RESULTS AND DISCUSSION

Isolation and characterization of microsatellite sequences

Extraction of genomic DNA

Genomic DNA of neem was extracted by using DNeasy? Plant Mini Kit (Qiagen, Hilden). The DNA was of good quality as it was free from the contaminants such as proteins, RNA and polysaccharides as shown in Figure 16.

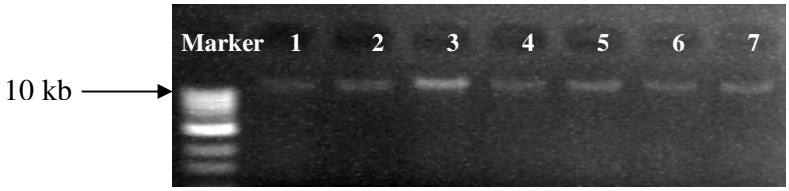

Figure 16 Genomic DNA of neem**.**

Digestion and ligation of genomic DNA

Digestion of neem genome with *Rsa*I produced fragments ranging from 200 to 1000 bp. Digested DNA fragments were ligated with 21-mer and 25-mer adapter. The success of digestion and ligation with 21-mer and 25-mer adapter to the restricted - genomic DNA was tested by using PCR (Figure 17).

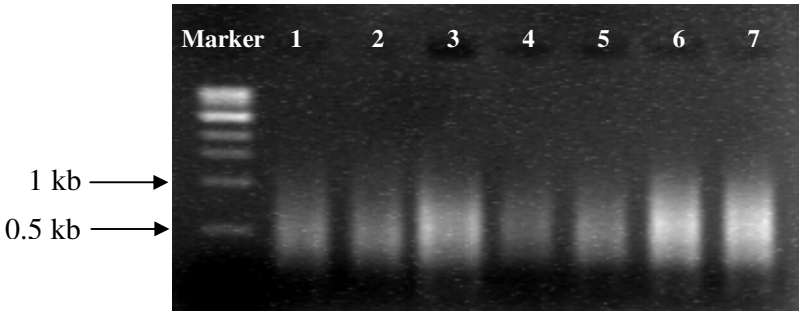

Figure 17 Photo showing the PCR products after digestion and ligation of neem genomic DNA.

Hybridization of oligonucleotide probe

The hybridization of artificial microsatellite oligonucleotide $(CT)_{10}$ with the restricted-ligated genomic DNA fragments was successful. The success of the enrichment was tested by using 21-mer oligonucleotide as primer. The PCR amplification test is shown in Figure 18. Fragments sizes distributed continuously (≤ 500 bp).

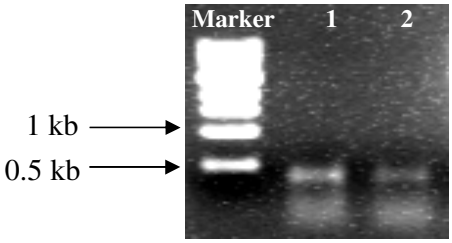

Figure 18 PCR products after enrichment of microsatellite**.**

Cloning into a plasmid vector

The pGEMT-easy vector (Promega) was ligated with the fragments containing microsatellites and transformed into competent cells of *E. coli* strain DH5α**.** After blue white colony selection, 68 white colonies were picked and transferred into a new LB plate. The colonies were amplified by colony PCR with universal vector primers (T7 and SP6) as shown in Figure 12. The clones insert sizes of the enriched microsatellites ranged from 300 to 700 base pairs with the average size of 437 base pairs.

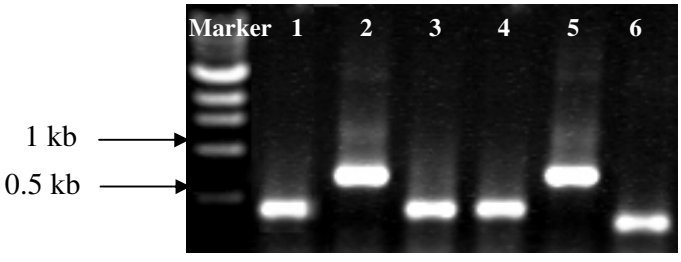

Figure 19 Picture of colony PCR.

Sequencing of DNA fragment and microsatellite identification

According to sequencing of all 68 colonies, the results showed that 47 sequences (69.1 %) contained microsatellite repeats. The analysis of these 47 sequences showed that 42 fragments contained one motif and other 5 fragments with 2 motifs. Base on the organization of the identified repeat motifs, microsatellite sequences were classified as 39 perfect and 15 imperfect repeats. Out of 52 motifs comprising of 35 (67%) AG/TC, 12 (23%) TG/AC and 5 (10%) TA/AT. Examples of microsatellite repeat motifs are shown in Figure 20.

The microsatellite length of repeat arrays varied. The shortest and the longest repeat motif were $(TA)_3$ and $(GA)_{24,}$ respectively. The average number of repeats motif was 11.87.

Designing primers

Out of 47 sequences, 21 sequences were not exploitable as the SSR markers, since repeat motifs were located close to the vector cloning site without enough flanking region to design primers. Over all, 26 unique sequences containing SSR were available for primer designing and were used to examine the amplification of microsatellite loci in 24 neem populations. The details of primer sequences, repeat motifs, complexity, type and expected size of 26 SSR loci were shown in Table 4.

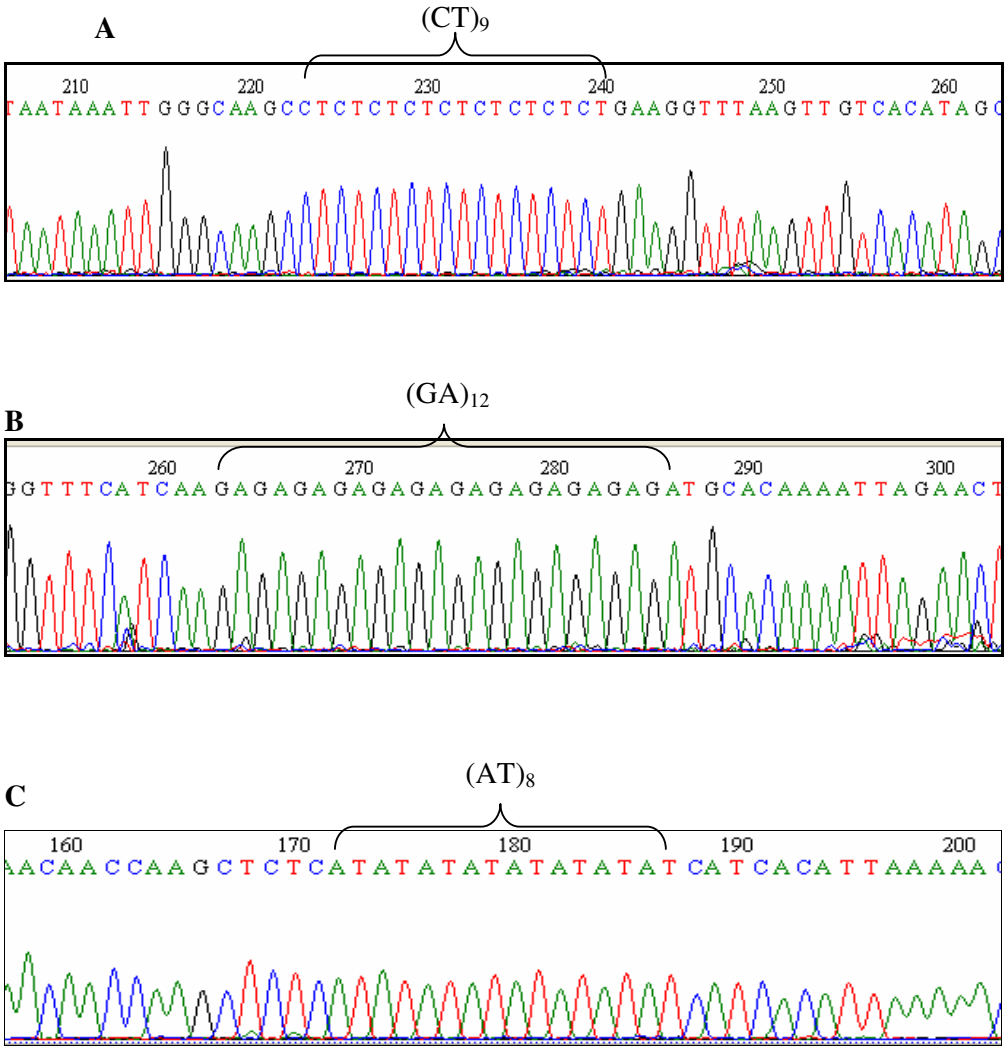

Figure 20 Example of a sequence containing microsatellite repeat motif in neem A. $(CT)_9$, B. $(GA)_{12}$ and C $(AT)_8$.

