CHAPTER IV

RESULTS

4.1 Species diversity from field work study

The plants were identified to genus and species based on flower, fruit (acorn), leaf and other vegetative characteristics (see key to genus in appendices). Due to the large variation in the plant phenology, specimens for taxonomic identification had to be collected during several visits, usually in the cool and dry season (December - February) to get flowers and some months later for acorns. Some species, especially *Lithocarpus*, flower in the summer (May - August). About half of the plants were identified to species using leaves and acorns, but the other half was identified based on acorns only, or leaves and/or flowers. The sample of Fagaceae tree, flowers, and acorns are shown in figure 4.1.

The Fagaceae genera under study can be recognized in the field (Gardner et al., 2000) for having the following common features: evergreen or deciduous trees without latex or colour sap, often with thick, deeply cracked bark; leaves simple, spirally arranged, pinnately nerved, margin entire or serrate; stipules caducous; flowers tiny, usually creamy-yellow, in slender clusters at end of twigs and upper leaf axils, males and females in separate flowers same of separate inflorescences but on the same tree (monoecious); male flowers with 6 (4-7) spreading sepals, without petals, 6-18 free stamens, female flowers similar but sepals closely pressed together around 3 styles; fruit a nut (acorn), partly or completely enclosed by cupule. The three genera can often be differentiated in the field. *Castanopsis*: mostly evergreen; leaves serrate or entire; inner bark smooth; inflorescence erect (flower clusters upright); many stamen; fruits usually covered by spiny cupules. *Lithocarpus*: mostly evergreen; leaves usually entire; inner bark with narrowed ridges; inflorescence erect; 6 stamen; fruits mostly or partly covered by cupules, not spiny. *Quercus*: often

deciduous; leaves usually serrate; inner bark with narrowed ridge; male catkins pendulous; 6 stamens; fruits partly covered by cupules, not spiny.

Species diversity of Fagaceae at *Khun Mae Kuong* Forest is shown in Table 4.1. From all of 146 trees examined in this study, 30 different species were identified. The number of species in *Castanopsis* and *Quercus* was similar, i.e. 12 and 11 species, respectively, but *Lithocarpus* had only 7 species. High species diversity within a forest was also visible, especially in Hill evergreen forests, whereby 11-12 species were identified from 23-24 trees in each of the four sites investigated. Several species were represented by only one individual, and within a distance of 50-100 m there were often more than one species. Nevertheless, certain species were more prevalent than the others, for example *C. acuminatissima* was clearly the most common species in Hill evergreen forests and at one site (KNK) half of the trees (12 out of 24) belonged to this species alone. In the dry deciduous forests, *Q. kerrii* was found to be common (18 out of 50 trees) and tended to form large stands.

Among these three genera, *Castanopsis* was found most frequently in the Hill evergreen forests, *Quercus* was most common in deciduous forests at low altitudes, whereas *Lithocarpus* was found scattering in a wide variety of forest types. The genera *Castanopsis* and *Quercus* mostly occupied separate locations and habitats. In the *Quercus*-rich deciduous forests, none of the Fagaceae trees examined was identified to *Castanopsis*. In the Hill evergreen forests there was no *Quercus* tree among the samples, except at the hill sites with pine. Evergreen species of *Lithocarpus*, such as *L. elegans* and *L. harmandianus*, were identified in the Evergreen forests, but some species like *L. ceriferus* and *L. sootepensis* were found in deciduous and pine-evergreen forests together with several species of *Quercus*.

Figure 4.1: Fagaceae trees (a - c), flowers (c - d) and acorns (e - g). Typical acorns of (e) *Castanopsis*, (f) *Lithocarpus* and (g) *Quercus*.

Table 4.1: The species diversity study, number of trees examined and samples used in the DNA analysis.

Plant species			Number of trees from each species at each study site						No. of samples	
			Hill evergreen forest		Hill evergreen forest with pine		Deciduous Dipterocarp forest		rDNA-RFLP	ISSR
			KPA	KNK	KBA	KRD	KPS	KHH		
Castanopsis	*C. acuminatissima* (Blume) A.DC.	Ca	-	12	3	3	-	-	9	11
	C. argentea (Blume) A.DC.	Cag	1	1	-	1	-	-	1	2
	C. armata (Roxb.) Spach	Car	1	1	-	-	-	-	-	1
	C. calathiformis (Skan.) Rehder & Wilson	Cc	3	2	-	-	-	-	2	3
	C. cerabrina (Hickel & A. Camus) Barnett	Cce	1	1	-	1	-	-	-	1
	C. crassifolia Hickel & A. Camus	Ccr	1	1	-	-	-	-	-	1
	C. diversifolia (Kurz) King & Hook.f.	Cd	4	2	-	-	-	-	2	3
	C. echinocarpa A.DC.	Ce	-	-	1	1	-	-	-	-
	C. ferox (Roxb.) Spach	Cfe	4	1	-	1	-	-	2	2
	C. fissa (Champ) Rehder & Wilson	Cfi	2	-	-	-	-	-	1	1
	C. indica (Roxb.) A.DC.	Ci	6	-	-	-	-	-	-	2
	C. tribuloides (Sm.) A.DC.	Ct	-	-	-	8	-	-	1	7
Lithocarpus	*L. ceriferus* (Hickel & A. Camus) A. Camus	Lc	-	-	1	1	4	-	1	1
	L. elegans (Blume) Hatus ex Soepadmo	Le	-	1	1	1	-	-	4	4
	L. harmandianus (Hickel & A. Camus) A. Camus	Lh	1	1	3	-	-	-	4	5
	L. polystachyus (A. DC.) Rehder	Lp	-	-	1	2	-	-	2	2
	L. recurvatus Barnett	Lr	-	-	1	-	-	-	1	1
	L. sootepensis (Craib) A. Camus	Ls	-	-	2	2	2	-	4	3
	L. vestitus (Hickel & A. Camus) A. Camus)	Lv	1	1	-	-	-	-	-	1
Quercus	*Q. brandisianus* Kurz	Qb	-	-	3	2	4	-	2	4
	Q. fleuryi Hickel & A.Camus	Qf	-	-	-	-	-	1	1	1
	Q. helferianus A.DC.	Qh	-	-	-	-	2	-	-	-
	Q. kerrii Craib	Qk	-	-	4	-	9	9	6	7
	Q. kingianus Craib	Qki	-	-	2	-	-	2	2	4
	Q. lenticellatus Barnett	Ql	-	-	-	1	-	1	-	-
	Q. lineatus Blume	Qli	-	-	-	-	-	3	1	2
	Q. mespilifolius Wall. ex DC.	Qm	-	-	-	-	4	2	3	3
	Q. myrsinaefoius Blume	Qmy	-	-	1		-	-	-	1
	Q. quangtriensis Hickel & A. Camus	Qq	-	-	-	-	-	5	2	2
	Q. rex Hemsl.	Qr	-	-	-	-	-	2	1	1
	Total number of trees (146)		**25**	**24**	**23**	**24**	**25**	**25**	52	76

The present study supports that northern Thailand, as represented by *Khun Mae Kuong* Forest in Chiang Mai province, still maintains high species diversity which is part of diversity centre of this group of Fagaceae. Soepadmo (1972) emphasized that Southeast Asia, Indochina in particular, maintained the greatest assemblage and most primitive forms of *Castanopsis*, *Lithocarpus* as well as *Quercus*: subg. *Cyclobalanopsis*. Many of these species, especially in the genus *Castanopsis*, form part of the montane forest distribution east of Himalaya including eastern Nepal, north-eastern India, northern Myanmar, northern Thailand, southern China and western part of Indochina. Among the *Castanopsis* spp., *C. acuminatissima* is considered to be the most wide spread in Southeast Asia (Barnett, 1944). Other species of *Castanopsis* identified in this study are not as common. The genera *Quercus* and *Lithocarpus* are also known to be highly diversified in Southeast Asia and occur in the same range of distribution as *Castanopsis*, but *Quercus* reaches much further north and east into Korea and Japan and *Lithocarpus* extends far south into Indonesia and coastal East Asia (Barnett, 1944; Soepadmo, 1972). A number of Fagaceae species are endemic to Thailand, and they are from all three genera (Phengklai, 2004) including *L. sootepensis* and *Q. lenticellatus* found in this study.

4.2 Genetic diversity

4.2.1 Molecular diversity inferred from rDNA-RFLP

The RFLP variation in the major (18S-25S) ribosomal genes among Fagaceae species understudy was obtained from hybridising the whole 9 kb ribosomal gene from wheat (pTa71) with *Eco*RI, *Bam*HI and *Hind*III-digested genomic DNA of 52 samples from 21 Fagaceae species (Table 4.2). A repeat unit representing whole ribosomal gene in these three Fagaceae genera appeared to be about 10-12 kb in size: 10.5 and 11 kb-*Eco*RI; 9.4 and 10 kb-*Bam*HI; 10, 11 and 12 kb-*Hind*III fragments (see examples in Figure 4.2). Other size classes were most likely products of mutations that change restriction sites. Conserved fragments in all three genera were 4 kb-*Eco*RI, 2 kb- and 1 kb-*Bam*HI fragments, indicating common ancestry. A single tree possessed one or two sets of this major ribosomal gene family. Most of these

Fagaceae species have two pairs of major loci in the nuclear genome (unpublished results), and possibly additional minor loci. European species of *Quercus* were reported to have one pair major and one pair minor loci (Zoldos et al., 1999). Major chromosomal loci are those coinciding with the nucleolar organizer regions (NOR) where the ribosomal genes are transcribed and nucleolus is formed.

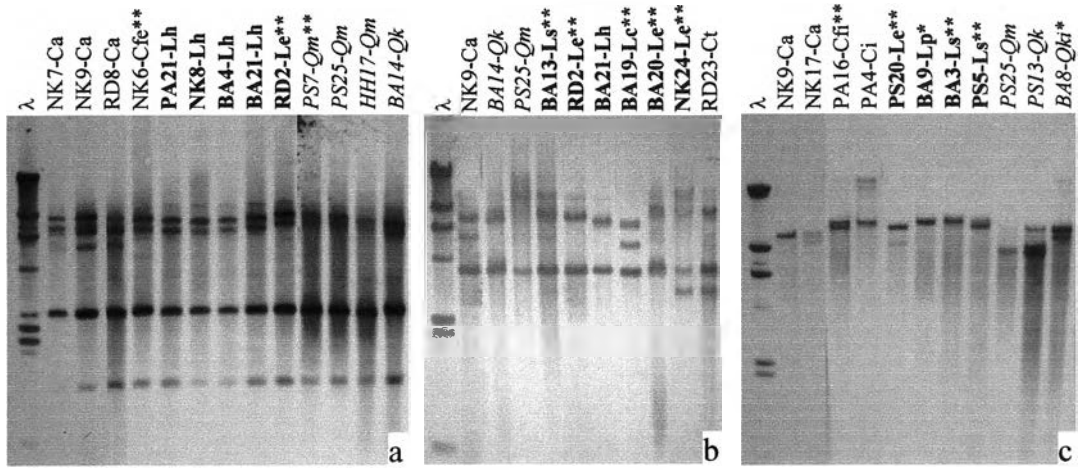

Figure 4.2: Luminographs of ribosomal RFLP after Southern hybridisation with *Bam*HI (a), *Eco*RI (b) and *Hind*III (c) restricted genomic DNA.

