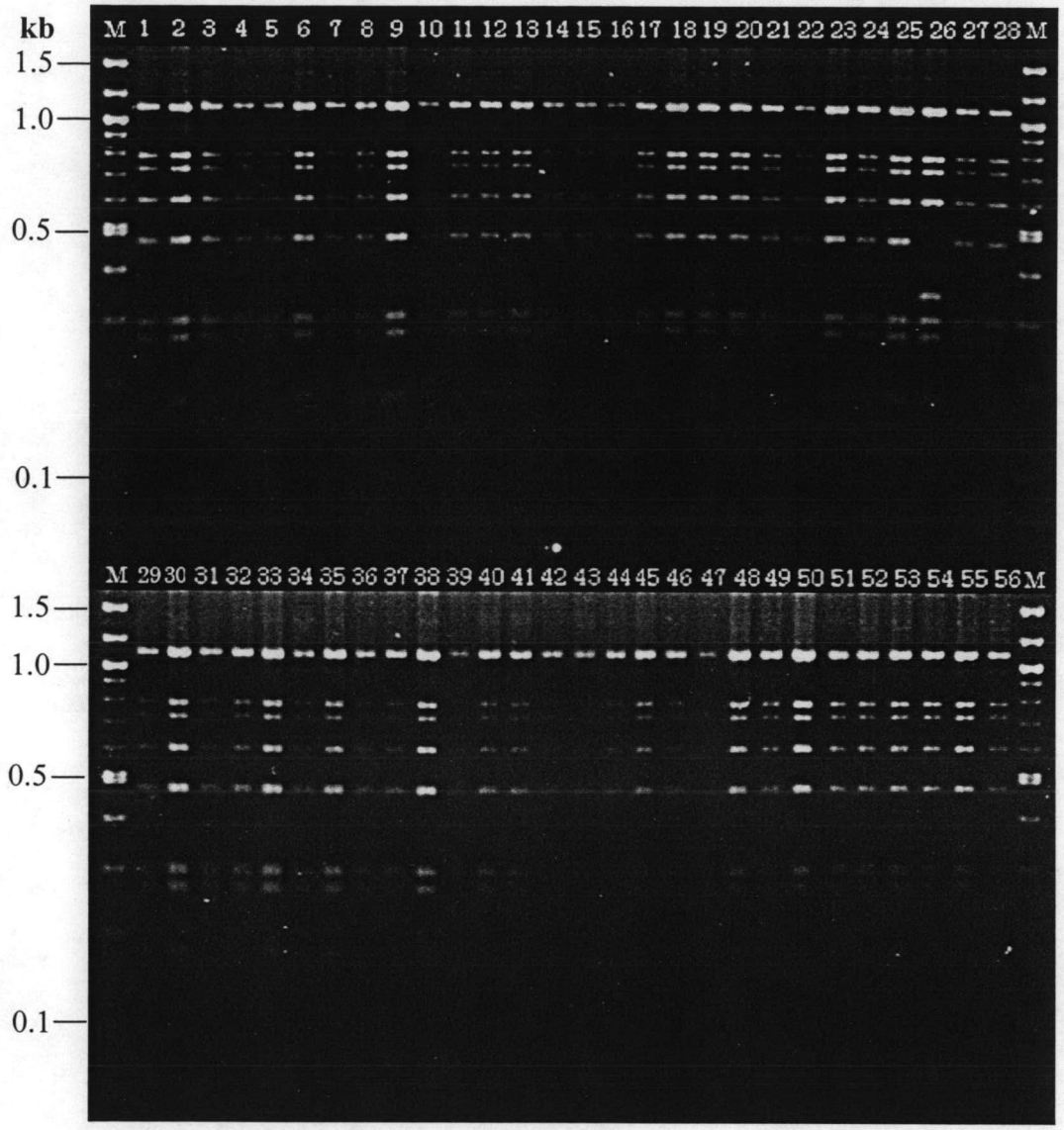

Figure 5.12 PCR-RFLP in C_1C region of cpDNA of "*Hoya parasitica* complex" digested with *Msp* I. Lane M: 100 bp DNA ladder, Lane 1-56: Samples of *H. parasitica*.