Table 4 Primer sequences, repeat motif, complexity, type, melting temperature (T_m) and expected product size of 26 SSR loci developed in neem

Primer Name	Repeat Type	Complexity	Type	Primer Sequences	Tm (^oC)	Exp. Size (bp)
NpCT_4_Left	$(GA)_7$	simple	perfect	TGGTAACCAATCTGTGTGTGC	59	224
NpCT_4_Right				CGGTTCCTGGTTTCTTTTGG	61	
NpCT_5_Left	$(CA)_{15}$	simple	imperfect	GAAAGGAGGGTTTTCAAATCA	59	151
NpCT_5_Right				TCGGCCGAACACAATTTTA	60	
NpCT_6_Left	$(CT)_{22}$	simple	imperfect	ACAAAATTTTTCCCGTCGAG	59	150
NpCT_6_Right				AGAGCTATGAATGGTGGACTCAC	59	
NpCT_7_Left	$(CT)_7$	simple	perfect	AACTATGGAGAATTCTGGAATCTTG	59	101
NpCT_7_Right				TTATCCATCTGGAGAATCAGAAA	57	
NpCT_8_Left	$(CT)_9$	simple	imperfect	AACATGGCCATTTGTTCCTC	59	154
NpCT_8_Right				GACTGATTCCGGGGGTAAAG	60	
NpCT_11_Left	$(GA)_{19}$	simple	imperfect	GCATCAGTCAGCCATAGTGC	59	206
NpCT_11_Right				TTGAAAAATCCTGGCGAGTG	60	
NpCT_12_Left	$(CT)_9$	simple	perfect	ACAAACAATCAAAAATCAACTGG	58	100
NpCT_12_Right				TGCAAATTTTAAGATCCCAAGC	60	
NpCT_13_Left	$(CT)_8$	simple	perfect	CCACAAACAAATGGGAAACC	60	158
NpCT_13_Right				CCCTTATTACAAAAGAAGAGGGAAG	59	
NpCT_14_Left	$(CT)_{10}$	simple	perfect	GTCCACGCAAACAGAGACAC	59	232
NpCT_14_Right				TTGGCTTGGCTTTCTCTTTC	59	
NpCT_15_Left	$(CA)_{10}$	simple	perfect	TTCATCATAACACCCCTGACTC	59	183
NpCT_15_Right				TTTGTATTGATACCGAGCAAGC	59	
NpCT_21_Left	$(CT)_9$	simple	perfect	CATGTGGATCGGACAATACG	59	187
NpCT_21_Right				TGGGTTTCACTCACACATGG	60	
NpCT_22_Left	$(GA)_8$	simple	perfect	TCCGATTCCAACTCAAAAGG	60	236
NpCT_22_Right				CCGTAGCCTCCCTATATAAATCC	59	
NpCT_23_Left	$(GT)_{13}$	simple	perfect	GGGAAGTTAGGATCATTTTATGC	58	167
NpCT_23_Right				GACTCATGAGGCTTTGTGTTTG	59	
NpCT_26_Left	$(GA)_{12}$	simple	perfect	AATTTTCAGTTAAGAGTTCTGGTTCC	59	151
NpCT_26_Right				ACTGGTATTCAAAGTGACAAAGC	58	
NpCT_28_Left	$(GA)_8$	simple	perfect	CCTCCGATTCCACTCAAAAG	59	237
NpCT_28_Right				CCGTAGCCTCCCTATATAAATCC	59	
NpCT_30_Left	$(CA)_{18}$	simple	imperfect	TGTTTTTCTTCTCTTCCTTCCTTC	59	151
NpCT_30_Right				TTTGAAATCCATTTTGCACAG	58	
NpCT_34_Left	$(GA)_{18}$	simple	perfect	ATTTGTGTGTGCGTGCTAGG	59	156
NpCT_34_Right				CGAGGAACTGAGACTCCTGAA	59	

Table 4 (Continued)

Primer Name	Repeat Motif	Complexity	Type	Primer Sequences	Tm (^{O}C)	Exp.Size (bp)
NpCT_40_Left	$(CT)_9$	simple	perfect	TGTGGATCGGACAATACGAA	59	185
NpCT_40_Right				TGGGTTTCACTCACACATGG	60	
NpCT_41_Left	$(CT)_{10}$	simple	imperfect	GGCGTGAAGCTCACTCTGAT	60	168
NpCT_41_Right				CCCATTGCAGTCTCTTTCTCT	58	
NpCT_43_Left	$(CA)_{18}TA)_4$	compound	perfect	TTCAGTGTTCGAAGACATAGATCC	59	171
NpCT_43_Right				CTACAATTTCACGCCACACAC	59	
NpCT_45_Left	$(GT)_{14}$	simple	perfect	TCCGAAAGGAAAACGAATTAAG	59	124
NpCT_45_Right				AACCTTGCCATCTTTCCTTG	59	
NpCT_48_Left	$(CA)_{10}$	simple	perfect	TCCCAGTTATTCAACGTAGGC	59	104
NpCT_48_Right				TCTTAATCATGGATTGCTTCACA	59	
NpCT_49_Left	$(CT)_{11}$	simple	imperfect	TGGAACTCACTCTGATAAAAATCAA	59	163
NpCT_49_Right				TGGATACCCATGCAGTTCTTT	59	
NpCT_52_Left	$(GA)_{25}$	simple	imperfect	AATTCGTGGTTCTTCAGTTGG	59	161
NpCT_52_Right				TGAGCAACTTTACTCATTGTTGTTT	59	
NpCT_53_Left	$(GA)_{10}$	simple	perfect	ATTTCGCATTGCTTTTGCTT	59	163
NpCT_53_Right				CGGATTCTCGCAACATTAAA	58	
NpCT_59_Left	$(GA)_8$	simple	perfect	AGTGCAGCTGAAGGAGGAAG	59	212
NpCT_59_Right				TTGGCACAAAGTGGTTTCAG	59	
NpCT_63_Left	$(CT)_{19}$	simple	imperfect	TCAACCTACTTTTAGTCAAGCACAAG	60	150
NpCT_63_Right				CTTTCCATATGGTCGACTGC	58	
NpCT_68_Left	$(CT)_{11}$	simple	perfect	TCGTCATGACCTCCCTCTTC	60	153
NpCT_68_Right				TCTTGCTTACGCGTGGATAAC	60	

Screening primers

The 26 primer pairs obtained from the neem genomic library were used to test in 24 neem populations as shown in Figure 21**.** Out of 26 primers, 8 primer pairs failed to amplify fragments. Eighteen primer pairs could amplify, but 10 primer pairs were able to amplify the expected sizes. However, only 8 primer pairs as shown in Table 5 produced clear polymorphic and easily scorable bands and can be used to analyze genetic relationship of both Thai neem and Indian neem.