Among the three genera, *Quercus* seemed to be the least variable in the rDNA-RFLPs. Besides the conserved fragments common to all species, *Quercus* had only 4-5 fragments by each restriction enzyme, whereas *Castanopsis* was the most variable and having all the RFLPs in this study. A complete lack of fragment classes 5-6 kb-*Eco*RI and 5-7 kb-*Bam*HI in *Quercus* may indicate a significant evolutionary distance between *Quercus* and the other two genera. Between *Castanopsis* and *Lithocarpus* species in this study, *Castanopsis* was more polymorphic and appeared to have fragments that occurred only in this genus such as the 3.3 kb-*Eco*RI fragment. Such RFLPs could be useful in tracing genetic relationships or gene flow that may occur via hybridisation.

The genetic distance study based on rDNA-RFLPs (Figure 4.3) was obtained from 25 *Bam*HI, *Hind*III and *Eco*RI restricted fragments in the 18S-25S ribosomal genes described above. The dendrogram revealed relatively good separation of the three genera and grouping of the samples within species, supporting broad taxonomic identification. The *Castanopsis* group (I) contained nearly all samples of the most common species *C. acuminatissima* regardless of location or forest type. The *Quercus* group (II) contained nearly all samples of the most common species *Q. kerrii* and other species of *Quercus* especially those from the KHH forest. *Lithocarpus*, on the other hand formed two small groups (III and IV), both of which had the same species members.

Considerable diversion from this main grouping was shown by the variation in the ribosomal repeats. A number of species, although represented by only one or two samples, were not placed with species of the same genus and some were not in any groups. Several *Castanopsis* samples fell outside *Castanopsis* group, and some of them were included within *Lithocarpus*, e.g. *C. fissa* (PA16) and *C. diversifolia* (NK4). On the other hand, all four samples of *L. harmandianus* got included in the *Castanopsis* group. *Quercus* although most homogeneous in this respect had certain species grouping with *Lithocarpus*, for example *Q. brandisianus* and *Q. quangriensis*, seemed to share the ribosomal gene RFLPs with *L. harmandianus*. Trees of *Q. kerrii* and *Q. kingianus* that were found outside the KHH forest, i.e. PS13 and BA8, were grouped with *Lithocarpus* also. The explanation for these diversions could only be gene flow or gene introgression within forests. Introgressive hybridisation, recent or ancient, must have occurred between *Castanopsis* and *Lithocarpus*, and between *Lithocarpus* and *Quercus*. In the present study *Castanopsis* and *Quercus* were relatively separate, geographically or ecologically, i.e. no *Castanopsis* was discovered from deciduous forests and no *Quercus* was found in the Hill evergreen forests.

Table 4.2: RFLP fragments (in kb) generated by Southern hybridization using 18S-5.8S-25S ribosomal clone pTa71.

	Ca	Cag	Cc	Cd	Cfe	Cfi	Ct	Lc	Le	Lh	Lp	Lr	Ls	Qb	Qf	Qk	Qki	Qli	Qm	Qq	Qr
No. trees	9	1	2	2	2	1	1	1	4	4	2	1	4	2	1	6	2	1	3	2	1
EcoRI																					
0(12)			12.0	12.0																	
1(10.5)	11.0		11.0	11.0	11.0				11.0	11.0		11.0	11.0	11.0	11.0	11.0	11.0	11.0	11.0	11.0	11.0
2(8.0)	8.0				8.0		8.0		8.0				8.0	8.0		8.0	8.0	8.0	8.0		8.0
3(7.0)	7.0									7.0	7.0			7.0	7.0	7.0		7.0	7.0		
4(6.8)					6.8			6.8		6.8		6.8		6.8			6.8			6.8	
5(6.0)	6.0																				
6(5.0)				5.0	5.0			5.0			5.0	5.0	5.0								
7(4.0)	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0
8(3.3)	3.3		3.3	3.3	3.3		3.3														
BamHI																					
1(10.0)	10.0	10.0		10.0	10.0	10.0	10.0		10.0	10.0	10.0		10.0		10.0	10.0	10.0	10.0	10.0	10.0	10.0
2(9.4)	9.4	9.4	9.4	9.4	9.4	9.4			9.4	9.4			9.4	9.4		9.4	9.4		9.4		
3(9.0)	9.0		9.0	9.0	9.0	9.0	9.0	9.0	9.0	9.0	9.0	9.0	9.0		9.0	9.0	9.0	9.0	9.0	9.0	9.0
4(8.0)	8.0		8.0		8.0	8.0	8.0		8.0	8.0		8.0	8.0	8.0		8.0	8.0			8.0	
5(7.0)	7.0				7.0	7.0	7.0	7.0		7.0	7.0	7.0									
6(6.6)		6.6		6.6		6.6	6.6	6.6	6.6		6.6		6.6								
7(6.0)	6.0																				
8(5.0)		5.0		5.0		5.0	5.0	5.0			5.0	5.0	5.0								
9(3.0)	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0
10(1.0)	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
HindIII																					
1(12.0)						12.0			12.0				12.0			12.0	12.0				
2(11.0)	11.0	11.0		11.0	11.0		11.0	11.0	11.0						11.0	11.0	11.0	11.0	11.0		11.0
3(10.0)	10.0		10.0		10.0				10.0	10.0	10.0	10.0		10.0						10.0	
4(9.4)				9.4				9.4			9.4	9.4	9.4			9.4					
5(8.0)	8.0		8.0	8.0	8.0										8.0	8.0	8.0	8.0	8.0		8.0
6(7.0)			7.0																		

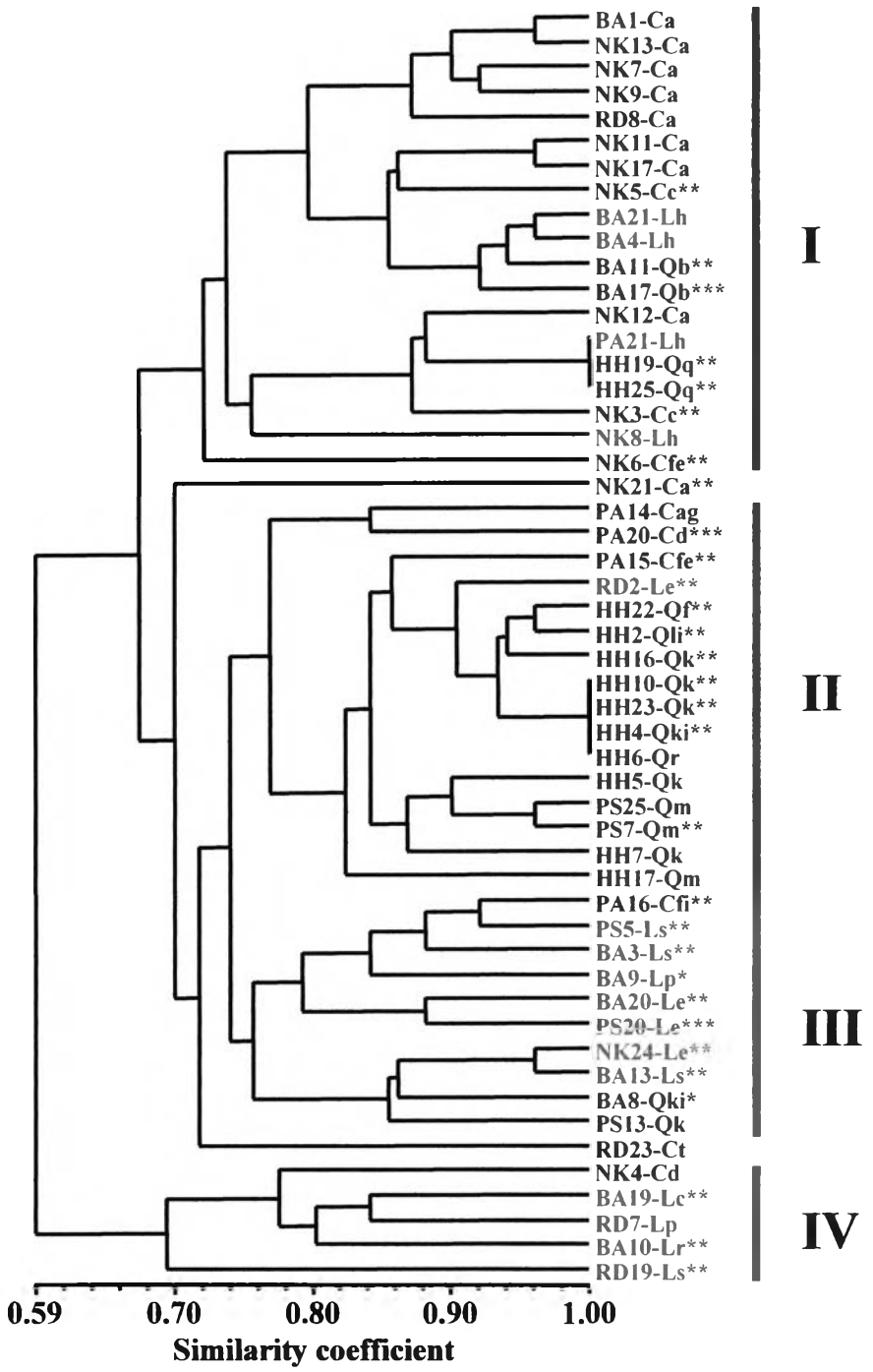

Figure 4.3: Genetic relationships in Fagaceae at *Khun Mae Kuong* Forest, based on restriction fragment length polymorphism (RFLP) in the 18S-25S ribosomal genes (rDNA). The rDNA-RFLP phenogram was constructed from 25 *Eco*RI, *Bam*HI and *Hind*III fragments in 52 trees from 21 species (Table 3). The scale shows similarity coefficient. The plants were identified to species using leaves and acorns, except where indicated with *(acorns only), **(leaves only) and ***(leaves and flowers).

4.2.2 Molecular diversity inferred from ISSR

ISSR-PCR amplification was performed to assess the level of polymorphism in 76 samples from 27 species in these three Fagaceae genera. After an initial screening using 32 ISSR primers, 10 most polymorphic primers were selected (Table 4.3). Typical banding patterns are presented in Figure 4.3. These 10 ISSR primers generated 173 bands ranging in size from 400 to 2800 bp, corresponding to an average of 17 bands per primer. A number of amplified fragments were genus- or species specific. From all these 173 bands, similarity coefficients were calculated and a UPGMA dendrogram was generated (Figure 4.5). The three genera were clearly separated except only a few diversions. Samples from individual species also grouped together, supporting taxonomic classification.