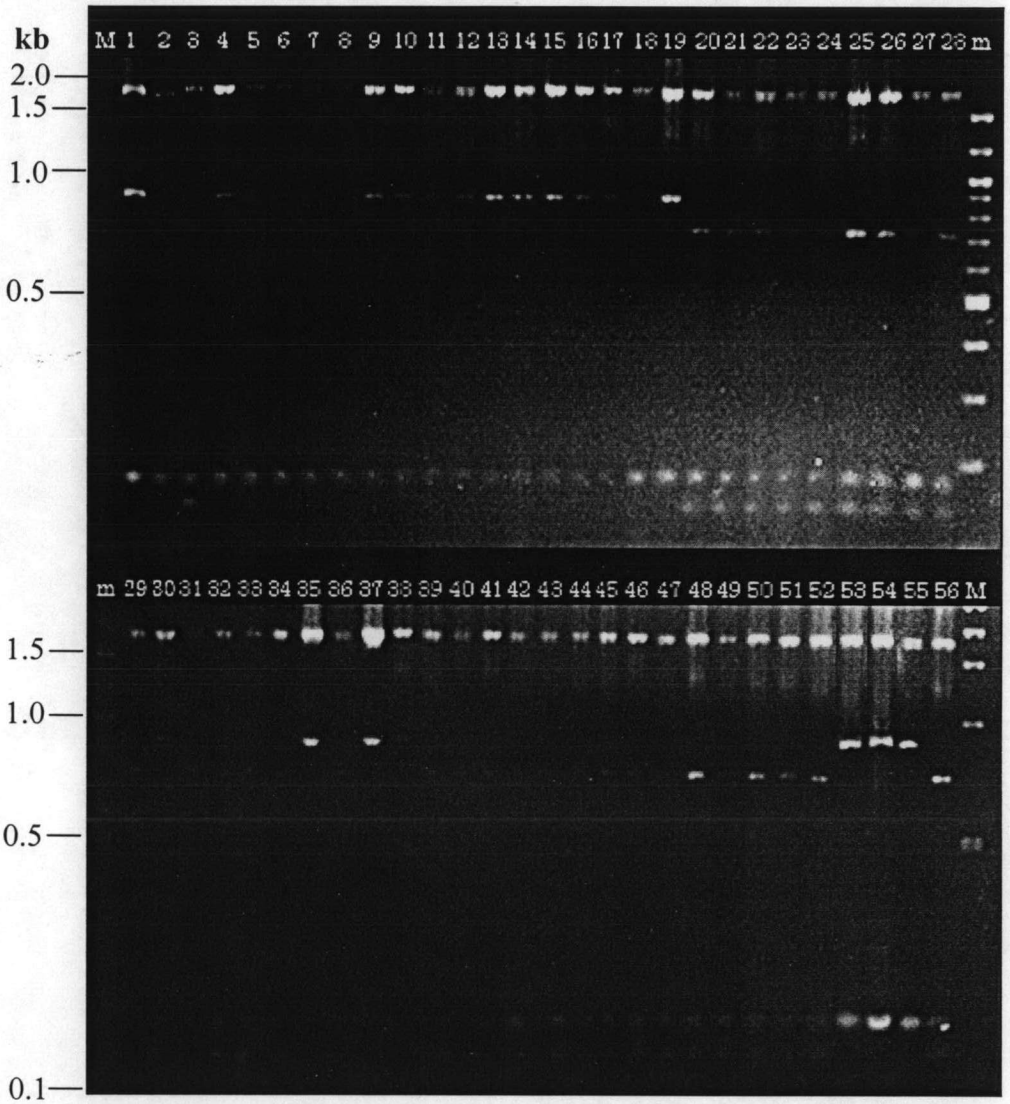

Figure 5.13 PCR-RFLP in CD region of cpDNA of "*Hoya parasitica* complex" digested with *Ase* I. Lane M: 1kb DNA ladder, Lane m: 100 bp DNA ladder, Lane 1-56: Samples of *H. parasitica*.

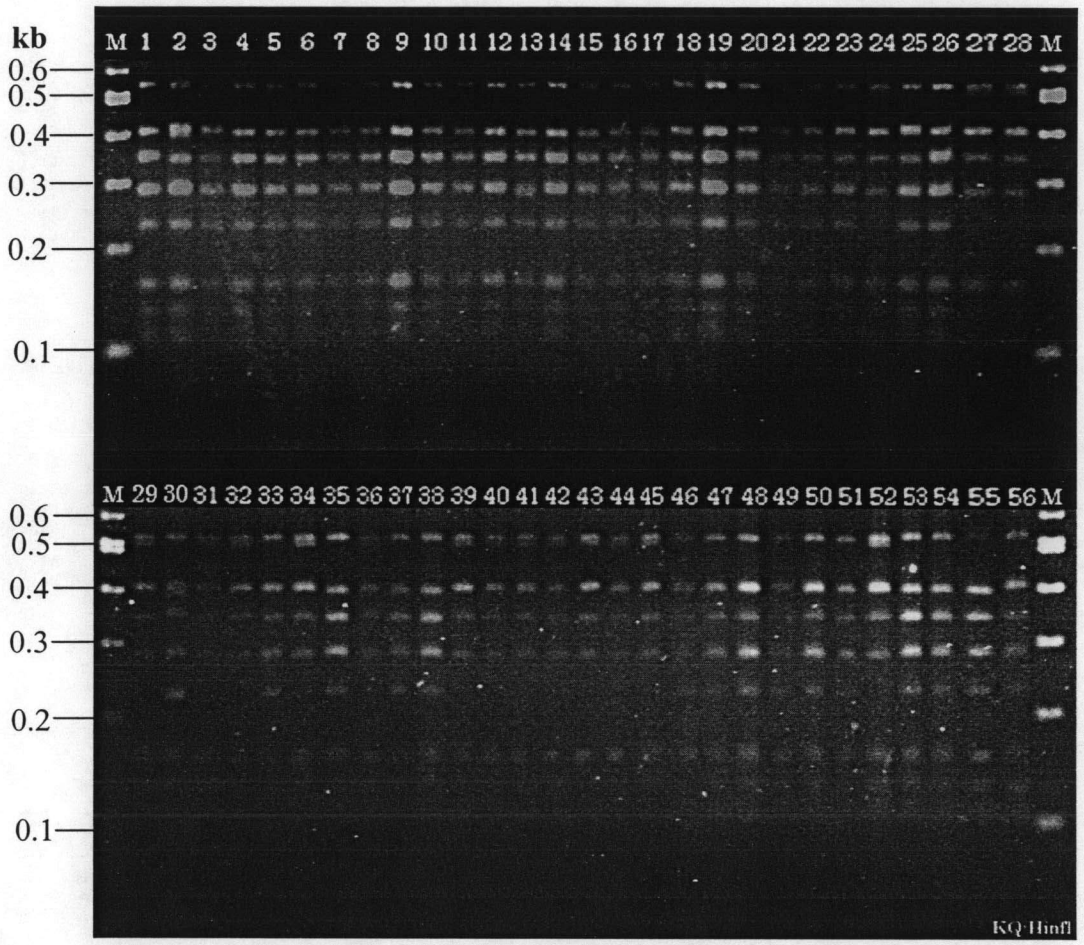

Figure 5.14 PCR-RFLP in K_2Q region of cpDNA of "*Hoya parasitica* complex" digested with *Hin*f I. Lane M: 100 bp DNA ladder, Lane 1-56: Samples of *H. parasitica*.