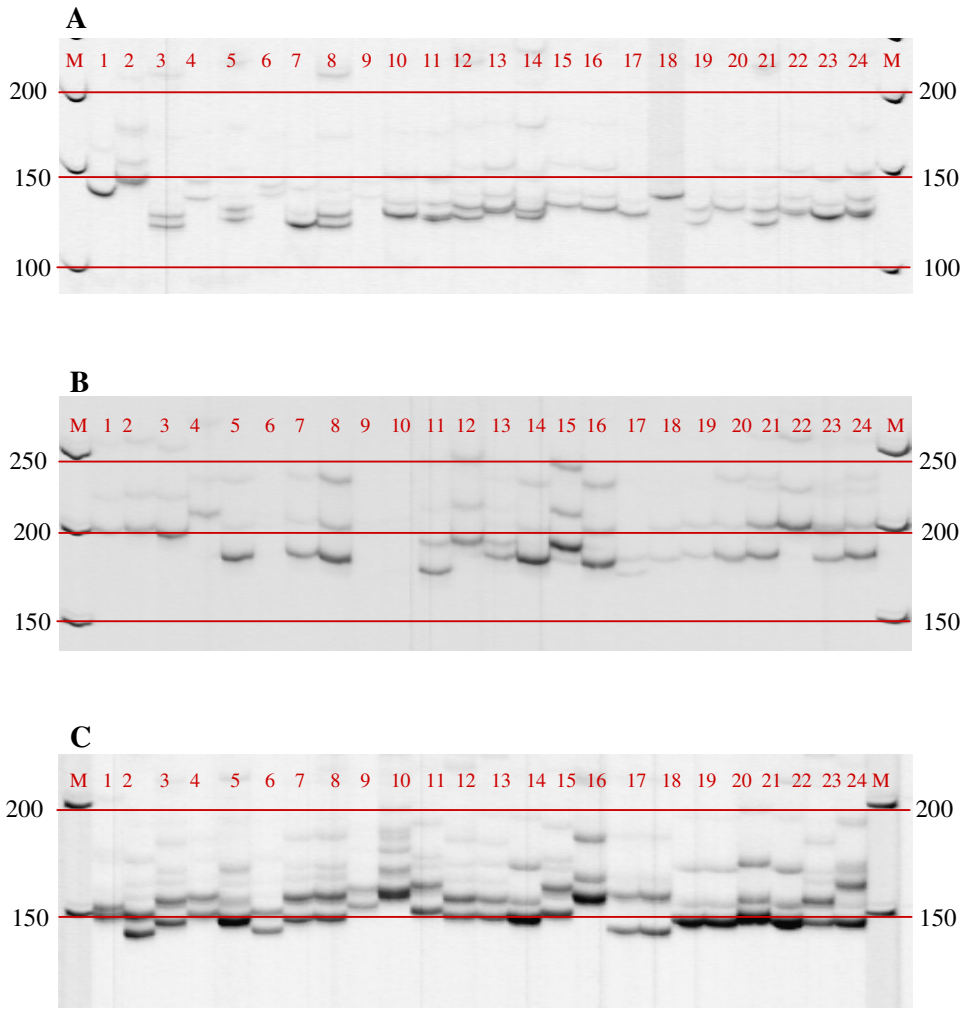

Figure 21 Primer screening of 24 populations of neem. (A) Primer NpCT_6, (B) Primer NpCT_11 and (C) Primer NpCT_48. Lanes M : 50 bp ladder.

Remark: Population names of 1-24 were shown in Table 3.

Table 5 Eight microsatellite markers selected for determination of genetic variation in neem populations

Primer/Locus name	Number of alleles per locus	Observed product size (bp)
NpCT_4	4	220-250
NpCT_5	9	130-170
NpCT_6	6	120-170
NpCT_11	8	140-200
NpCT_13	7	100-150
NpCT_14	5	150-200
NpCT_34	8	130-170
NpCT_48	9	100-150
Total	**56**	
Mean	**7**	

Application of microsatellite markers in determination of genetic variation in neem populations

Eight microsatellite markers which were developed previously were used in this study. The results of variation of DNA fragments at each locus derived from PCR products using those 8 primers were scored and statistically analysed as follows;

Number of alleles per locus

Total number of alleles per locus in Indian neem and Thai neem were shown in Table 6. All eight primer pairs produced a low to moderate level of polymorphisms. The number of alleles per locus was observed in 24 neem populations. The number of alleles ranged from 4 (NpCT_4) to 9 (NpCT_5 and NpCT_48) with an average of 7 alleles per locus. Observed PCR products size differences from all the polymorphic loci ranged from 100 to 250 bp.

Both Indian neem and Thai neem showed number of alleles per locus range from 4 to 9 alleles (Table 6). At loci NpCT_4, NpCT_5 and NpCT_34, it revealed that the number of alleles per locus were equal both in Indian neem and Thai neem. At locus NpCT_4, the lowest (4) number of alleles was found in both Indian neem and Thai neem.

The number of alleles per locus in Thai neem was lower than in Indian neem at locus NpCT_13, NpCT_14 and NpCT_48. The number of alleles found in Indian neem (53 alleles) was slightly higher than in Thai neem (49 alleles). Accordingly, the average number of alleles observed in Indian neem was 6.624 and Thai neem was 6.125.

Effective number of alleles

The effective number of alleles found in Indian neem and Thai neem is presented in Table 6. Out of 8 loci, only at loci NpCT_6 and NpCT_14 in Indian neem showed lower effective number of alleles than in Thai neem. Indian neem showed the lowest (2.74) and the highest (7.77) effective number of alleles at locus NpCT_14 and NpCT_5, respectively. Thai neem showed the lowest (1.59) and the highest (5.16) at locus NpCT_13 and NpCT_34, respectively. The total effective number of alleles in Indian neem (38.16) was higher than in Thai neem (29.64). Accordingly, the average effective number of alleles in Indian neem was 4.77 and in Thai neem was 3.71.

Table 6 Number of alleles per locus and effective number of alleles in Indian neem and Thai neem

Primer/Locus	Number of alleles per locus (n_a)		Effective number of alleles (n_e)	
	Indian neem	**Thai neem**	**Indian neem**	**Thai neem**
NpCT_4	4	4	3.59	3.20
NpCT_5	9	9	7.77	4.49
NpCT_6	4	6	3.40	4.25
NpCT_11	7	8	4.76	4.14
NpCT_13	7	4	3.89	1.59
NpCT_14	5	4	2.74	3.11
NpCT_34	8	8	5.25	5.16
NpCT_48	9	6	6.76	3.70
Total	**53**	**49**	**38.16**	**29.64**
Mean	**6.625**	**6.125**	**4.77**	**3.71**

The results of the alleles presenting at each locus and each population of Indian neem and Thai neem is shown in Table 7. The lowest (1) number of alleles was detected in population 3 (Sunyani, Ghana) at locus NpCT_4 and the highest (9) was detected in population 16 (Annur, Tamil Nadu, India) and population 18 (Khao Laung, Thailand) at locus NpCT_5.

For all populations, population 13 (Ghaati Subramanya, Karnataka, India) showed the highest (5.325) mean number of alleles and population 22 (Multan, Cantonment Area, Pakistan) was the lowest (3.250). For all loci, locus NpCT_4 showed the lowest (2.79) mean number of alleles per locus and locus NpCT_48 showed the highest (5.79).

Table 7 Number of alleles per locus and mean of alleles per population in Indian neem and Thai neem

Population	Number of alleles per locus in Indian neem								Mean number of alleles per Population
	NpCT_4	NpCT_5	NpCT_6	NpCT_11	NpCT_13	NpCT_14	NpCT_34	NpCT_48	
Pop. 1 ★	2	5	3	5	3	2	5	4	3.625
Pop. 2 ★	2	4	2	5	3	2	5	4	3.375
Pop. 3	1	4	3	4	4	2	2	7	3.375
Pop. 4 ★	4	5	2	4	4	4	6	4	4.125
Pop. 5 ★	3	3	4	5	4	2	6	6	4.125
Pop. 6 ★	2	4	3	5	3	2	5	4	3.500
Pop. 7	2	8	3	6	6	3	7	7	5.250
Pop. 8	3	7	3	4	3	2	5	7	4.250
Pop. 9 ★	3	5	4	4	3	4	5	4	4.000
Pop. 10	3	6	3	6	4	2	3	7	4.250
Pop. 11	2	6	3	6	4	3	7	6	4.625
Pop. 12	2	5	3	4	4	4	6	8	4.500
Pop. 13	3	8	3	5	6	5	6	7	5.375
Pop. 14	2	4	3	4	5	3	6	8	4.375

Table 7 (Continued)

Population	Number of alleles per locus in Indian neem								Mean number of alleles per Population
	NpCT_4	NpCT_5	NpCT_6	NpCT_11	NpCT_13	NpCT_14	NpCT_34	NpCT_48	
Pop. 15	2	5	4	5	3	3	3	5	3.750
Pop. 16	2	9	3	5	5	3	7	8	5.250
Pop. 17	2	4	2	4	4	4	5	5	3.750
Pop. 18 ★	3	9	3	7	3	4	6	3	4.750
Pop. 19	2	5	4	7	5	3	2	7	4.375
Pop. 20	2	5	3	5	3	2	3	4	3.375
Pop. 21	3	5	4	4	4	4	3	4	3.875
Pop. 22	3	5	3	2	2	3	3	5	3.250
Pop. 23	3	5	4	5	5	3	3	8	4.500
Pop. 24	3	6	3	5	6	4	5	7	4.875
Mean	2.46	5.5	3.13	4.83	4.0	3.04	4.75	5.79	

Remark: Thai neem ★).