Table 4.3: ISSR primer sequences, number of fragments scored and approximate size range (in base pairs) of the fragments resulted from each primer probing 76 samples from 27 species of Fagaceae in northern Thailand.

Primer from UBC set 9		No. of fragments scored	Fragment size range (bp)
Code	Sequence*		
810	$(GA)_8T$	17	450-1500
825	$(AC)_8T$	19	500-1800
834	$(AG)_8YT$	16	500-1500
835	$(AG)_8YC$	10	450-1500
840	$(GA)_8YT$	10	500-1300
857	$(AC)_8YG$	23	450-2000
868	$(GAA)_6$	12	550-1800
873	$(GACA)_4$	26	600-2800
876	$(GATA)_2(GACA)_2$	21	400-1900
881	$(GGGTG)_3$	19	400-2000

*Y stands for pyrimidine.

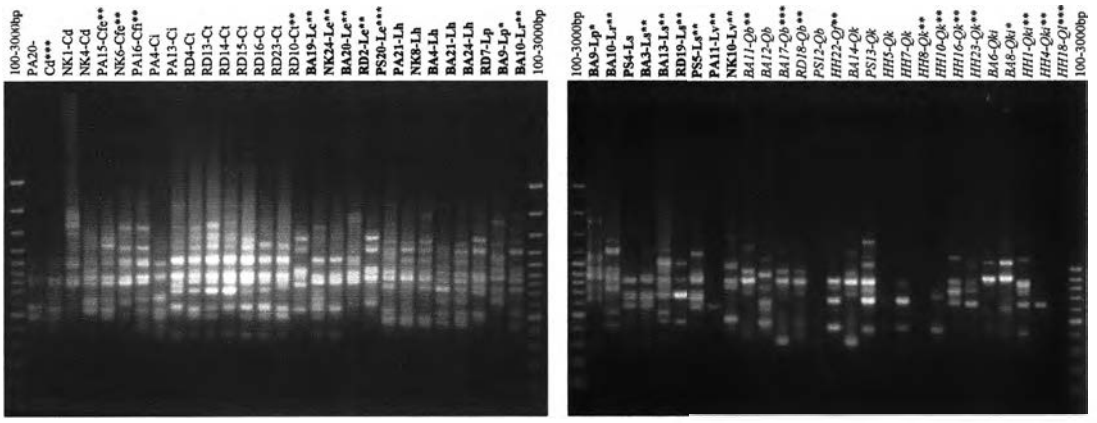

Figure 4.4: Agarose gel electrophoresis showing inter-simple sequence repeats (ISSR) bands generated by PCR reactions using USB primer no. 857.

The *Castanopsis* group (I) consisted of all samples of *Castanopsis* except only three samples that did not belong to any genus-group. These are *C. calathiformis* (NK3 and NK5) and *C. cerabrina* (NK23), both species have non-spiny acorns which is an unusual character for *Castanopsis*. The main *Castanopsis* group could be considered dividing further into two subgroups, one consisting of all samples of *C. acuminatissima* and several other species of *Castanopsis* essentially from the Hill evergreen forests, while the other consisting exclusively of *C. tribuloides*. This species was found to be common in Hill evergreen forest with pine, a somewhat patchy or disturbed habitat. Not only that *C. tribuloides* had clearly been differentiated from other *Castanopsis* species, it was also genetically closer to *L. elegans* which was found in the same type of forest. A few *Lithocarpus* samples were included in the main *Castanopsis* group, e.g. *L. sootepensis* which was also from the Hill evergreen forests with pine. This is indicate either (a) gene flow between species belonging to different genera, i.e. between *Castanopsis* and *Lithocarpus*, or (b) that the species in *Lithocarpus*, for example *L. sootepensis*, could be more taxonomically and correctly placed in the genus *Castanopsis*.

The *Quercus* group (II) included nearly all samples belonging to this genus. The species differentiation was also in good agreement with the taxonomic classification. Nevertheless, geographical structure regarding forest types could be seen again, i.e. trees of different species growing in the same forest appeared to be related to one another. For example, *Q. kerrii* sample BA14 was more related to *Q. brandisianus* in the KBA/KRD forests than to most *Q. kerrii* trees in the deciduous forests KHH/KPS. As seen before this was a clear indication of gene flow within forests. The *Quercus* group did not contain all species of *Quercus*, for example *Q. kingianus*, *Q. myrsinaefoius* and *Q. quangtriensis* were more associated with *Lithocarpus*. This could again indicate either (a) gene flow between species belonging to different genera, i.e. between *Lithocarpus*and *Quercus* especially in the Dry deciduous forests, or (b) that certain species of *Quercus* could be more correctly placed in *Lithocarpus.*

The *Lithocarpus* group (III) by itself contained mostly species from the Hill evergreen forests including all samples of *L. harmandianus*. Other species have either grouped with *Castanopsis* or were related to *Quercus*, making the genus *Lithocarpus* extremely variable.

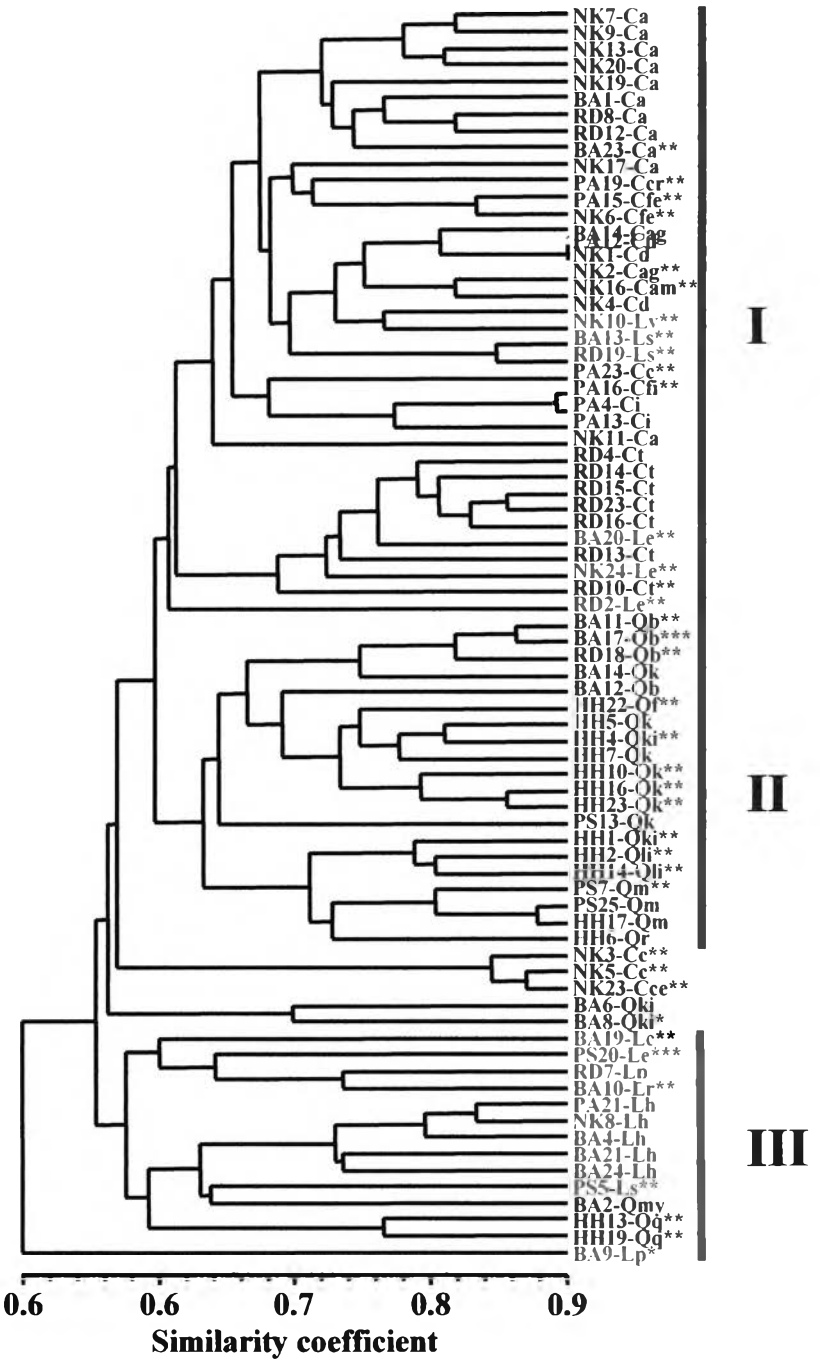

Figure 4.5: Genetic relationships in Fagaceae at *Khun Mae Kuong* Forest, based on inter-simple sequence repeats (ISSR). The ISSR phenogram was constructed from 173 polymorphic bands generated by 10 primers (Table 4.2) in 76 samples from 27 species. The scale shows similarity coefficient. The plants were identified to species using leaves and acorns, except where indicated with *(acorns only), **(leaves only) and ***(leaves and flowers).

4.3 Genomic diversity

4.3.1 Chromosome number and karyotype analysis

Chromosomes were mostly isolated from leaves using protoplast dropping technique. Chromosome counts were successfully made from 24 out of 30 taxonomically identified species belonging to all three genera, i.e. *Castanopsis*, *Lithocarpus* and *Quercus* (Table 4.4; Figure 4.6). Karyotypes were constructed from 19 species: nine from *Castanopsis*, five from *Lithocarpus* and five from *Quercus* (Figures 4.7 – 4.9). Most of the species had diploid (2n = 24) chromosome number, with the basic number being 12. Meiotic chromosomes from flower buds were examined in two species of *Castanopsis* and 12 bivalents were observed, confirming the diploid status 2n = 2x = 24 (Figure 4.10). Metaphase chromosomes of these Fagaceae species were relatively small, about 2-5 μm in size, and all were metacentric and sub-metacentric chromosomes. Karyotypes of the species examined were very similar. There was usually one pair of chromosomes with secondary constriction, so called satellite (SAT-chromosomes). Although it was generally difficult to recognize the SAT-chromosomes, cells of certain species showed apparent secondary constrictions (e.g. Figures 4.6b, 4.7d). A significant variation from the diploid number of 24 was detected in one species, i.e. *Q. lenticellatus*. One sample of this species had 14 chromosomes in mitotic metaphases (Figure 4.11) or seven homologous chromosome pairs, indicating a diploid status 2n = 2x = 14 with the basic number apparently being seven.

Table 4.4: Chromosome numbers of Fagaceae species.