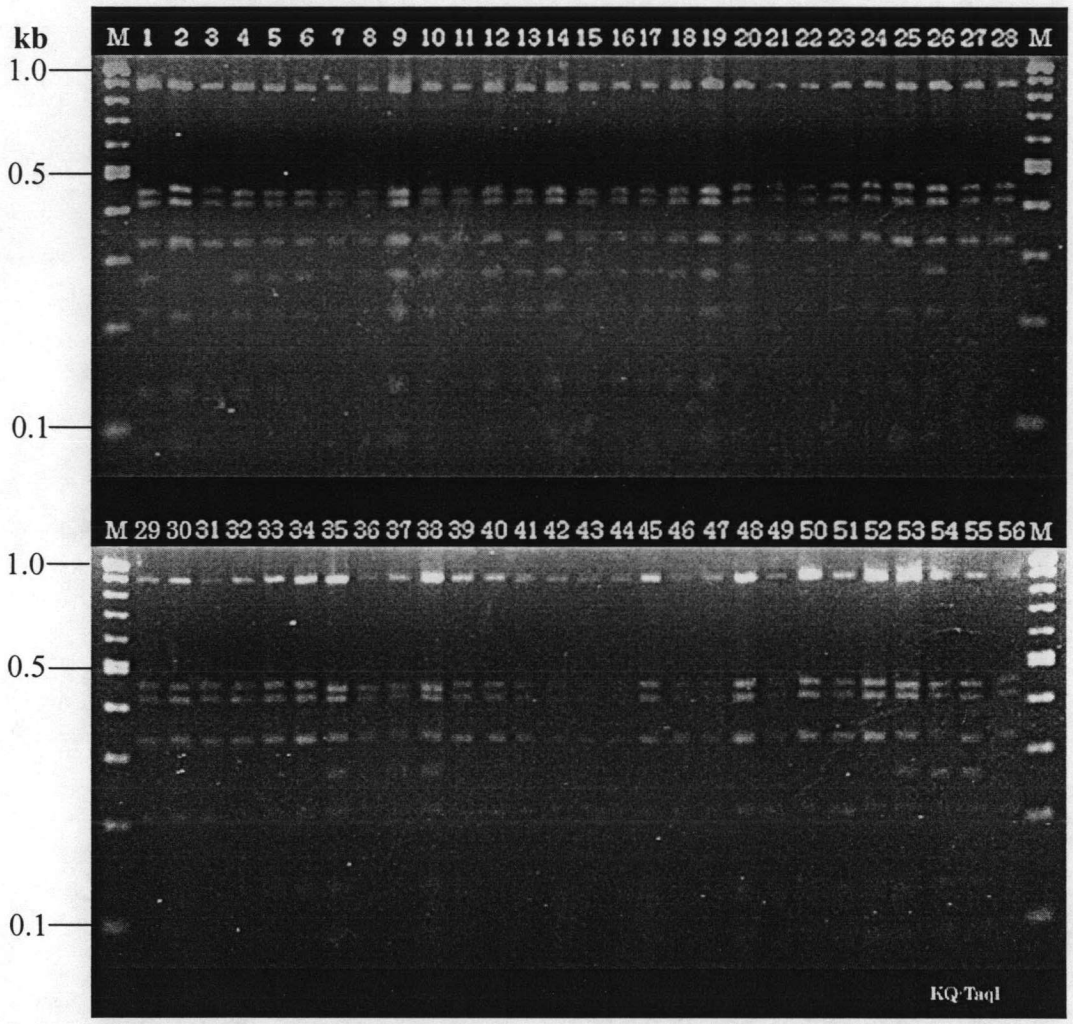

Figure 5.15 PCR-RFLP in K_2Q region of cpDNA of "*Hoya parasitica* complex" digested with *Taq* I. Lane M: 100 bp DNA ladder, Lane 1-56: Samples of *H. parasitica*.

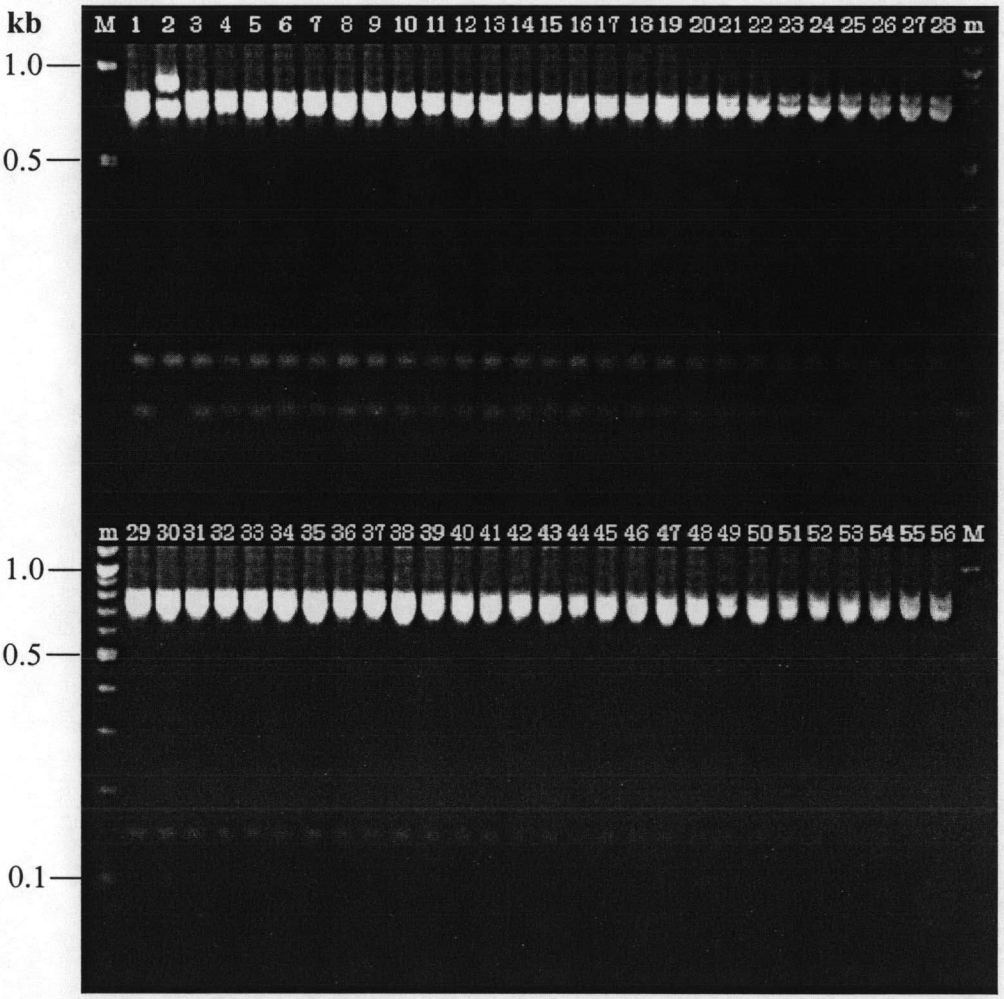

Figure 5.16 PCR-RFLP in KK region of cpDNA of "*Hoya parasitica* complex" digested with *Ase* I. Lane M: 1kb DNA ladder, Lane m: 100 bp DNA ladder, Lane 1-56: Samples of *H. parasitica*.