Allele frequencies of 24 neem populations

Allele frequencies of 24 neem populations are shown in Table 8. The results of the allele frequencies revealed that some alleles only found in Indian neem or Thai neem. For instance, at locus NpCT_6, the allele 5, 6 were found only in Thai neem and allele 1, 2, 3 found only in Indian neem. Furthermore, at locus NpCT_6 different common alleles (allele 5, 6) were found in Thai neem population 1 (Ban Bo, Kalasin, Thailand) and population 2 (Ban Nong Hoi, Kanchanaburi, Thailand), respectively. At locus NpCT_11 the alleles 5-8 were distributed in all Thai neem populations while the alleles 1-6 were present in all Indian neem populations.

Table 8 Allele frequency of Indian neem and Thai neem

Primer/ Locus	Allele	Allele frequency of neem population							
		Pop.1 ★	Pop.2 ★	Pop.3	Pop.4 ★	Pop.5 ★	Pop.6 ★	Pop.7	Pop.8
NpCT_4	1	-	-	-	0.0556	0.1739	-	-	0.0526
	2	-	-	-	0.1111	-	0.8750	-	-
	3	0.5333	0.4688	-	0.7222	0.1087	-	0.5833	0.4474
	4	0.4667	0.5312	1.000	0.1111	0.7174	0.1250	0.4167	0.5000
NpCT_5	1	0.0667	0.0625	-	0.0556		-	0.0556	0.0526
	2	-	-	-	-	-	-	-	-
	3	0.1333	0.3438	-	0.3333	-	0.2500	0.1667	0.0526
	4	0.2667	0.0312	0.2353	0.2778	-	-	0.1667	-
	5	-	-	-	-	0.0217	0.1667	0.0278	0.1316
	6	0.5000	0.5625	0.3824	0.3056	0.4565	-	0.2222	0.2368
	7	0.0333	-	0.0882	0.0278	0.5217	-	0.1667	0.2105
	8	-	-	0.2941	-	-	0.5417	0.1389	0.1053
	9	-	-	-	-	-	0.0417	0.0556	0.2105
NpCT_6	1	-	-	0.2059	-	0.0652	-	0.5556	0.5789
	2	-	-	0.3529	-	0.6304	-	0.3889	0.3684
	3	-	-	0.4412	-	0.2609	-	0.0556	0.0526
	4	0.0333	-	-	-	0.0435	0.0833	-	-
	5	0.7000	0.2500	-	0.5000	-	0.5000	-	-
	6	0.2667	0.7500	-	0.5000	-	0.4167	-	-
NpCT_11	1	0.1000	0.1526	0.0588	0.0833	0.2174	0.1667	0.0556	0.1316
	2	-	-	0.2941	-	0.3478	-	0.4722	0.5263
	3	-	-	-	-	0.0870	-	0.1111	-
	4	-	-	0.1471	-	0.0217	0.2917	0.0556	0.1053
	5	0.5333	0.5000	0.5000	0.5000	0.3261	0.3333	0.2500	0.2368
	6	0.2000	0.1875	-	0.3333	-	0.1667	0.0556	-
	7	0.1000	0.0938	-	0.0833	-	0.0417	-	-
	8	0.0667	0.0625	-	-	-	-	-	-
	9	-	-	-	-	-	-	-	-
NpCT_13	1	0.1667	0.1250	0.1765	0.0556	0.0435	0.1667	0.0556	-
	2	-	-	-	-	-	-	-	-
	3	0.8000	0.7812	-	0.7778	0.8913	0.6667	0.1667	0.5526
	4	0.0333	0.0938	0.2647	0.1111	0.0217	0.1667	0.4167	0.3684
	5	-	-	0.5000	0.0556	0.0435	-	0.1389	0.0789
	6	-	-	0.0588	-	-	-	0.1389	-
	7	-	-	-	-	-	-	0.0833	-

Table 8 (Continued)

Primer/ Locus	Allele	Allele frequency of neem population							
		Pop.1 ★	Pop.2 ★	Pop.3	Pop.4 ★	Pop.5 ★	Pop.6 ★	Pop.7	Pop.8
NpCT_14	1	0.4333	0.5625	0.3235	0.0833	-	0.6250	0.2500	0.8421
	2	0.5667	0.4375	0.6765	0.6111	0.0652	0.3750	0.5833	0.1579
	3	-	-	-	0.2778	0.9348	-	-	-
	4	-	-	-	0.0278	-	-	0.1667	-
	5	-	-	-	-	-	-	-	-
NpCT_34	1	-	-	-	0.0556	0.0435	0.3333	0.0556	0.0789
	2	0.3333	0.1562	-	0.1389	-	0.2917	0.1111	-
	3	0.0333	-	-	0.0556	0.1957	-	0.5000	0.3947
	4	-	-	0.7941	0.0556	0.3696	0.2083	0.0278	0.0789
	5	0.2000	0.5000	-	0.3889	0.2826	0.0833	0.0833	0.3947
	6	0.3667	0.2188	0.2059	0.3056	0.0435	0.0833	0.1389	-
	7	0.0667	0.0938	-	-	0.0652	-	0.0833	0.0526
	8	-	0.0312	-	-	-	-	-	-
NpCT_48	1	0.2000	0.2188	0.0588	0.3056	0.0870	0.2917	0.1111	0.2105
	2	0.5333	0.5312	-	0.3611	0.3478	0.2500	-	-
	3	0.2333	0.2188	0.3529	0.2222	0.0870	0.2500	-	-
	4	0.0333	0.0312	0.1765	-	0.1957	-	0.1667	0.1316
	5	-	-	0.1471	0.1111	0.1957	-	0.2222	0.1053
	6	-	-	0.0588	-	0.0870	0.2083	0.2222	0.1053
	7	-	-	0.0882	-	-	-	0.0833	0.1579
	8	-	-	0.1176	-	-	-	0.0556	0.2105
	9	-	-	-	-	-	-	0.1389	0.0789

Table 8 (Continued)

Primer/ Locus	Allele	Allele frequency of neem population							
		Pop.9 ★	Pop.10	Pop.11	Pop.12	Pop.13	Pop.14	Pop.15	Pop.16
NpCT_4	1	0.1750	0.7083	-	-	0.0476	0.7333	0.6250	0.7368
	2	0.5750	0.2708	0.7000	0.9250	0.4762	0.2667	0.3750	0.2632
	3	0.2500	0.0208	0.3000	0.0750	0.4762	-	-	-
	4	-	-	-	-	-	-	-	-
NpCT_5	1	-	0.0417	-	0.2000	0.0714	0.1000	-	0.1579
	2	0.0500	-	-	0.1000	0.1667	-	0.1250	0.0526
	3	0.2000	0.1667	0.1500	0.2000	0.1429	0.3667	-	0.1842
	4	0.0250	0.1250	-	0.2000	0.0476	0.1667	-	0.0789
	5	-	0.2708	0.0500	0.2500	0.1190	0.3667	-	0.1053
	6	0.6250	-	0.2000	0.2500	0.2857	-	0.0417	0.1579
	7	0.1000	-	-	-	0.1429	-	0.2917	0.1053
	8	-	0.2708	0.5250	-	0.0238	-	0.4583	0.0263
	9	-	0.1250	0.0750	-	-	-	0.0833	0.1316
NpCT_6	1	-	0.3958	0.3750	0.1250	0.0952	0.0333	0.1667	0.3684
	2	-	0.5625	0.2000	0.2500	0.6667	0.6667	0.6667	0.5526
	3	0.5500	0.0417	0.4250	0.6250	0.2381	0.3000	0.0833	0.0789
	4	0.2000	-	-	-	-	-	0.0833	-
	5	0.1250	-	-	-	-	-	-	-
	6	0.1250	-	-	-	-	-	-	-
NpCT_11	1	0.3500	0.2083	0.2250	0.1750	0.2857	0.1000	0.0833	0.2365
	2	-	0.3958	0.1000	-	0.1905	0.3000	0.2083	0.2895
	3	-	0.0625	0.3250	0.4750	0.3333	-	0.1250	0.1053
	4	0.1500	0.1458	0.1250	0.2250	0.1190	0.4000	0.5000	0.2368
	5	0.3000	0.1458	0.1750	0.1250	0.0714	0.2000	0.0833	0.1316
	6	0.2000	0.0417	0.0500	-	-	-	-	-
	7	-	-	-	-	-	-	-	-
	8	-	-	-	-	-	-	-	-
	9	-	-	-	-	-	-	-	-
NpCT_13	1	0.1000	-	-	0.1000	0.1905	0.1333	-	0.2632
	2	-	-	0.0250	-	0.0476	-	-	0.0789
	3	0.8250	0.2917	0.3000	0.4000	0.0714	0.4333	0.5000	0.4474
	4	0.0750	0.3542	0.2250	0.4750	0.1905	0.1667	0.0833	0.1842
	5	-	0.2500	0.4500	0.0250	0.2619	0.2333	0.4167	0.0263
	6	-	0.1042	-	-	0.2381	0.0333	-	-
	7	-	-	-	-	-	-	-	-