Species Name	Chromosome number	Number of samples/trees used in this study
C. acuminatissima (Blume) A.DC.	24	3
C. argentea (Blume) A.DC.	24	2
C. armata (Roxb.) Spach	24	1
C. calathiformis (Skan.) Rehder & Wilson	24, 28	2
C. cerabrina (Hickel & A. Camus) Barnett	24	2
C. diversifolia (Kurz) King & Hook.f.	24	1
C. echinocarpa A.DC.	24	1
C. ferox (Roxb.) Spach	24	2
C. fissa (Champ) Rehder & Wilson	24	2
C. indica (Roxb.) A.DC.	24	6
C. tribuloides (Sm.) A.DC.	24	2
L. ceriferus (Hickel & A. Camus) A. Camus	24	2
L. elegans (Blume) Harus ex Soepadmo	24	4
L. harmandianus (Hickel & A. Camus) A. Camus	24	2
L. polystachyus (A. DC.) Rehder	24	1
L. recurvatus Barnett	24	1
L. vestitus (Hickel & A. Camus) A. Camus)	24	2
Q. brandisianus Kurz	24	4
Q. kerrii Craib	24	2
Q. kingianus Craib	24	2
Q. lenticellatus Barnett	14, 24	2
Q. mespilifolius Wall. ex DC.	24	3
Q. myrsinaefoius Blume	24	1
Q. rex Hemsl.	24	1

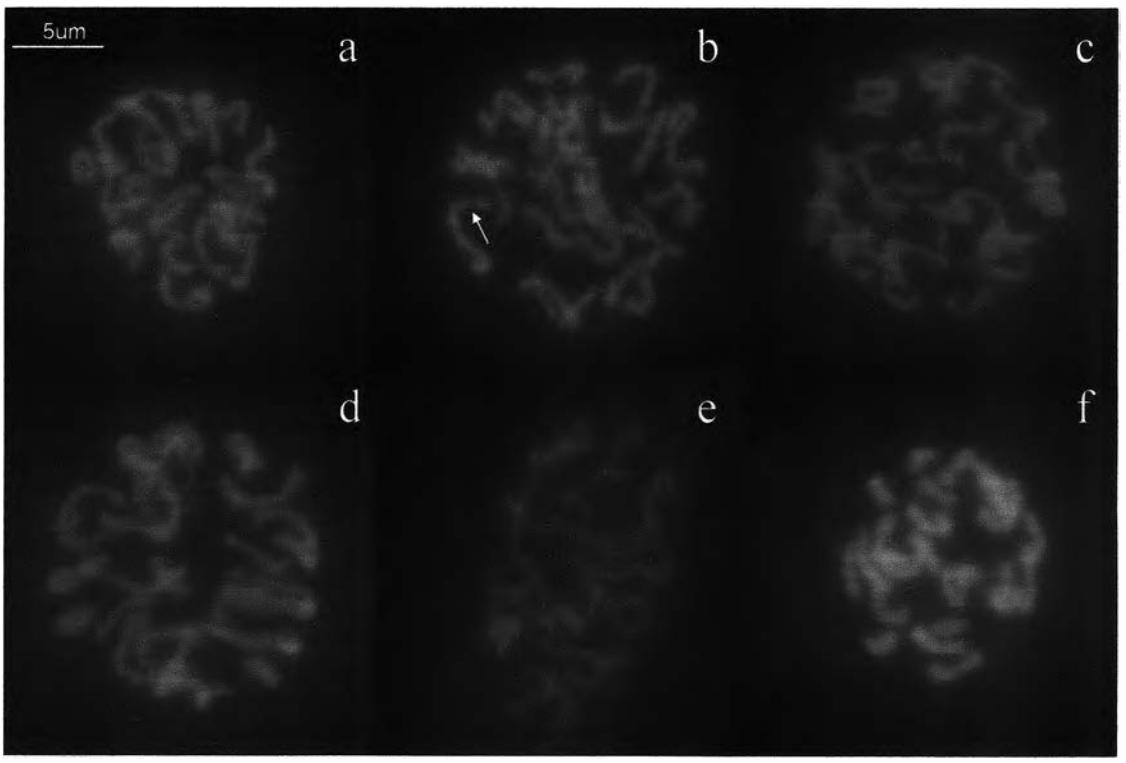

Figure 4.6: Metaphase chromosomes of (a) *C. echinocarpa* A.DC., (b) *C. ferox* (Roxb.) Spach, (c) *L. polystachyus* (A. DC.) Rehder, (d) *Q. kingianus* Craib, (e) *Q. lenticellatus* Barnett (sample HH18), and (f) *Q. myrsinaefoius* Blume. Arrow in (b) show secondary constriction.

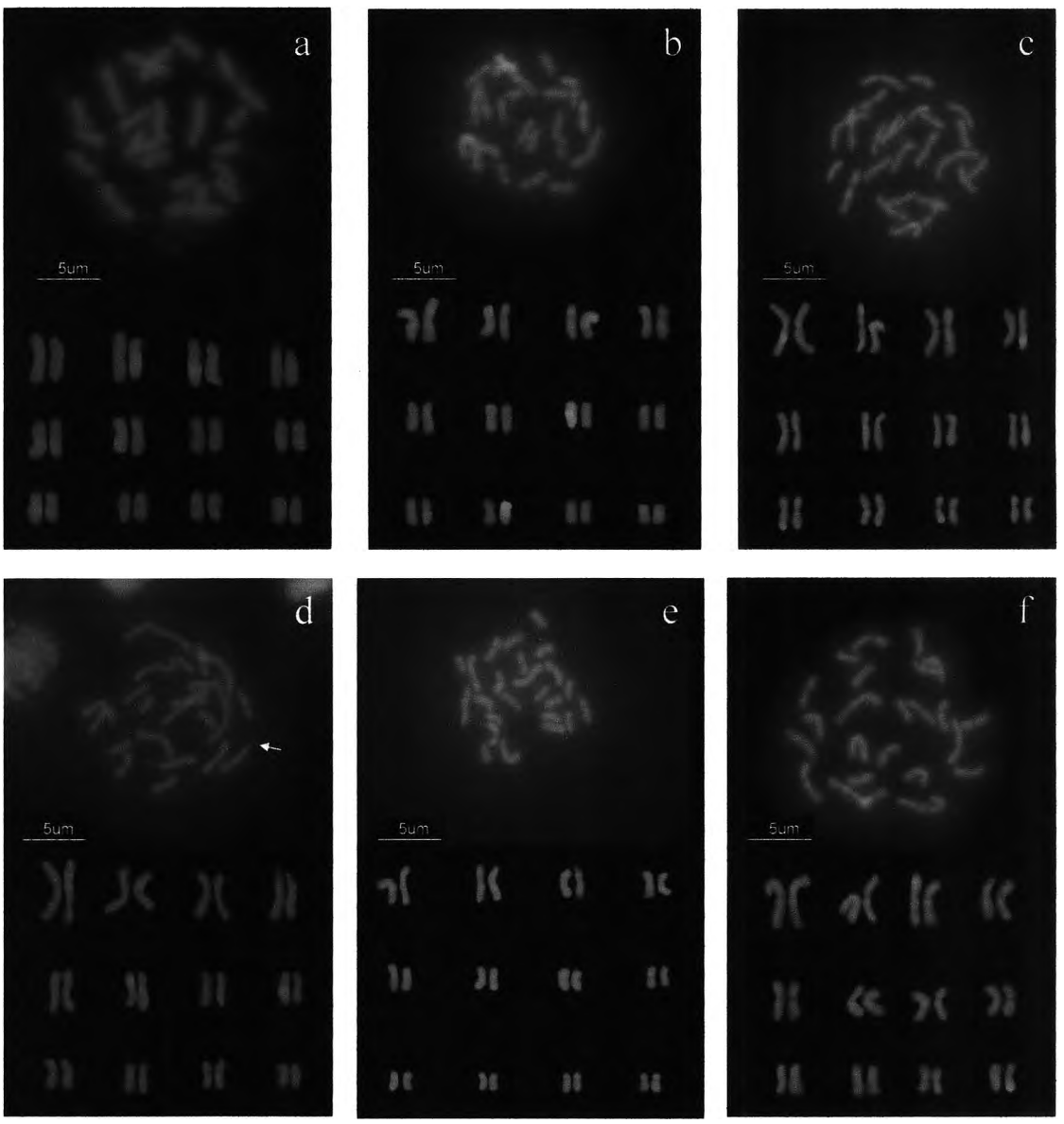

Figure 4.7: Mitotic metaphases and karyotypes of (a) *C. acuminatissima* (Blume) A.DC., (b) *C. argentea* (Blume) A.DC., (c) *C. armata* (Roxb.) Spach, (d) *C. calathiformis* (Skan.) Rehder & Wilson, (e) *C. cerabrina* (Hickel & A. Camus) Barnett, and (f) *C. diversifolia* (Kurz) King & Hook.f. Arrow in (d) show secondary constriction.