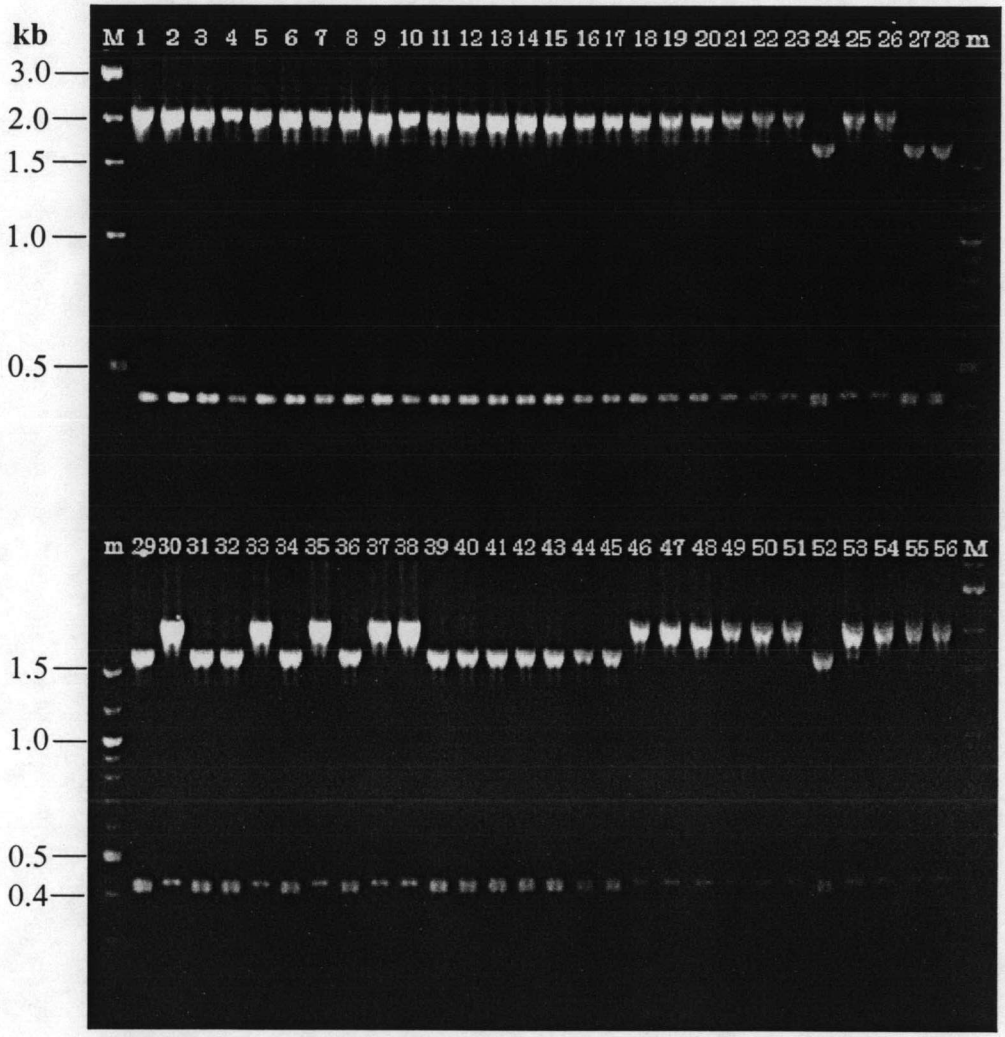

Figure 5.17 PCR-RFLP in KK region of cpDNA of "*Hoya parasitica* complex" digested with *Eco*RI. Lane M: 1kb DNA ladder, Lane m: 100 bp DNA ladder, Lane 1-56: Samples of *H. parasitica*.

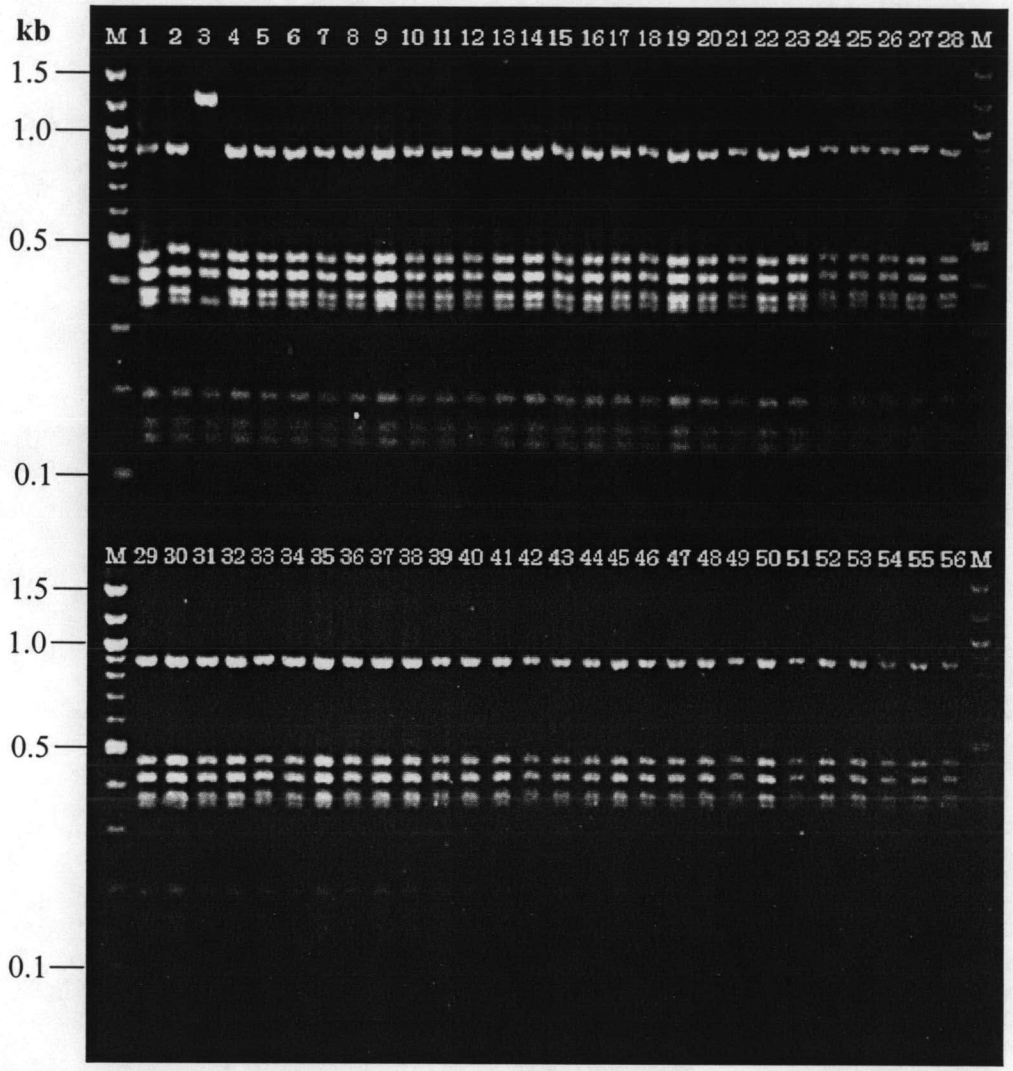

Figure 5.18 PCR-RFLP in TC region of cpDNA of "*Hoya parasitica* complex" digested with *Taq* I. Lane M: 100 bp DNA ladder, Lane 1-56: Samples of *H. parasitica*.