Table 8 (Continued)

Primer/ Locus	Allele	Allele frequency of neem population							
		Pop.9 ★	Pop.10	Pop.11	Pop.12	Pop.13	Pop.14	Pop.15	Pop.16
NpCT_14	1	0.0750	0.0625	0.3750	0.4750	0.0714	0.4000	0.0833	0.1316
	2	0.2250	0.9375	0.4500	0.4500	0.3333	0.5000	0.2083	0.3684
	3	0.5000	-	0.1750	0.0500	0.3333	0.1000	0.7083	0.5000
	4	0.2000	-	-	0.0250	0.2381	-	-	-
	5	-	-	-	-	0.0238	-	-	-
NpCT_34	1	-	-	0.0500	-	-	0.0333	-	0.0263
	2	0.1000	-	-	-	-	-	-	-
	3	0.1500	0.1667	0.0250	0.0250	0.0238	0.4333	0.7917	0.0789
	4	0.3750	0.1667	0.5000	0.3250	0.2857	-	0.1667	0.2632
	5	0.3250	0.6667	0.1750	0.3000	0.1905	0.0333	0.0417	0.0263
	6	0.0500	-	0.1250	0.2500	0.2857	0.2333	-	0.1579
	7	-	-	0.1000	0.0500	0.1429	0.1000	-	0.3158
	8	-	-	0.0250	0.0500	0.0714	0.1667	-	0.1316
NpCT_48	1	0.6250	0.0417	0.1000	-	0.1429	0.1333	-	0.2632
	2	0.2250	-	-	0.0250	-	-	-	-
	3	0.1250	-	0.2250	0.1250	0.0476	0.1333	0.0417	0.0263
	4	0.0250	0.1042	-	0.2000	-	0.0333	0.0417	0.2105
	5	-	0.0417	0.0750	0.3000	0.1905	0.1333	0.0417	0.0263
	6	-	0.0625	0.1000	0.1250	0.1667	0.2667	0.1250	0.0263
	7	-	0.1667	0.1750	0.1750	0.1190	0.0667	0.7500	0.0789
	8	-	0.5000	0.3250	0.0250	0.0714	0.0667	-	0.3158
	9	-	0.0833	-	0.0250	0.2619	0.1667	-	0.0526

Table 8 (Continued)

Primer/ Locus	Allele	Allele frequency of neem population							
		Pop.17	Pop.18 ★	Pop.19	Pop.20	Pop.21	Pop.22	Pop.23	Pop.24
NpCT_4	1	0.7308	0.0435	0.7273	0.3421	0.5000	-	0.2692	0.0909
	2	0.2692	-	0.2727	-	0.4091	0.4167	0.4615	0.6136
	3	-	0.4565	-	-	0.0909	0.4167	0.2692	0.2955
	4	-	0.5000	-	0.6579	-	0.1667	-	-
NpCT_5	1	-	0.3043		-	-	-	0.0769	0.3409
	2	0.1154	0.0870	0.2045	0.0263	0.1818	-	-	0.1364
	3	-	0.2174	-	-	0.0682	-	0.1154	-
	4	-	0.0217	-	-	-	-	0.2692	-
	5	-	0.0870	0.1136	0.1316	0.1136	0.3750	0.3846	-
	6	0.3462	0.1739	0.1364	0.2105	0.4545	0.0417	-	0.2727
	7	0.1538	0.0217	0.4773	0.5789	-	-	-	0.0227
	8	0.3846	0.0435	-	0.0526	0.1818	0.3333	0.1538	0.1818
	9	-	0.0435	0.0682	-	-	0.2500	-	0.0455
NpCT_6	1	-	0.3478	0.2045	-	0.4545	-	0.2308	0.2045
	2	-	-	0.4773	0.2105	0.0227	0.0417	0.5000	0.5000
	3	0.5385	-	0.1818	0.1053	0.4545	0.7500	0.2308	0.2955
	4	0.4615	-	0.1364	0.6842	0.0682	0.2083	0.0385	-
	5	-	0.3913	-	-	-	-	-	-
	6	-	0.2609	-	-	-	-	-	-
NpCT_11	1	0.2692	0.0870	0.2955	-	0.1364	-	0.1154	0.0455
	2	0.5385	0.0652	0.1591	0.6316	0.3864	0.2917	0.4615	0.3636
	3	-	-	0.0682	0.0526	0.1364	-	0.1923	
	4	0.0769	0.0217	0.0682	0.0263	-	-	0.1538	0.2045
	5	0.1154	0.4348	0.0455	0.1579	0.3409	0.7083	0.0769	0.3182
	6	-	0.0870	0.3409	0.1316	-	-	-	0.0682
	7	-	0.2609	0.0227	-	-	-	-	-
	8	-	0.0435	-	-	-	-	-	-
	9	-	-	-	-	-	-	-	-
NpCT_13	1	-	0.1304	0.1364	-	0.1364	-	0.1538	0.1364
	2	0.1923	-	0.1818	-	-	-	-	0.0227
	3	0.6923	0.6739	0.0227	0.0263	0.3636	0.0833	0.3077	0.0909
	4	0.0385	0.1957	0.6136	0.9474	0.2727	0.9167	0.1154	0.5227
	5	0.0769	-	0.0455	0.0263	0.2273	-	0.3462	0.2045
	6	-	-	-	-	-	-	0.0769	0.0227
	7	-	-	-	-	-	-	-	-

Table 8 (Continued)

Primer/ Locus	Allele	Allele frequency of neem population							
		Pop.17	**Pop.18** ★	**Pop.19**	**Pop.20**	**Pop.21**	**Pop.22**	**Pop.23**	**Pop.24**
NpCT_14	1	0.3077	0.1957	0.2273	0.3158	0.2273	0.1250	0.1154	0.4091
	2	0.3077	0.0870	0.7500	0.6842	0.3864	0.5000	0.6923	0.3636
	3	0.2692	0.6739	-	-	0.3636	0.3750	0.1923	0.2045
	4	0.1154	0.0435	0.0227	-	0.0227	-	-	0.0227
	5	-	-	-	-	-	-	-	-
NpCT_34	1	-	0.1522	-	-	-	0.1667	-	0.0455
	2	-	0.1304	-	0.1053	-	0.0833	0.6923	-
	3	0.5385	0.0870	0.1818	0.8684	-	0.7500	-	0.4318
	4	-	0.4348	-	-	0.9091	-	0.1923	-
	5	0.1154	0.1522	-	-	0.0682	-	0.1154	0.1818
	6	0.0769	0.0435	0.8182	0.0263	0.0227	-	-	0.1591
	7	0.2308	-	-	-	-	-	-	0.1818
	8	0.0385	-	-	-	-	-	-	-
NpCT_48	1	0.2308	0.2826	0.0909	0.0526	-	-	0.1538	0.1364
	2	-	0.3913	-	-	-	-	-	-
	3	-	0.3261	-	-	-	0.1250	0.0385	-
	4	-	-	0.0682	-	-	-	0.0769	0.1591
	5	-	-	0.0455	-	-	-	0.0769	0.0682
	6	0.1538	-	0.2045	-	0.1364	0.1250	0.2308	0.0455
	7	0.1154	-	0.4091	0.2895	0.3182	0.2083	0.1923	0.0455
	8	0.3846	-	0.1591	0.4737	0.4318	0.2917	0.0385	0.2273
	9	0.1154	-	0.0227	0.1842	0.1136	0.2500	0.1923	0.3182

Observed heterozygosity and expected heterozygosity

The mean observed heterozygosity (*Ho*) (Table 9) of the Indian neem (0.4639) was slightly higher than Thai neem (0.4193). However, the lowest (0.1969) and the highest (0.6299) *Ho* was detected in Thai neem at locus NpCT_13 and NpCT_34, respectively. The average expected heterozygosity (*He*) in Indian neem (0.7674) was higher than in Thai neem (0.6994).