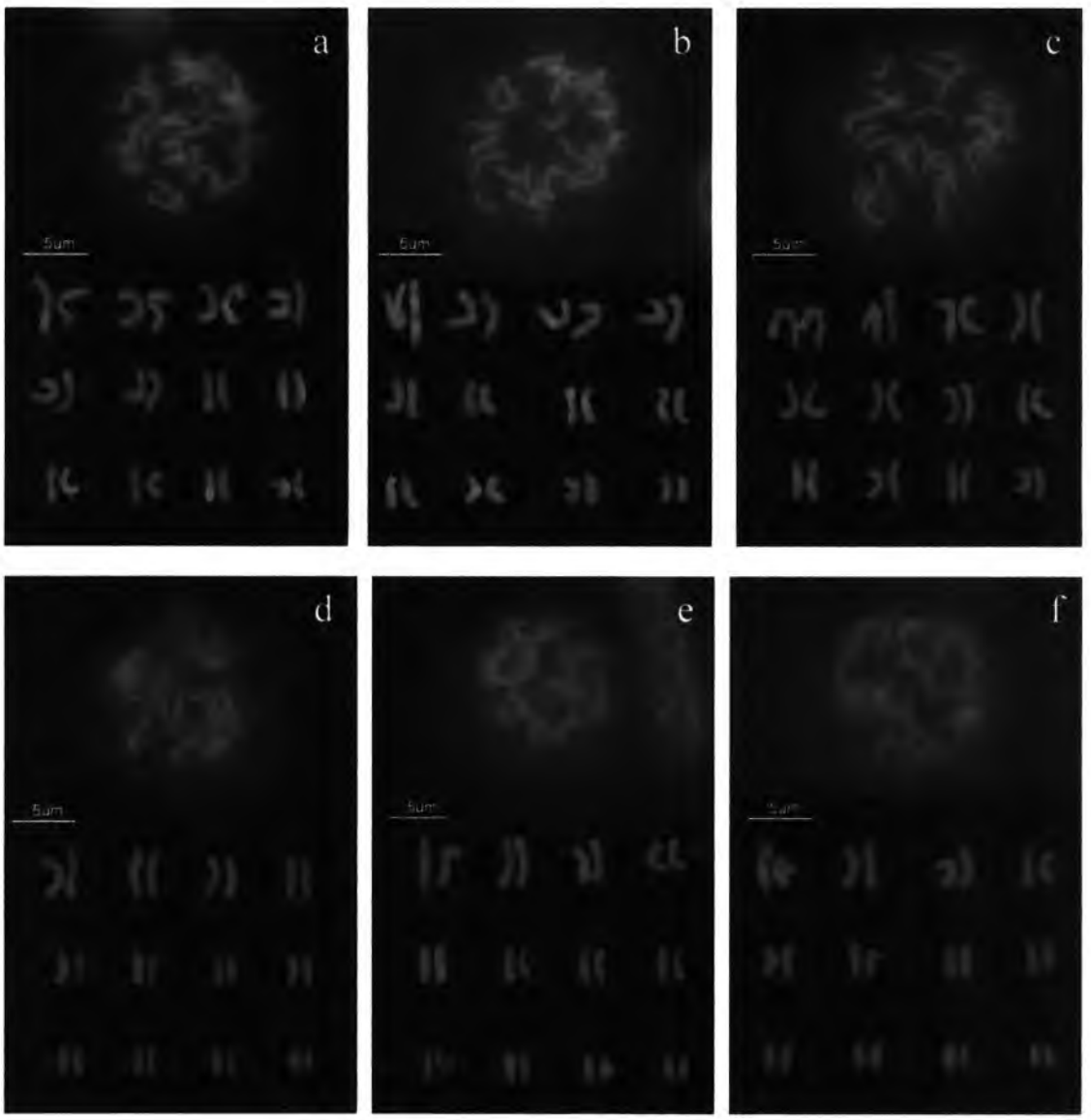

Figure 4.8: Mitotic metaphases and karyotypes of (a) *C. fissa* (Champ) Rehder & Wilson, (b) *C. indica* (Roxb.) A.DC., (c) *C. tribuloides* (Sm.) A.DC., (d) *L. ceriferus* (Hickel & A. Camus) A. Camus, (e) *L. elegans* (Blume) Harus ex Soepadmo, and (f) *L. harmandianus* (Hickel & A. Camus) A. Camus.

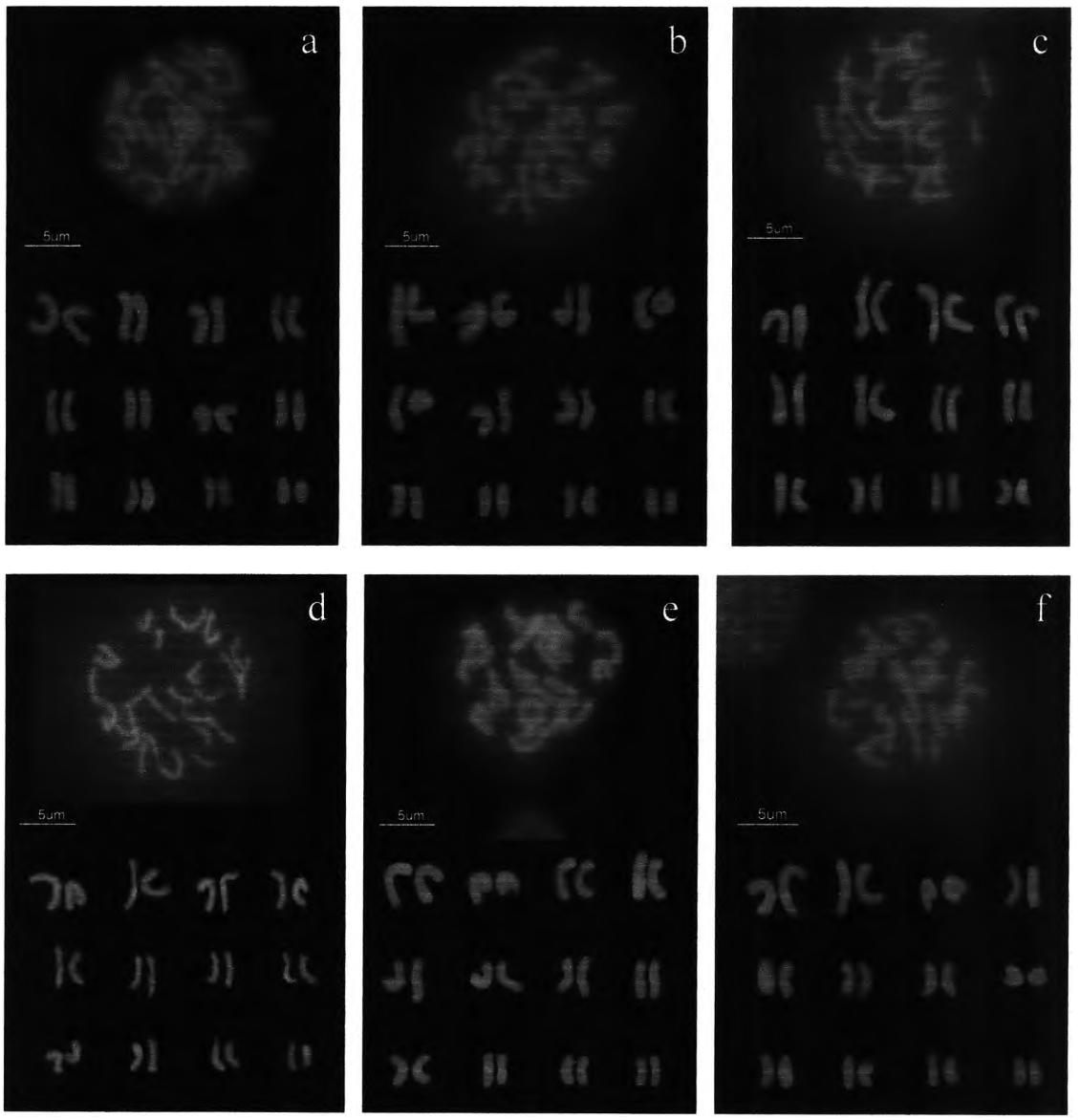

Figure 4.9: Mitotic metaphases and karyotypes of (a) *L. recurvatus* Barnett, (b) *L. vestitus* (Hickel & A. Camus) A. Camus), (c) *Q. brandisianus* Kurz, (d) *Q. kerrii* Craib, (e) *Q. mespilifolius* Wall. ex DC., and (f) *Q. rex* Hemsl.

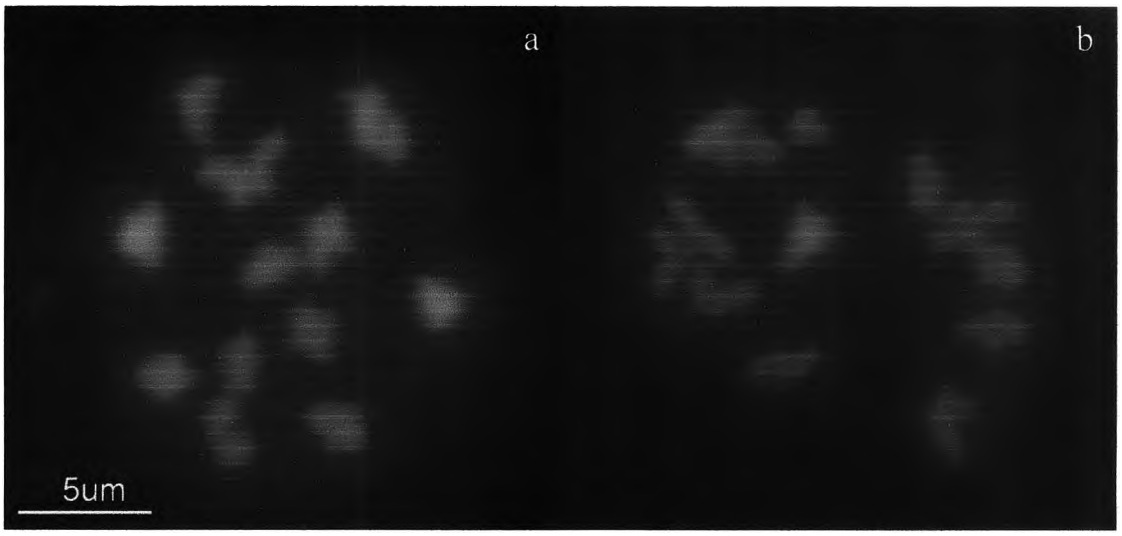

Figure 4.10: Meiotic chromosomes of (a) *C. indica* and (b) *C. tribuloides*, showing 12 bivalents.

Figure 4.11: Mitotic metaphase and karyotype of *Q. lenticellatus* Barnett (Sample RD17), showing 7 pairs of chromosomes.

4.3.2 Fluorescence *in situ* hybridization

Molecular cytogenetic mapping of the highly repetitive 18S-25S ribosomal gene (clone pTa71) on chromosomes by fluorescence *in situ* hybridisation (FISH) was performed successfully on 16 species: 9 *Castanopsis*, 4 *Lithocarpus* and 3 *Quercus* species. In addition, the mapping of 5S ribosomal repeats (clone pTa794) was performed successfully on 14 of these species. The numbers of the 18S-25S and 5S ribosomal loci were presented in Table 4.5. Almost all of these species showed four sites (two pairs) of 18S-25S rRNA gene and two sites (one pair) of 5S rRNA gene (Figures 4.12 – 4.17). Three species, i.e. *C. armata*, *C. indica* and *L. elegant*, showed two major and two minor sites of 18S-25S rRNA gene (Figures 4.12, 4.14 and 4.16). Two species, i.e. *C. argentea* and *Q. brandisianus*, showed two sites (one pair) of 18S-25S rRNA gene (Figures 4.12 and 4.17). On the other hand, two species, i.e. *C. calathiformis* (two samples) and *L. vestitus* (one sample), showed odd number of (unpaired) rDNA sites, indicating hybrid origin of species or individual trees. The two samples of *C. calathiformis* showed three sites of 18S-25S rRNA gene and two sites (one pair) of the 5S rRNA gene (Figure 4.18). Six sites (five major and one minor sites) of the 18S-25S rRNA gene were observed in *L. vestitus*, together with three unpaired sites of the 5S rRNA gene (Figure 4.19). Molecular cytogenetic mapping by FISH was also performed successfully with *Q. lenticellatus* which had the chromosome number 2n = 14. The results showed four sites (two pairs) of 18S-25S rRNA gene and two sites (one pair) of 5S rRNA gene (Figure 4.20), confirming that this is not a haploid plant.