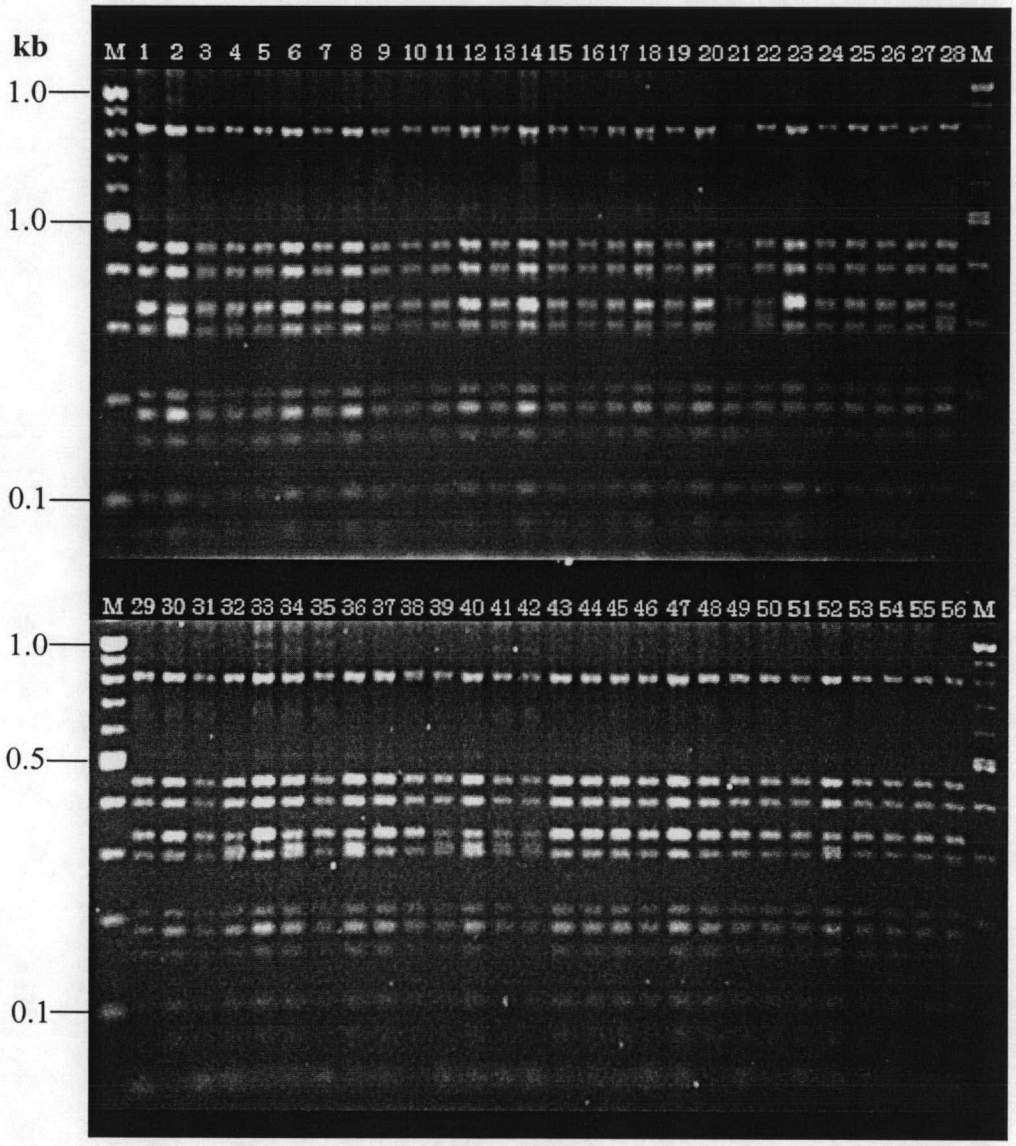

Figure 5.19 PCR-RFLP in VL region of cpDNA of "*Hoya parasitica* complex" digested with *Rsa* I. Lane M: 100 bp DNA ladder, Lane 1-56: Samples of *H. parasitica*.

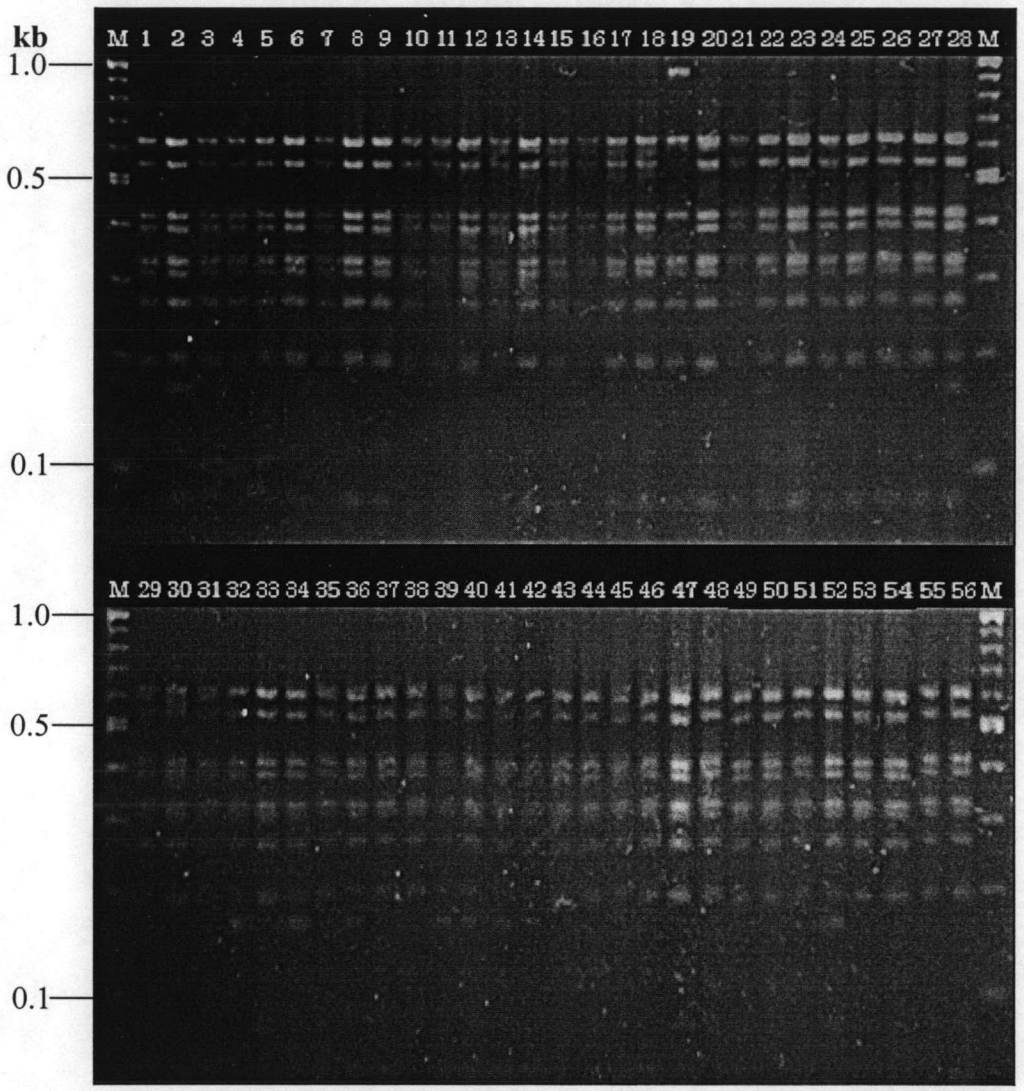

Figure 5.20 PCR-RFLP in VL region of cpDNA of "*Hoya parasitica* complex" digested with *Taq* I. Lane M: 100 bp DNA ladder, Lane 1-56: Samples of *H. parasitica*.