For all loci in both Indian and Thai populations, the observed heterozygosity was lower than expected heterozygosity. The highest deviation of the observed from the expected heterozygosity was found at locus NpCT_5 (*Ho* = 0.3669, *He* = 0.8727) in Indian neem and at locus NpCT_14 (*Ho* = 0.2362, *He* = 0.6812) in Thai neem.

Table 9 Observed heterozygosity (*Ho*) and expected heterozygosity (*He*) in Indian neem and Thai neem

Primer/Locus	Indian neem		Thai neem	
	H_o	H_e	H_o	H_e
NpCT_4	0.4188	0.7229	0.2913	0.6904
NpCT_5	0.3669	0.8727	0.5039	0.7805
NpCT_6	0.5779	0.7069	0.4567	0.7677
NpCT_11	0.5747	0.7914	0.4331	0.7613
NpCT_13	0.4383	0.7443	0.1969	0.3726
NpCT_14	0.3052	0.6366	0.2362	0.6812
NpCT_34	0.4708	0.8107	0.6299	0.8095
NpCT_48	0.5584	0.8535	0.6063	0.7324
Mean	**0.4639**	**0.7674**	**0.4193**	**0.6994**

Test of Hardy-Weinberg equilibrium

Test of Hardy-Weinberg equilibrium in Indian neem is shown in Table 10. Out of 17 populations of Indian neem, 7 populations (population 3, 8, 12, 13, 14, 15 and 23) showed significant departure from the equilibrium at locus NpCT_14, while 6 populations showed slight departure from the equilibrium at loci NpCT_5 and NpCT_6. Only locus NpCT_34 confirmed the Hardy-Weinberg equilibrium in all populations of Indian neem.

In individual population of Indian neem, population 3 (Sunyani, Ghana) showed the highest significant departure from the Hardy-Weinberg equilibrium in 4 (50%) loci out of 8. Population 17 (Allahabad Town, Uttar Pradesh, India), population 19 (Lamahi, Nepal) and population 24 (Bandia, Senegal) did not show any departure from the equilibrium in all loci.

The results of testing Hardy-Weinberg equilibrium in Thai neem are shown in Table 11. In individual population, tests for the departure from the equilibrium showed significant deviation for 2 loci in 4 populations out of 7. Like in Indian neem, locus NpCT_14 also showed the highest significant departure from the equilibrium in 3 Thai neem populations (population 4, 5, and 6). Locus NpCT_11 did not show departure from the equilibrium in all populations of Thai neem. At all loci, population 18 (Khao Laung, Nakhon Sawan, Thailand) did not show departure from the equilibrium.

Table 10 The *p*-value from Hardy-Weinberg equilibrium in Indian neem

Population /Locus	*p*-value of Indian neem							
	NpCT_4	**NpCT_5**	**NpCT_6**	**NpCT_11**	**NpCT_13**	**NpCT_14**	**NpCT_34**	**NpCT_48**
Pop. 3	**	0.0003*	0.0114*	0.0149*	0.5704 ns	0.0005*	0.0956 ns	1.000 ns
Pop. 7	1.0000ns	0.0138*	0.0225*	0.6352ns	0.1379ns	1.0000ns	0.3280 ns	0.1995 ns
Pop. 8	0.3698ns	0.2596ns	1.0000ns	1.0000ns	1.0000ns	0.0004*	0.3530 ns	0.0208*
Pop. 10	0.0545ns	0.0022*	0.0486*	0.0168*	0.3906ns	1.0000ns	0.3516 ns	0.2071 ns
Pop. 11	0.0012*	**	0.7644ns	1.0000ns	1.0000ns	0.0812ns	0.3788 ns	0.6122 ns
Pop. 12	1.0000ns	0.0888ns	0.0155*	0.3428ns	0.3698ns	0.0018*	0.1138 ns	1.0000 ns
Pop. 13	0.0221*	0.0264*	1.0000ns	1.0000ns	0.2604ns	0.0006*	1.0000 ns	0.2542 ns
Pop. 14	1.0000ns	0.2964ns	0.6162ns	0.1324ns	1.0000ns	0.0403*	0.1254 ns	0.5079 ns
Pop. 15	0.0061*	0.0044*	0.5176ns	0.5427ns	0.5626ns	0.0114*	0.4037 ns	1.0000 ns
Pop. 16	1.0000ns	0.0956ns	0.0216*	0.5778ns	0.3198ns	0.0693ns	0.2925 ns	1.0000 ns
Pop. 17	0.4991ns	1.0000ns	1.0000ns	1.0000ns	0.5079ns	1.0000ns	0.6020 ns	0.5635 ns
Pop. 19	1.0000ns	0.4133ns	**	0.1840ns	0.6069ns	1.0000ns	**	0.6496 ns
Pop. 20	1.0000ns	0.6427ns	0.2925ns	1.0000ns	0.0027*	1.0000ns	0.2587 ns	0.0216*
Pop. 21	1.0000ns	0.0385*	0.0063*	0.6527ns	0.0154*	0.0604ns	1.0000 ns	0.6616 ns
Pop. 22	0.2893ns	1.0000ns	1.0000ns	1.0000ns	0.0435*	1.0000 ns	0.0526 ns	0.0014*
Pop. 23	0.6020ns	0.2257ns	0.3004ns	1.0000ns	0.2145ns	0.0334*	0.2443 ns	1.0000 ns
Pop. 24	0.6096ns	0.0989ns	0.0842ns	0.0780ns	0.3530ns	0.6427 ns	1.0000 ns	1.0000 ns

Remark: ns = Non Significant.

* = Significant at $p < 0.05$.

** = Only 1 allele at locus. Calculations not performed.

Table 11 The *p*-value from Hardy-Weinberg equilibrium in Thai neem

Population /Locus	*p*-value of Thai neem							
	NpCT_4	**NpCT_5**	**NpCT_6**	**NpCT_11**	**NpCT_13**	**NpCT_14**	**NpCT_34**	**NpCT_48**
Pop. 1	0.6035^{ns}	0.0403*	0.5394^{ns}	0.1145^{ns}	0.0044*	0.2939^{ns}	0.5778 ^{ns}	1.0000 ^{ns}
Pop. 2	0.0149*	0.2889^{ns}	0.1995^{ns}	0.1145^{ns}	1.000^{ns}	0.1331^{ns}	0.6163 ^{ns}	1.0000 ^{ns}
Pop. 4	0.1774^{ns}	1.0000^{ns}	0.1757^{ns}	1.0000^{ns}	1.0000^{ns}	0.0403*	0.324 ^{ns}	0.0391*
Pop. 5	1.0000^{ns}	0.0762^{ns}	0.0063*	0.6605^{ns}	0.3109^{ns}	0.0249*	0.6069 ^{ns}	1.0000 ^{ns}
Pop. 6	1.0000^{ns}	0.0044*	0.0764^{ns}	1.0000^{ns}	1.0000^{ns}	0.0061*	1.0000 ^{ns}	0.1331 ^{ns}
Pop. 9	1.0000^{ns}	0.6473^{ns}	1.0000^{ns}	0.1116^{ns}	1.0000^{ns}	0.1637^{ns}	0.0155*	1.0000 ^{ns}
Pop.18	0.0989^{ns}	0.2339^{ns}	0.6000^{ns}	0.3800^{ns}	0.2138^{ns}	0.4889^{ns}	0.6752 ^{ns}	0.3729 ^{ns}

Remark: ns = Non Significant.

* = Significant at $p < 0.05$.

** = Only 1 allele at locus. Calculations not performed.

Genetic distance and dendrogram of 24 neem populations

DNA polymorphism detected by 8 microsatellite markers allowed to estimate the genetic distance among populations. The genetic distance was calculated for each pair of populations to estimate the extent of their divergence in Indian neem and Thai neem which is shown in Table 12 and Table 13 respectively.