The 5S rRNA gene map seems to be conserved – nearly all species had the same number of loci and position. These samples had one pair of paracentromeric major sites. The number and position of 18S-25S rRNA gene is much more variable. The first major pair is subtelomeric (subterminal) in most species observed, while the second pair is intercallary except in *Q. kerrii* where both pairs are subterminal. When examined both sequences together, the ribosomal gene maps were significantly genus-specific (Figure 4.21). In most *Castanopsis*, the 18S-25S and the 5S ribosomal genes were localized on different chromosome pairs, but *Lithocarpus* typically had 18S-25S

and 5S genes localizing next to each other on the same chromosome arms. In *Quercus*, the maps were variable, including both rRNA genes on different chromosomes (e.g. *Q. brandisianus*) and on the same chromosome arms with subterminal 18S-25S rDNA loci and paracentromeric 5S rDNA loci (e.g. *Q. kerrii*). Based on these rDNA maps, there is a good indication of introgressive hybridisation or gene flow between species belonging to different genera, or that we have genetic evidence supporting that certain species could be more correctly placed in another genus. For example, *C. fissa* has the typical rDNA map of *Lithocarpus*, i.e. the two ribosomal genes are locating next to each other. Other evidences, molecular and morphological, also indicate that *C. fissa* is more closely related to *Lithocarpus* than to other species of *Castanopsis*. The *Q. lenticellatus* tree (sample RD17) that has chromosome number 2n = 14 also showed the localization of 5S and 18S-25S rRNA genes next to each other, indicating that this sample is more closely related to *Lithocarpus*.

Table 4.5: Number of 18S-25S and 5S rDNA loci on chromosomes of Fagaceae spp.

Species	Sample	Chro. no.	18S-25S rDNA	5S rDNA	Note
Ca	BA22	24	4 sites (2 pairs)	2 sites (1 pair)	
Cag	PA14	24	2 sites (1pair)	2 sites (1 pair)	a
Cam	PA17	24	4 sites (2 pairs)	2 sites (1 pair)	d
Cc	NK5	24	3 sites	2 sites (1 pair)	
Cc	PA25	24, 28	3 sites	2 sites (1 pair)	a, b
Cce	NK23	24	4 sites (2 pairs)	2 sites (1 pair)	
Cd	NK1	24	4 sites (2 pairs)	2 sites (1 pair)	
Cfi	PA9	24	4 sites (2 pairs)	2 sites (1 pair)	b
Ci	PA18	24	4 sites (2 pairs)	2 sites (1 pair)	d
Ct	RD14	24	4 sites (2 pairs)		c
Lc	RD1	24	4 sites (2 pairs)		c
Le	NK24	24	4 sites (2 pairs)	2 sites (1 pair)	a, d
Lp	RD7	24	4 sites (2 pairs)	2 sites (1 pair)	
Lv	PA11	24	6 sites	3 sites	e
Qb	PS9	24	2 sites (1pair)	2 sites (1 pair)	b
Qk	HH9	24	4 sites (2 pairs)	2 sites (1 pair)	
Ql	RD17	14	4 sites (2 pairs)	2 sites (1 pair)	

Notes: a: From interphase only.
b: From only one cell.
c: Signal from thick metaphase preparations.
d: One pair major and one pair minor sites of 18S-25S
e: Five major and one minor sites of 18S-25S

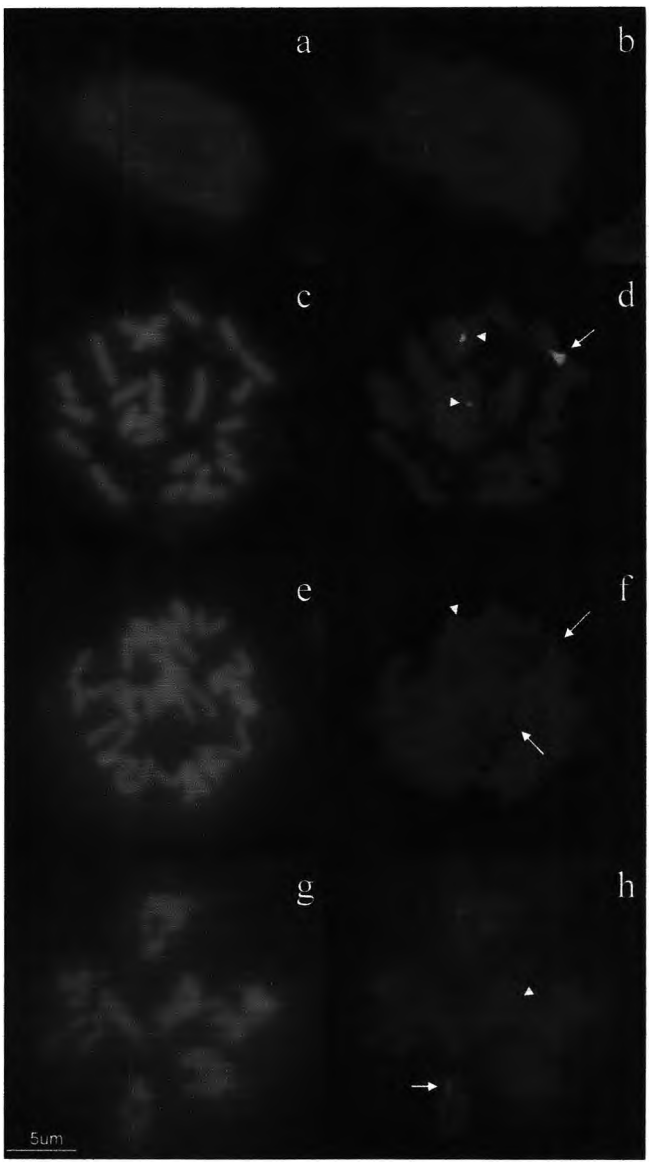

Figure 4.12: Fluorescence *in situ* hybridisation of the 18S-25S rDNA (Clone pTa71) and the 5S rDNA (clone pTa794) to interphase chromatin (a, b), mitotic metaphase chromosomes (c-f) and meiotic chromosomes (g, h) of *C. argentea* (Blume) A.DC. (a, b), *C. acuminatissima* (Blume) A.DC. (c, d), and *C. indica* (Roxb.) A.DC. (e-h), counterstained with DAPI. Green-FITC signals represent the 18S-25S rDNA while red fluorescent signals represent the 5S rDNA. These three species show two sites (one pair) of the 5S rDNA loci on mitotic chromosomes and one meiotic bivalent. There are four sites (two pairs) of the 18S-25S rDNA in *C. acuminatissima* (d), the first pair is at subtelomeric region (arrows) and the second pair is on chromosome arm (arrow heads). There are two sites (one pair) of the 18S-25S rDNA in *C. argentea* (b). *C. indica* show two major sites at subtelomeric region (arrows) and two minor sites (arrowheads) of the 18S-25S rDNA (f, h).

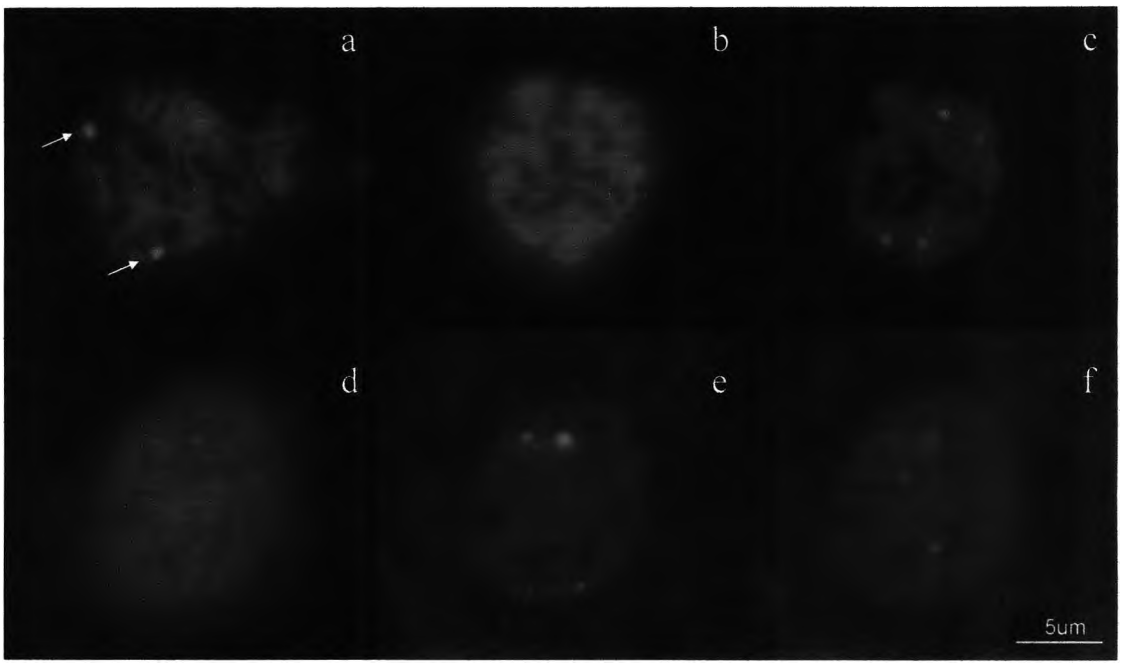

Figure 4.13: Fluorescence *in situ* hybridisation of the 18S-25S rDNA (Clone pTa71) and the 5S rDNA (clone pTa794) to mitotic metaphase chromosomes (a-c) and interphase chromatin (d-f) of *C. cerabrina* (Hickel& A. Camus) Barnett, counterstained with DAPI. There are four sites (two pairs) of the 18S-25S rDNA (red fluorescent signals in a, green-FITC in c and e), the first pair is at subtelomeric region (arrows in a) while the second pair is on chromosome arm. The red fluorescent signals in c and f correspond to two sites (one pair) of the 5S rDNA.