Twelve haplotypes were found among 10 taxa (9 forms of *H. parasitica* complex in Thailand; i.e. I, II, III, IV, V, VI, VII, VIII, IX and an individual of *H. parasiticxa* var. *citrina* (Ridl.) Rintz from Malaysia, X) collected from 46 wild populations consisting of the total 56 individuals (Table 5.7).

Table 5.7 Haplotype frequencies and composition of "*H. parasitica* complex" in Thailand.

Haplotypes	**Form**										**form/**
	I	II	IIII	IV	V	VI	VII	VIII	IX	X	**haplotype**
H1	1	3	1	-	4	1	3	-	9	-	7
H2	-	-	-	-	-	-	-	-	1	-	1
H3	-	-	-	-	-	-	-	-	1	-	1
H4	-	-	-	-	-	-	-	-	1	-	1
H5	-	-	-	-	-	-	-	-	1	-	1
H6	1	-	-	-	-	-	-	-	-	-	1
H7	-	-	-	-	-	-	-	7	2	1	3
H8	-	-	-	-	-	-	-	-	1	-	1
H9	-	-	-	-	-	-	-	-	1	-	1
H10	-	-	-	1	-	-	-	3	5	-	3
H11	-	-	-	-	-	-	-	4	3	-	2
H12	1	-	-	-	-	-	-	-	-	-	1
haplotype / form	3	1	1	1	1	1	1	3	10	1	

PCR-RFLP patterns found in form II, III, V, VI, and VII were monomorphic, which was the haplotype H1, while those found in form IV and X (var. *citrina* from Malaysia) were the haplotype H10 and H7, respectively. Three PCR-RFLP patterns were found in form I (*H. rigida* Kerr): haplotype H1 in the south west, haplotype H12 in the eastern and haplotype H6 in southern Thailand. The latter two were unique to form I. As many as ten patterns were found in form IX:-H1, H2, H3, H4, H5, H7, H8, H9, H10, and H11, of which six, namely H2, H3, H4, H5, H8, and H9, were unique to form IX (Table 5.6). Form VIII contained 3 haplotypes (H7, H10, and H11) and none was unique.

Of 12 haplotypes, four were considered major haplotypes, namely H1, H7, H10, and H11 while the remainders were restricted to only one individual or form. The former occurred in a wide geographical range, while the latter were confined to one area (Figure 5.21). The haplotype H1 is the most common haplotype, presented in 7 forms of "*H. parasitica* complex" in Thailand. It is mainly found in northern

Thailand and rarely occur in the northeast, southwest, and south of Thailand. The haplotype H7 mainly occurs in southernmost of the peninsular Thailand and rarely found in eastern and northeastern Thailand. The haplotype H10 is widely distributed in Southwestern, Eastern, and Southern Thailand, while the haplotype H11 is found in the East, and South of Thailand.

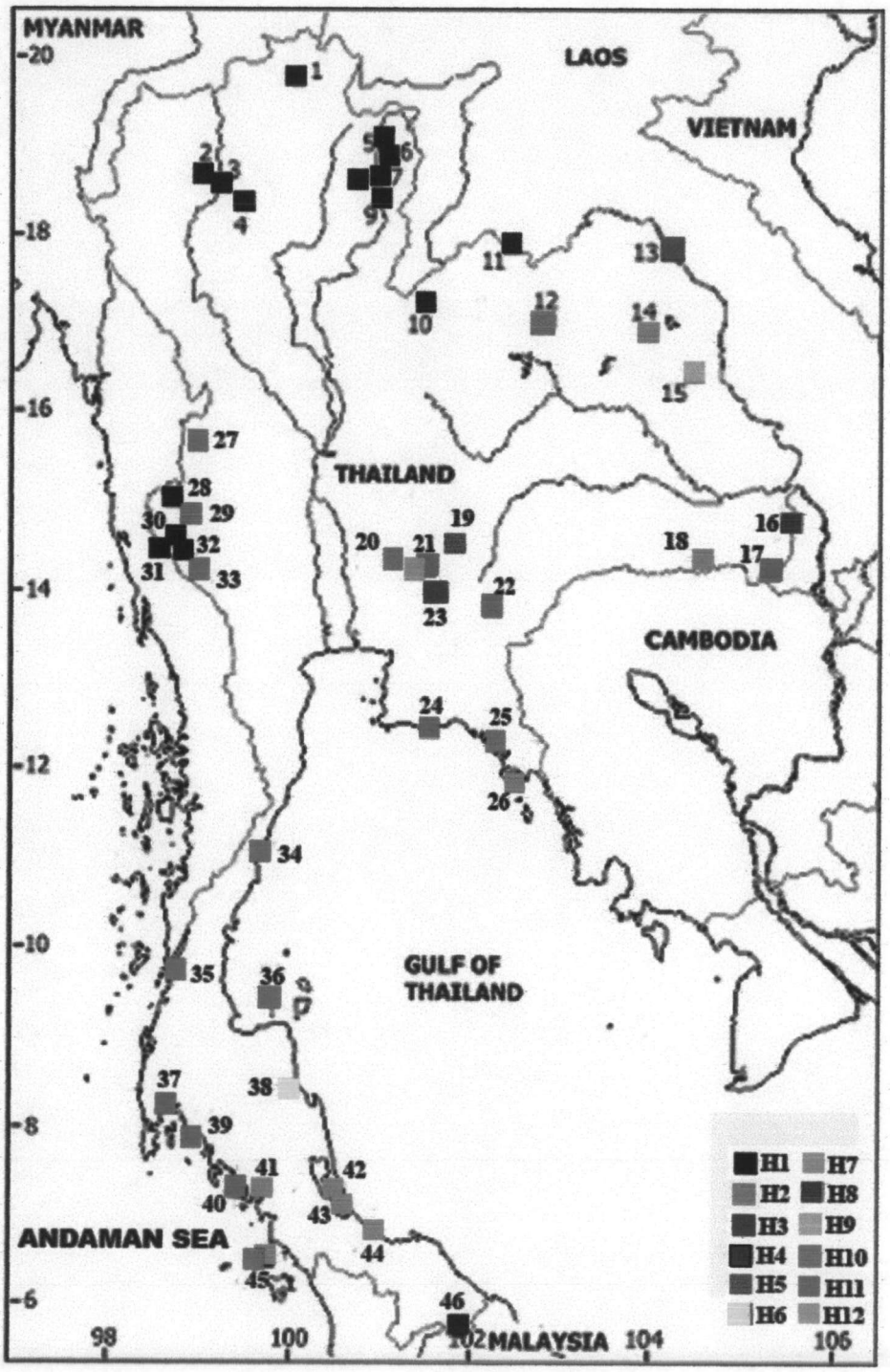

Figure 5.21 The distribution of twelve haplotypes of "*Hoya parasitica* complex" in Thailand.