The lowest genetic distance (0.031) among Indian neem populations was found between population 11 (Sagar, Chanatoria Madhya Pradesh, India) and population 20 (Geta, Nepal). The greatest genetic distance was found between population 17 (Allahabad Town, Uttar Pradesh, India) and population 3 (Sunyani, Ghana). Thai neem showed the lowest genetic distance (0.064) between population 1 (Ban Bo, Kalasin, Thailand) and population 2 (Ban Nong Hoi, Kanchanaburi, Thailand) and the highest genetic distance was found between population 1 (Ban Bo, Kalasin, Thailand) and population 4 (Doi Tao, Chiang Mai, Thailand).

Dendrograms resulted from UPGMA (Unweighted Pair Group Methods Analysis) cluster analysis using the program TFPGA separated 24 populations of neem into 2 main groups based on the taxon clearly. The first group consisted of the 7 populations of Thai neem (*A. indica* var. *siamensis*). Within the first group, population from the Central, North and North-East of Thailand were grouped together and population Tung Laung (Surat thani, Thailand) was distinct. The population Vientiane, Lao P.D.R was distinct from the others, similar to geographically location of seed source.

The second group consisted of the populations of Indian neem. In this group, 16 populations were clustered together while population Sunyani from Ghana was more distant. However, the population from diverse location such as Ramannaguda from Orissa, India and Yezin from Myanmar and population from Multan from Cantonment Area, Pakistan and Bandia from Senegal were also grouped together.

Table 12 Genetic distance between Indian neem

Population	Pop.3	Pop.7	Pop.8	Pop.10	Pop.11	Pop.12	Pop.13	Pop.14	Pop.15	Pop.16	Pop.17	Pop.19	Pop.20	Pop.21	Pop. 22	Pop.23	Pop.24
Pop. 3																	
Pop. 7	0.328																
Pop. 8	0.447	0.158															
Pop. 10	0.610	0.448	0.531														
Pop. 11	0.381	0.539	0.553	0.403													
Pop. 12	0.704	0.677	0.709	0.569	0.206												
Pop. 13	0.580	0.386	0.701	0.520	0.274	0.345											
Pop. 14	0.644	0.499	0.550	0.261	0.562	0.432	0.451										
Pop. 15	0.859	0.797	0.712	0.519	0.552	0.814	0.560	0.308									
Pop. 16	0.717	0.571	0.538	0.242	0.458	0.560	0.442	0.190	0.299								
Pop. 17	0.893	0.726	0.573	0.468	0.566	0.825	0.817	0.339	0.388	0.288							
Pop. 19	0.743	0.558	0.849	0.379	0.715	0.618	0.547	0.379	0.603	0.423	0.623						
Pop. 20	0.550	0.366	0.462	0.577	0.031	0.076	0.655	0.639	0.717	0.736	0.443	0.412					
Pop. 21	0.428	0.705	0.694	0.423	0.171	0.445	0.437	0.597	0.562	0.289	0.351	0.656	0.816				
Pop. 22	0.665	0.404	0.604	0.703	0.528	0.567	0.690	0.605	0.685	0.825	0.498	0.710	0.337	0.546			
Pop. 23	0.539	0.405	0.613	0.264	0.320	0.387	0.281	0.253	0.485	0.360	0.569	0.593	0.819	0.405	0.644		
Pop. 24	0.526	0.254	0.366	0.403	0.268	0.355	0.225	0.377	0.488	0.407	0.461	0.480	0.476	0.432	0.227	0.380	

Table 13 Genetic distance between Thai neem

Population	Pop.1	Pop.2	Pop.4	Pop.5	Pop.6	Pop.9	Pop.18
Pop. 1							
Pop. 2	0.064						
Pop. 4	0.733	0.073					
Pop. 5	0.569	0.540	0.599				
Pop. 6	0.361	0.413	0.409	0.643			
Pop. 9	0.387	0.355	0.268	0.413	0.376		
Pop. 18	0.252	0.273	0.188	0.329	0.486	0.288	

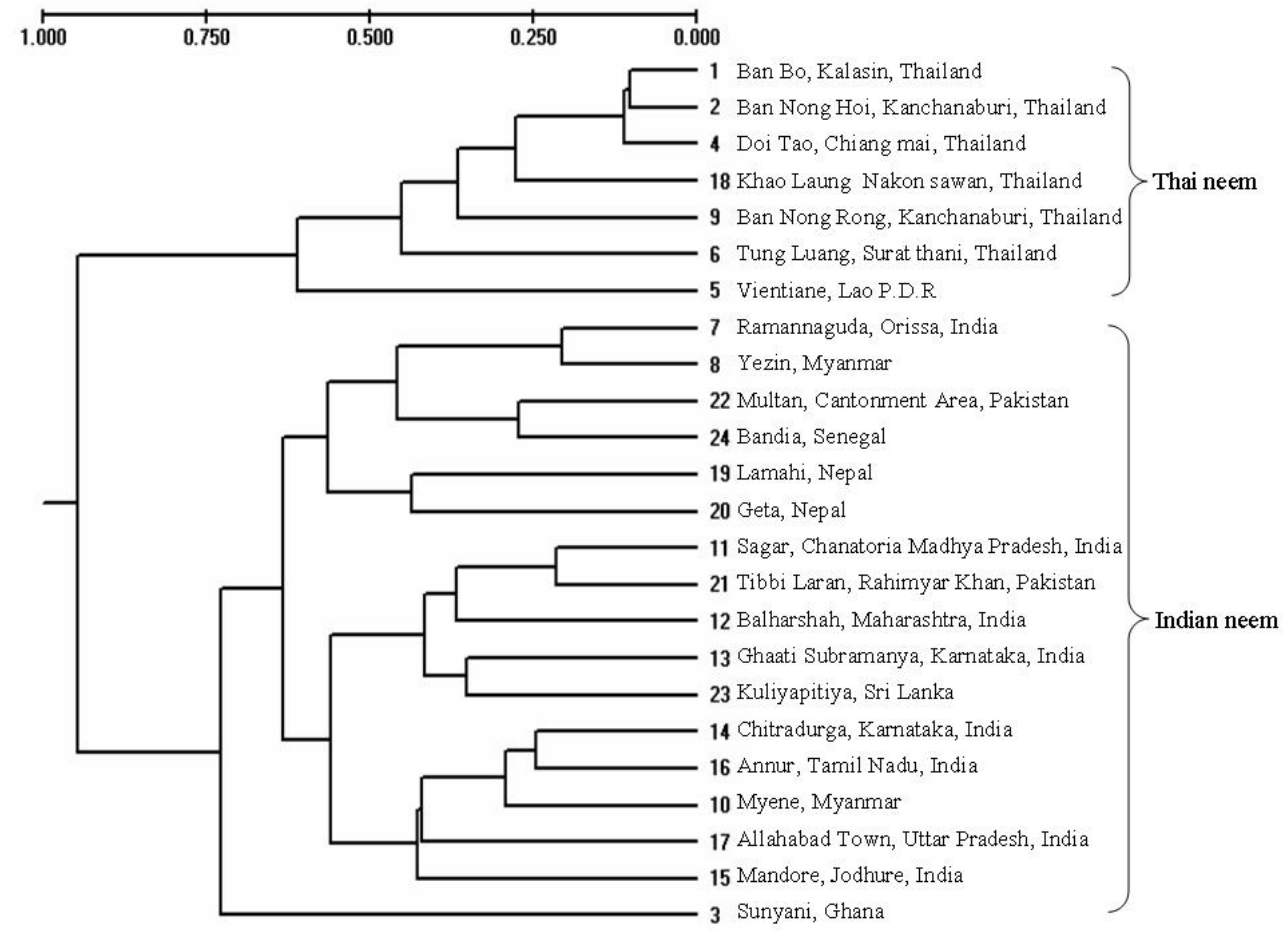

Figure 22 Dendrogram of 24 neem populations constructed by Unweighted Pair Group Methods. (Miller, 1997).

F-coefficient of Indian neem and Thai neem

F-coefficient of Indian neem and Thai neem is shown in Table 14. According to the table, F_{is} is the inbreeding co-efficient of an individual relative to its own population, while F_{it} is the overall inbreeding co-efficient of an individual relative to the whole set of populations. An average F_{it} in Indian neem (0.4009) was slightly higher (0.3936) than in Thai neem which indicated that overall loci of Thai neem had a deficiency of heterozygotes than Indian neem.