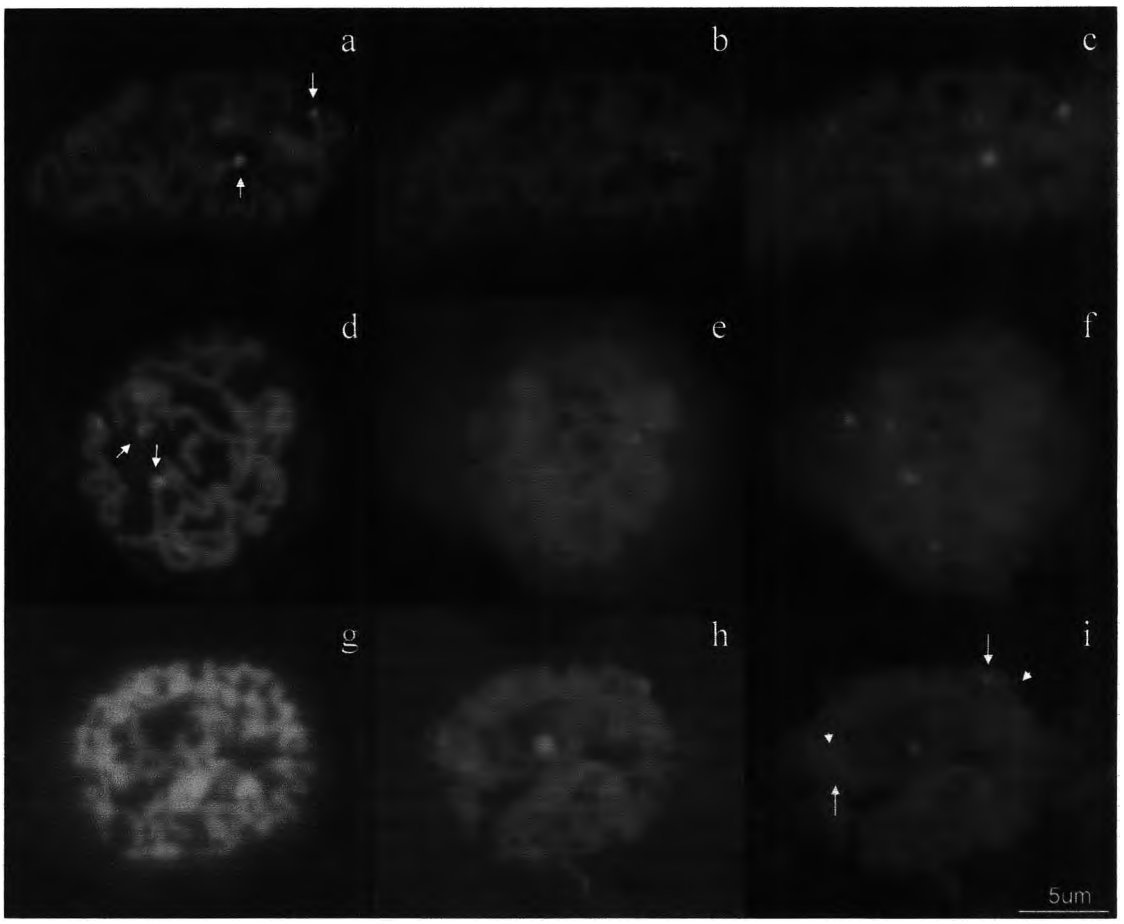

Figure 4.14: Fluorescence *in situ* hybridisation of the 18S-25S rDNA (Clone pTa71) and the 5S rDNA (clone pTa794) to mitotic metaphase chromosomes of *C. armata* (Roxb.) Spach (a-c), *C. diversifolia* (Kurz) King & Hook.f. (d-f), and *C. fissa* (Champ) Rehder& Wilson (g-h), counterstained with DAPI. There are four sites (two pairs) of the 18S-25S rDNA (red fluorescentsignals in a, c, d, and f, green-FITC in h and i) and two sites (one pairs) of the 5S rDNA (green-FITC in b and e, red fluorescent signals in i). The major pair and the first pair of the 18S-25S rDNA in *C. armata* and *C. diversifolia* respectively, are at subtelomeric region (arrows in a and d). *C. fissa* show that the 5S rDNA (arrowheads in i) and the 18S-25S rDNA (arrows in i) are on the same chromosome.

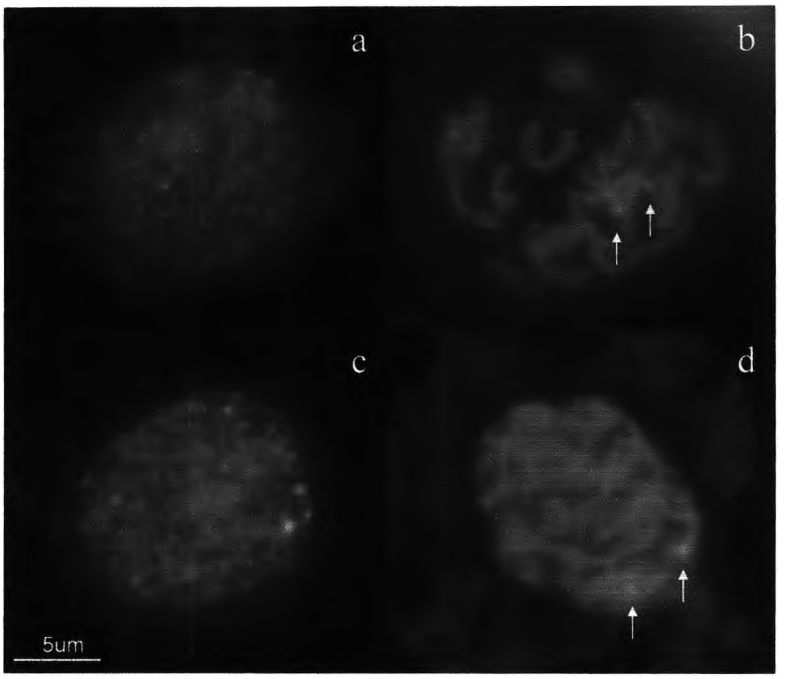

Figure 4.15: Fluorescence *in situ* hybridisation of the 18S-25S rDNA (Clone pTa71) to interphase chromatin (a, c) and mitotic metaphase chromosomes (b, d) of *C. tribuloides* (Sm.) A.DC. (a, b) and *L. ceriferus* (Hickel& A. Camus) A. Camus (c, d), counterstained with DAPI. There are four sites (two pairs) of the 18S-25S rDNA (red fluorescent signals), the first pair is at subtelomeric region (arrows).

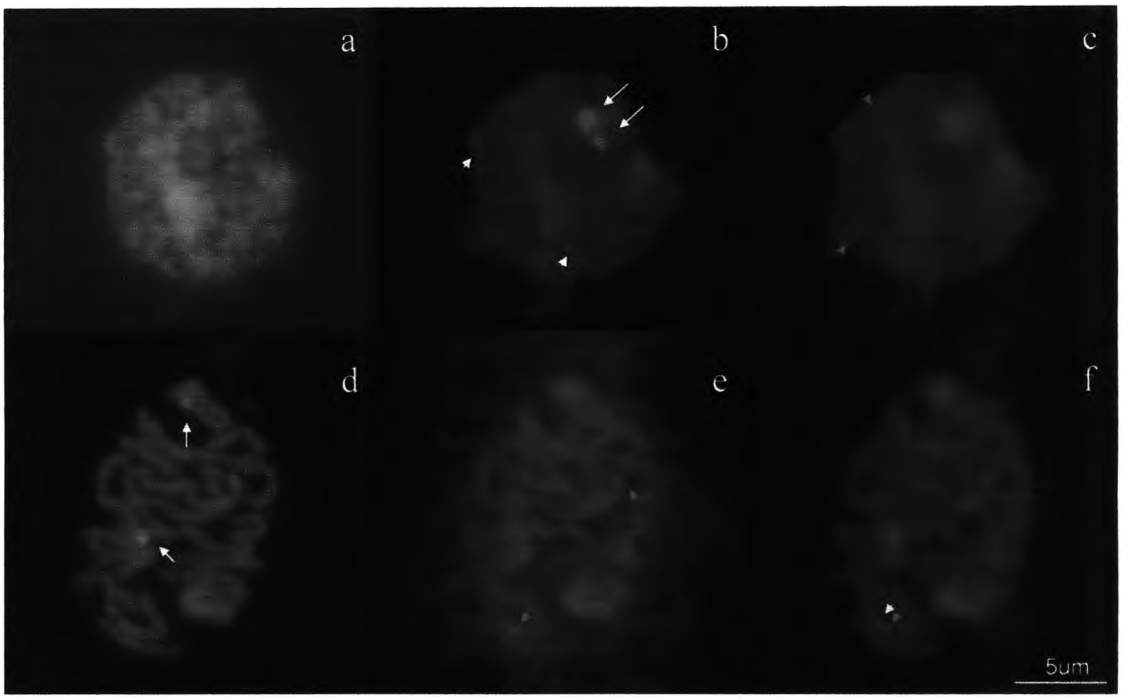

Figure 4.16: Fluorescence *in situ* hybridisation of the 18S-25S rDNA (Clone pTa71) and the 5S rDNA (clone pTa794) to interphase chromatin of *L. elegans* (Blume) Harusex Soepadmo (a-c) and mitotic metaphase chromosomes of *L. polystachyus* (A. DC.) Rehder (d-f), counterstained with DAPI. There are four sites (two pairs) of the 18S-25S rDNA (green-FITC signals in b and c, red fluorescent signals in d and f) and two sites (one pair) of the 5S rDNA (red arrowheads; red fluorescent signals in c, green-FITC in e and f). *L. elegants* show two major sites (white arrows in b) and two minor sites (white arrowheads in b) of the 18S-25S rDNA. The first pair of the 18S-25S rDNA in *L. polystachyus* is at subtelomeric region (white arrows in d), while the second pair is next to the 5S rDNA on the same chromosome (red and white arrowheads in f).

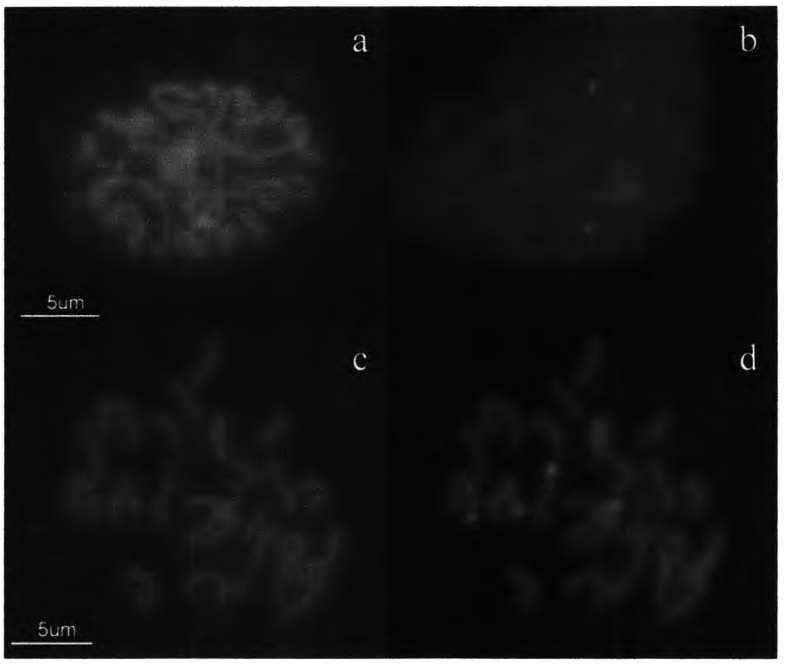

Figure 4.17: Fluorescence *in situ* hybridisation of the 18S-25S rDNA (Clone pTa71) and the 5S rDNA (clone pTa794) to mitotic metaphase chromosomes of *Q. brandisianus* Kurz (a, b) and *Q. kerrii* Craib (c, d), counterstained with DAPI. *Q. brandisianus* show two sites (one pair) of both the 18S-25S rDNA (green-FITC signals) and the 5S rDNA (red fluorescent signals). There are four sites (two pairs) of the 18S-25S rDNA (red fluorescent signals) and two sites (one pair) of the 5S rDNA (green-FITC signals) in *Q. kerrii*. All of the 18S-25S rDNA are at subtelomeric region, while the 5S rDNA is at paracentromeric region of the same chromosome as the first pair of the 18S-25S rDNA.