The UPGMA dendrogram of the PCR-RFLP bands scored spitted the 56 specimens into four similarly clustered groups (Figure 5.22). Specimens classified as group 1 consisted of members of haplotypes H1, H2, H3, H4, and H5. Specimens classified as group 2 consisted of members of haplotypes H6, H7, and H8. Specimens classified as group 3 encompassed two subgroups, subgroup A consisted of members of haplotypes H9 and H10, while subgroup B is haplotype H11. Group 4 is only haplotype H12.

The dendrogram demonstrates that haplotype H1 is similar to haplotype H7 and clearly separated from the haplotype H10 and H11. The other haplotypes appear to derive from these two haplotypes. Furthermore, the result did not show well-defined groups for all studied forms (I-X).

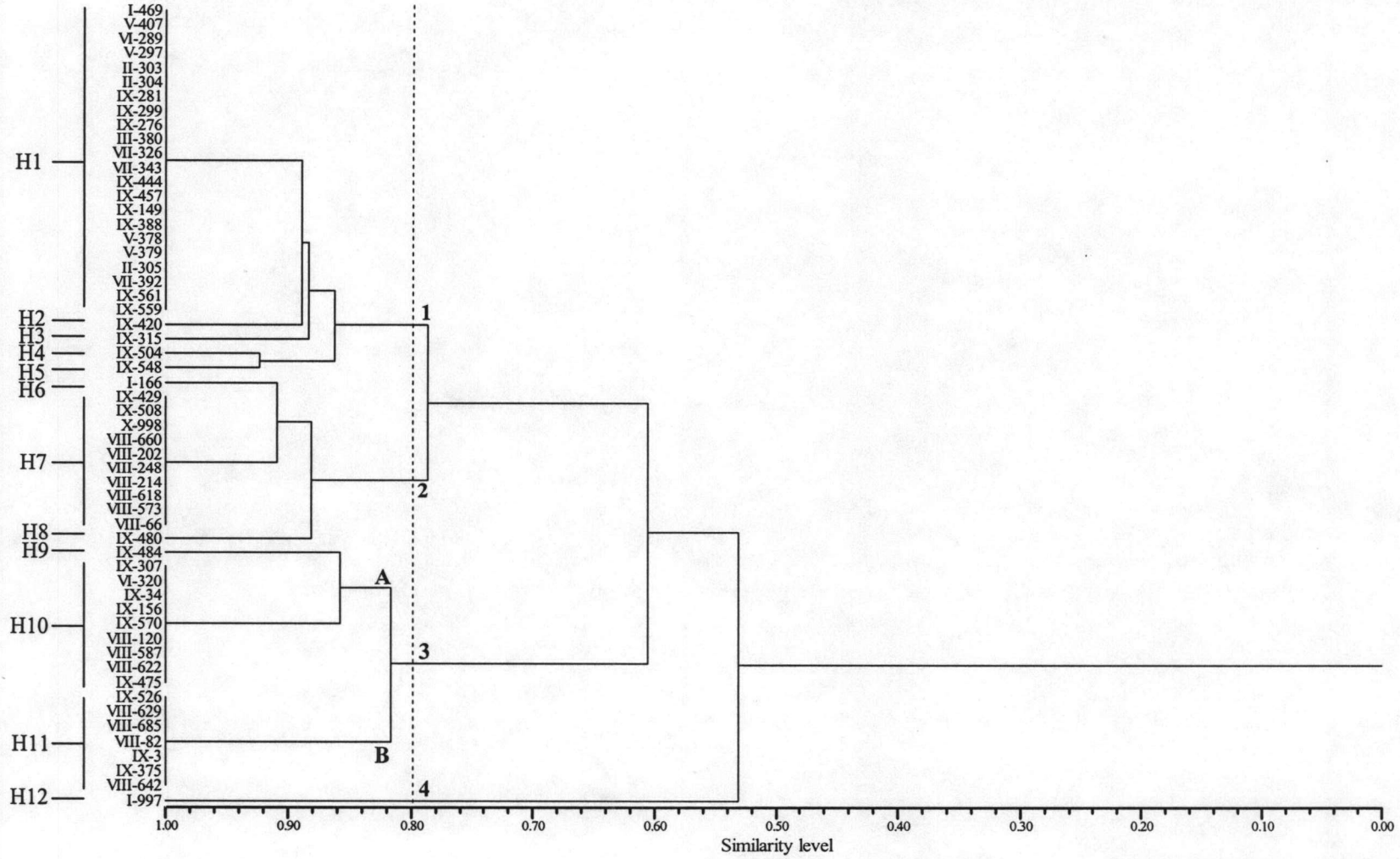

Figure 5.22 Dendrogram of 56 individuals of *H. parasitica* complex generated by cluster analysis using UPGMA based on PCR-RFLP. Each accession name, I to X are referred to morphological form. H1 to H12 are referred to haplotype 1-12.

Discussion and Conclusion

The result of digestion of the amplified products of each primer pair by fourteen restriction enzymes showed that 83 out of the 126 PCR-product / enzyme combinations had the recognition sites (Table 5.4). This RFLP survey using 4- and 6-cutter endonuclease provided information for nucleotides equivalent to DNA sequencing of about 1,561 bp. Only nine combinations showed polymorphic patterns being distributed over 12 haplotypes (H1, H2, H3, H4, H5, H6, H7, H8, H9, H10, H11, and H12) of "*Hoya parasitica* complex" in Thailand. Forms IX, VIII and I had a great variation in chloroplast DNA: 10 haplotypes (H1, H2, H3, H4, H5, H7, H8, H9, H10, and H11) being detected in form IX, 3 haplotypes (H7, H10, and H11) in form VIII and 3 haplotypes (H1, H6, H12) in form I. Six haplotypes (H2, H3, H4, H5, H8, and H9) were unique to form IX and 2 haplotypes were unique to form I (H6, H12). The rests were shared by the members of form VIII (H7, H10, and H11) or by the members of form I (H1). In forms II, III, V, VI, VII possessed the same haplotype and represented by the haplotype H1, while form IV and X (*H. parasitica* var. *citrina* (Ridl.) Rintz from Malaysia) were the haplotypes H10 and H7, respectively.

Four of twelve haplotypes (H1, H7, H10 and H11) occurred in high frequency and dominated all the wild populations. The wider geographical distributions along with the high frequency of these haplotypes reflected their ancient origin, whereas the low frequency of the unique haplotypes (H2, H3, H4, H5, H6, H8, H9, and H12) suggests that they might be the results of recent mutations (Mohanty et al., 2001).