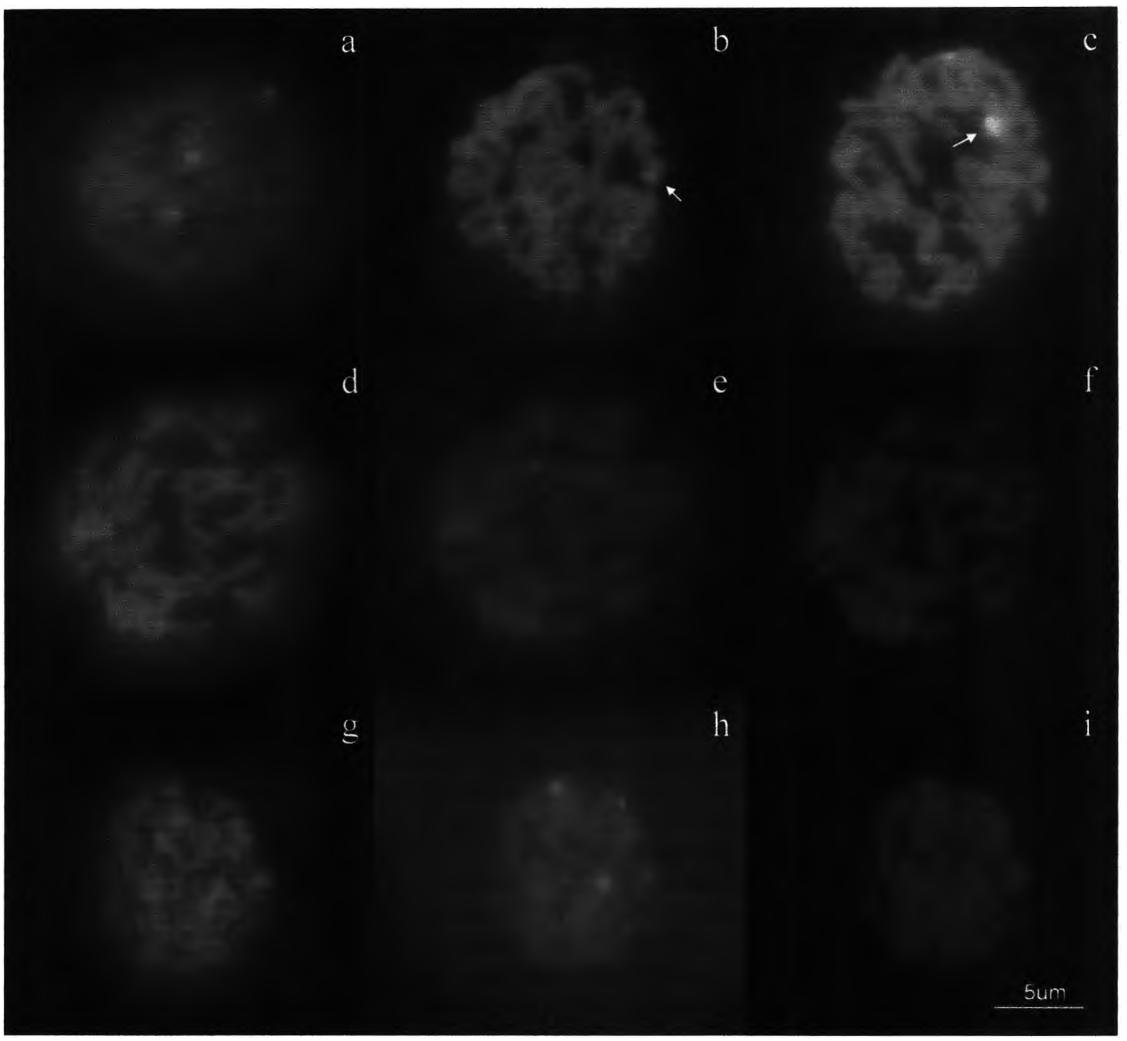

Figure 4.18: Fluorescence *in situ* hybridisation of the 18S-25S rDNA (Clone pTa71) and the 5S rDNA (clone pTa794) to interphase chromatin (a, g-i) and mitotic metaphase chromosomes (b-f) of *C. calathiformis* (Skan.) Rehder& Wilson, sample NK5 (a-f) and sample PA25 (g-i), counterstained with DAPI. There are three sites (un-pair) of the 18S-25S rDNA (red fluorescent signals in a-c, green-FITC signals in e and h) and two sites (one pair) of the 5S rDNA (red fluorescent signals in f and i). One site of the 18S-25S rDNA is at subtelocentric region (arrows in b and c).

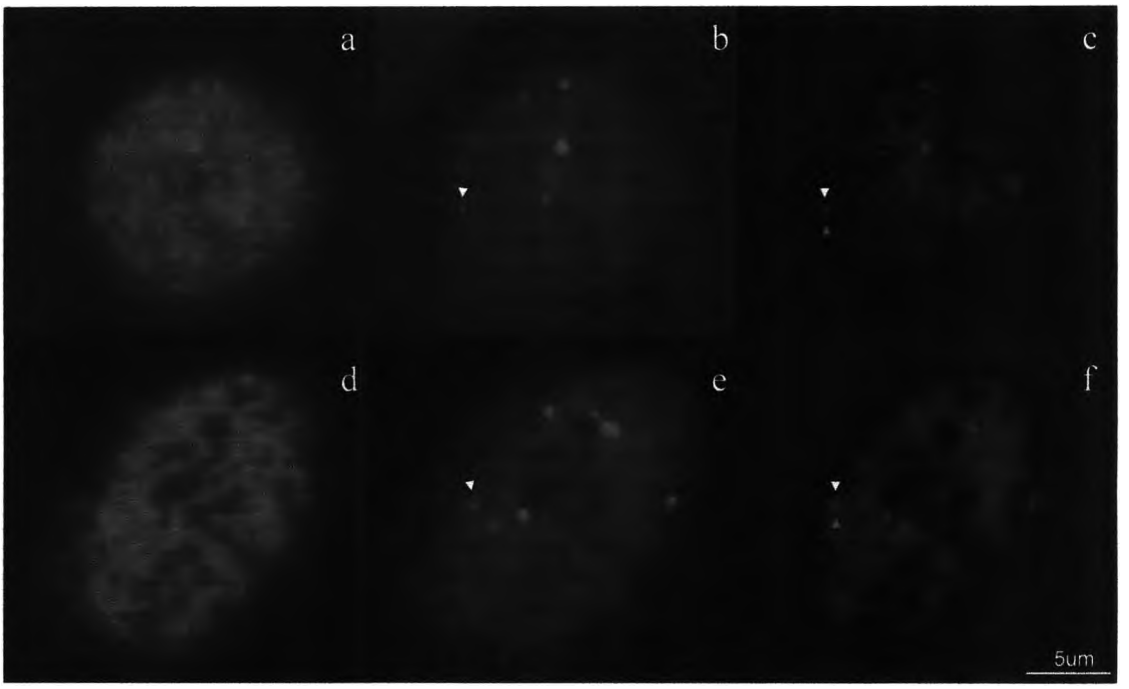

Figure 4.19: Fluorescence *in situ* hybridisation of the 18S-25S rDNA (Clone pTa71) and the 5S rDNA (clone pTa794) to interphase chromatin (a-c) and mitotic metaphase chromosomes (d-f) of *L. vestitus* (Hickel& A. Camus) A. Camus, counterstained with DAPI. There are five major sites of the 18S-25S rDNA (green-FITC signals) and one minor site of the 18S-25S rDNA (arrowheads), and 3 sites of the 5S rDNA (red arrowheads, red fluorescent signals). The minor site of the 18S-25S is next to one site of the 5S rDNA.

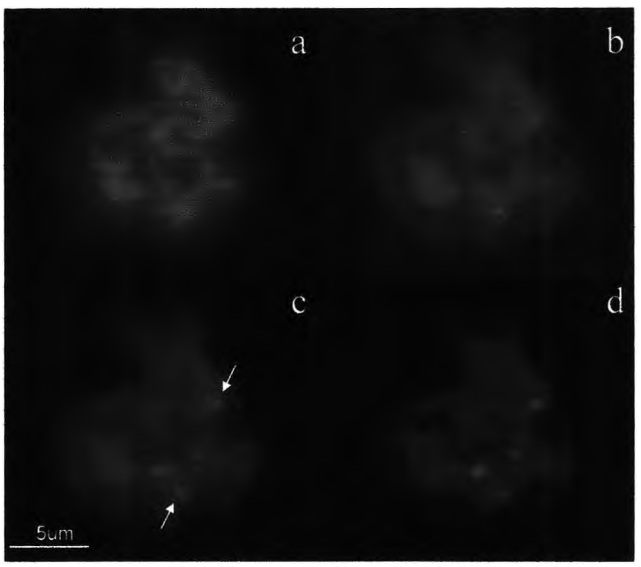

Figure 4.20: Fluorescence *in situ* hybridisation of the 18S-25S rDNA (Clone pTa71) and the 5S rDNA (clone pTa794) to mitotic metaphase chromosomes of *Q. lenticellatus* Barnett (sample RD17), counterstained with DAPI. There are four sites (two pairs) of the 18S-25S rDNA (red fluorescentsignals) and two sites (one pair) of the 5S rDNA (green-FITC signals). One pair of the 18S-25S is next to the 5S rDNA (arrows).

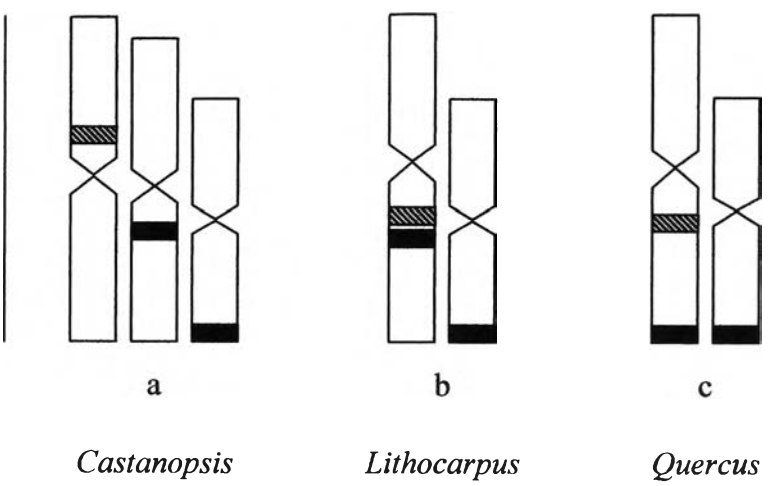

Figure 4.21: Idiogram showing localization of 18S-25S (black colour) and 5S (hatched) ribosomal genes on chromosomes of *Castanopsis* (a), *Lithocarpus* (b) and *Quercus* (c). Each chromosome represents a homologous pair. Vertical scale bar represents 5 μm.