There is a low significant difference of genetic structures between various localities (following floristic regions: N, NE, E, SE, C, SW and PEN) and habitats. It should be noted that in the northern populations the cpDNA pattern was unique representing by haplotype H1. The samples from the other localities shared the haplotypes with each other (Figure 5.21). Furthermore, the cpDNA patterns of the literal plants did not differ from the inland plants. These plants shared the haplotypes H7, H10, and H11. Such a haplotype distribution could be explained by the cytoplasmic gene flow (i.e. migration of seeds) between populations (Mohanty et al., 2001). The migration rate of seeds might be higher than expected. The seed with tufted hair could effectively facilitate a long-distanced dispersal (Vivian-Smith and

Panetta, 2002). Moreover, human activities have a profound effect on plant or haplotype migration, especially in economic plants, such as ornamental and medicinal species.

cpDNA variation among ten forms, nine (forms I-IX) in "*Hoya parasitica* complex" and one in *H. parasitica* var. *citrina* from Malaysia (i.e. form X) were small. The UPGMA dendrogram showed that the individuals studied did not form the isolated cluster with results to morphological form, but intermixed (Figure 5.22). This suggests a high genetic similarity and a close relationship among these ten forms.

H. rigida Kerr (form I), *H. ridleyi* King & Gamble (form VIII), and *H. citrina* Ridl. (form X) which included in this study are often treated taxonomically in different ways. Some authors assigned them as infra-specific categories of *H. parasitica* (Roxb.) Wall. ex Wight (Veldkamp et al., 1995; Rintz,1978, Kiew, 1995), while the others recognized them as distinct species (Kerr, 1939; Ridley, 1923). In this study, the result from PCR-RFLP of cpDNA showed that *H. parasitica* (Roxb.) Wall. ex Wight was closely related to these three taxa. The taxonomic status of these problematic taxa will be discussed below.

H. rigida Kerr was considered to be an endemic species in Thailand (Thaithong, 1995). This species grows in evergreen forest in eastern, western, and southern Thailand. The morphological and numerical studies indicated that *H. rigida* Kerr was clearly distinct from the other taxa. Nevertheless, in PCR-RFLP analysis, *H. parasitica* s.l. and *H. rigida* Kerr were found to be clustered together, suggesting that *H. rigida* Kerr was closely related to *H. parasitica* s.l. The *H. rigida* Kerr occurring in the eastern and southern Thailand had unique haplotype (H12 for the eastern population and H6 for the southern population), while the western population was haplotype H1 which shared by *H. parasitica* s.l. growing in the same area. The shared haplotype between *H. rigida* Kerr and *H. parasitica* s.l. in the western part of Thailand is probably the result of interspecific hybridization (♂*H. rigida* × *H. parasitica*♀). The alternative hypothesis is that *H. rigida* is in fact a member of *H. parasitica* complex with a unique morphological variant. Therefore, on the basis of PCR-RFLP data, *H. rigida* Kerr should be treated as an infraspecific taxon of *H.*

parasitica s.l. rather than a distinct species. Similar classification was also proposed by Veldkamp et al. (1995).

H. ridleyi King & Gamble was included in *H. parasitica* (Roxb.) Wall. ex Wight var. *parasitica* (Rintz, 1978). Morphologically, *H. ridleyi* differs from *H. parasitica* (Roxb.) Wall. ex Wight to some extent. Narrow leaves and obscure nerves when dry are found in *H. ridleyi* King & Gamble, whereas in *H. parasitica*, leaves are wider with conspicuous nerves (Ridley, 1923). However, the exhaustive morphological and numerical studies showed that *H. ridleyi* King & Gamble (form VIII) was not clearly distinct from the other forms (III-VII, IX) of "*Hoya parasitica* complex". The concurring result was also obtained from PCR-RFLP analysis. Therefore, *H. ridleyi* King & Gamble could be considered as an infraspecific taxon of *H. parasitica* s.l. as was treated by Veldkamp et al.(1995), Rintz (1978) and Kiew (1995).

H. citrina Ridl. is common to limestone hill in Malaysia and was treated as a distinct species (Ridley, 1923). The recent nomenclatural recombination was made by Rintz (1978) and this plant was cited as *H. parasitica* var. *citrina* (Ridl.) Rintz. The author also reported the presence of plant in the south of Thailand. In this study, we did not found *H. parasitica* var. *citrina* (Ridl.) Rintz in Thailand but obtained the rather similar specimens from southern Thailand. These samples were treated as form II. The intensive morphological and anatomical studies suggested that form II differed from *H. parasitica* var. *citrina* (Ridl.) Rintz. Moreover, the data of the cpDNA variation supported the dissimilarity between form II and *H. parasitica* var. *citrina* (Ridl.) Rintz. Therefore, the presence of *H. parasitica* var. *citrina* (Ridl.) Rintz in Thailand as mentioned by Rintz (1978) seems to be questionable.

The additional results from morphological and numerical studies also indicated that form II was a distinct form, dissimilar to the other eight forms of "*Hoya parasitica* complex" in Thailand. However, considering the genetic evidence, form II which occurred in the lowest part of southern Thailand shared the haplotype H1 with the other forms of "*Hoya parasitica* complex" occurring in northern or western Thailand. This indicated a close relationship among them and suggested that these plants might have been closely contact in the past, although they are clearly

geographically isolated at present, comparable to what was suggested by Parducii and Szmidt (1999) who worked on *Abies* species. Hence, the PCR-RFLP data pointed out that form II should be included in *H. parasitica* s.l., which do not correspond to the result from morphological and numerical studies. But, for *H. parasitica* var. *citrina* (Ridl.) Rintz (specimen from Malaysia, i.e. form X), it has the same haplotype (H7) with the forms VIII and IX of "*Hoya parasitica* complex" in Thailand. Thus this study confirms Rintz (1978) in reducing *H. citrina* Ridl. to a variety of *H. parasitica* var. *citrina* (Ridl.) Rintz.

The PCR RFLP of cpDNA provides informations on genetic variations in "*Hoya parasitica* complex" in Thailand. These variations supported the suggestions by Kerr (1951) and Thaithong (1995) that *H. parasitica* was extremely variable species in Thailand. The overall results showed a low potential utility of PCR-RFLP to distinguish the different morphological forms of *H. parasitica* complex. In general, at the infra-specific level, chloroplast DNA exhibits very low variations (Palmer, 1987). However, this kind of information can be either very useful (e.g. Mohanty et al., 2001; Xu et al., 2001) or not (e.g. Panda et al., 2003) for taxonomic investigations of various plant taxa. The taxonomic status of "*Hoya parasitica* complex" could not delimited by using only cpDNA variations. Thus, the diversity of cpDNA in "*Hoya parasitica* complex" should be considered cautiously for further studies involving this species.

Finally, this study showed that the cpDNA in the "*Hoya parasitica* complex" in Thailand were heterogeneous. Unfortunately, due to the limited sample sizes, the level of variation observed in the populations could not be quantified. It is likely that further studies, including larger available sample sizes and/or more powerful methods for detection of chloroplast DNA variation (for example, acrylamide gel is capable to detect small DNA fragments (< 100 bp) or to separate the bands which are not very different in sizes better than agarose gel which is more appropriate to detect large DNA fragments), would reveal additional variation and provide more promising evidence for clarify the confusing taxonomic problems of the "*Hoya parasitica* complex".