Within populations, an average of F_{is} in Indian neem (0.2366) was slightly lower than in Thai neem (0.2558). This means that the inbreeding in Indian neem is lower than in Thai neem populations.

Based on the F_{st} values obtained all over loci in Indian neem (0.2151) and Thai neem (0.1851) indicated that Indian neem had genetic differentiation among populations as compared to Thai neem.

Table 14 F-coefficient all loci of Indian neem and Thai neem

Primer/Locus	Indian neem			Thai neem		
	F_{is}	F_{it}	F_{st}	F_{is}	F_{it}	F_{st}
NpCT_4	0.0467	0.4247	0.3965	0.3700	0.5806	0.3343
NpCT_5	0.4999	0.5759	0.1519	0.2305	0.3780	0.1917
NpCT_6	-0.0308	0.2042	0.2279	0.1043	0.3598	0.2852
NpCT_11	0.1794	0.2897	0.1344	0.3532	0.4035	0.0779
NpCT_13	0.2762	0.4203	0.1992	0.4477	0.4647	0.0308
NpCT_14	0.3936	0.5084	0.1893	0.5087	0.6668	0.3218
NpCT_34	0.1269	0.4160	0.3310	0.1029	0.2014	0.1098
NpCT_48	0.2735	0.3563	0.1139	0.1114	0.1811	0.0785
Mean	**0.2366**	**0.4009**	**0.2152**	**0.2558**	**0.3936**	**0.1851**

Discussion

Microsatellite markers have been extensively used for DNA fingerprinting and elucidating genetic relationships within plant species (Ashkenazi *et al*., 2001). Although a number of population genetic studies have been conducted on *Azadirachta* species (Changtragoon *et al*., 1996, Singh *et al.,* 2002, Krisanapant, 2007), no microsatellite markers has been previously published on *Azadirachta* species. In this experiment, the protocol for development of microsatellite markers of Fischer and Bachmann (1998) was used. We used the only oligonucleotide $(CT)_{10}$ was used for hybridization in order to decreased a competition between many types of oligonucleotide probes. Condit and Hubbell (1992) and Wang *et al*. (1994) reported that among dinucelotide repeats, AG motif was found higher percentage than AC motif in tropical tree genomes, namely in *Caryocar brasiliense* (Collevatti *et al.,* 1999), in avocado (Ashworth *et al*., 2004). Furthermore, the study in Eucalyptus also indicated that AG repeats appear to be more abundant throughout the genome of *E. grandis* and *E. urophylla* than AC repeats (Brondani*, et al.,* 1998). But the result in hop indicated that both GA and GT repeats appeared to be highly abundant (Stajner *et al*., 2005). In *Citrus* species AG and AT were also found predominant in dinucleotide SSR (Dong *et al.,* 2006).

The efficiency of microsatellite hybridization in this study was 69.1% (out of 68 colonies sequenced, 47 contained the microsatellite repeats). In comparison to the efficiency level with original protocol, more than 60% of the sequenced clones contained at least one microsatellite sequence (Fischer and Bachmann, 1998). It means that this study shows a slightly good result.

Microsatellite markers from this study were low polymorphic with 30.77% (out of 26 primer pairs, produced 8 polymorphic loci) as comparing to other plant species, e.g. white clover, 48% (Jones, *et al.,* 2003), *Caryocar brasiliense*, 54% (Collevatti, 1999), hop 53% (Stajner *et al*., 2005), Quinoa 52% (Mason, 2005) and almond (80.65%) (Shiran *et al*., 2007).

Overall observed numbers of alleles in neem (4 to 9) was lower than in *Picea asperata*, one of the most important tree species used for the production of pulp wood and timber in western China, which contained the number of alleles per locus ranging from 13 to 25 (Wang *et al*., 2005). Likewise Eucalyptus (Brondani*, et al.,* 1998) and trembling aspen (*Populus tremuloides*) (Dayanandan *et al.,* 1998) also showed higher number of alleles than in neem ranging from 9 to 26 and 5 to 11 alleles, respectively .

However, a similar observation as in the present study has been reported in potato (Ashkenazi *et al.,* 2001) and apricot (Sanchez-Perez *et al*., 2005) with the number of alleles ranging from 1 to 9. Indian neem showed average number of alleles per locus and effective number of alleles higher than Thai neem. This may be due to lower number of populations were used in this study and geographical distribution in Thai neem populations is narrower than in Indian neem population.

The average observed heterozygosity in Indian neem (H_o = 0.46) and Thai neem (0.42) is lower than *Acer pseudoplatanus* (H_o = 0.55) as reported by Pandey (2005), *Caryocar brasiliense* (H_o = 0.73) (Collevatti, *et al.,* 1999), *Pinus pinaster* (H_o = 0.65) (Mariette*, et al*., 2001) and *Eucalyptus* species (H_o = 0.58) (Brondani*, et al.,* 1998). But higher than red clover (H_o =0.34) (Mosjidis and Klingler, 2006). A similar result was found in trembling aspen (*Populus tremuloides*) (H_o = 0.46) (Dayanandan *et al.,* 1998) and *Picia asperata* (H_o = 0.43) as reported by Wang *et al*., (2005).

The average expected heterozygosity in Indian neem (H_e = 0.77) and Thai neem (0.70) is higher than *A. pseudoplatanus* (H_e = 0.57) (Pandey, 2005), *Prunus persica* (H_e = 0.29) as reported by Bouhadida, *et al.,* (2007) and Madagascar periwinkle, *Catharanthus roseus*, (H_e = 0.56) (Shokeen *et al*., 2007). A higher of average expected heterozygosity was found in *Pinus pinaster* (H_e = 0.83) (Mariette*, et al*., 2001) and *Caryocar brasiliese* (H_e = 0.89) (Collevatte *et al*., 1999).

There has been the number of markers showing significant Hardy-Weinberg disequilibrium in Indian neem and Thai neem, especially in locus NpCT_14 showed deficits in both populations. There could be several reasons for the HW disequilibrium results. Firstly, the presence of homozygotes with null alleles can be the major cause. Secondly, the HWE estimation may not be reliable due to the small size of the samples used in some loci. Thirdly, the large number of loci with linkage disequilibrium suggests hidden population substructure (Li *et al*., 2007).

Changtragoon *et al.,* (1996) used 10 putative isozyme gene loci to identify and measuring genetic diversity in 3 *Azadirachta* species (*A. indica, A. indica* var. *siamensis* and *A. excelsa*). The three taxa were separated by a very high genetic distance and cluster analysis. Singh *et al*., (2002) used AFLP and SAMPL for assessment of intra-population genetic variation in Indian neem and Thai neem. The phenogram based on unweighted pair group method of averages (UPGMA) analysis depicted that the Kanpur accessions (Indian neem) were genetically distinct from the Thai accessions, similar to this results that the cluster analysis of Thai neem and Indian neem were separated clearly.

The F_{st} (Wright, 1978) values observed in Indian neem and Thai neem were between 0.1130 to 0.3965 and 0.0308 to 0.3343, respectively. The F_{st} values indicate the presence high genetic differentiation in neem as compared to Norway spruce (*Picea abies* (L.) (F_{st} between 0.012 and 0.029) (Maghuly*, et al*., 2006). An average of F_{st} in Indian neem (0.2152) and Thai neem (0.1851) was also higher than in *P. albies* (0.053) as reported by Lagercantz and Ryman (1990) and *Acer pseudoplatanus* (0.075) (Pandey, 2005). A similar F_{st} was found in *Picia asperata* (0.223) reported by Wang *et al.,* 2005. This is because, neem is insect pollinated species but *Picea abies* is wind pollinated. The higher of F_{st} in Indian neem than Thai neem may have resulted from the high differentiation of geographic distance between populations in this study.

CONCLUSION

Today neem is widespread and well known with their multiple uses and applications. South Asian and sub-Saharan Africa constitute the main areas of distribution. The neem tree has been used for various purposes, e.g. to manufacture bio-pesticides, medicinal purposes, as shade trees, and also planted to control the soil erosion. Despite, the usefulness of neem, the information on the genetic structure in Indian neem and Thai neem are still little. In order to understand the genetic variation and reproductive biology (mating system, gene flow) in neem, microsatellite markers are very useful tools. Therefore, eight polymorphic microsatellite markers were developed in neem and used to determine the genetic variation in Indian neem and Thai neem.

In this study the protocol developed by Fischer and Bachmann (1998) was used to develop microsatellite markers. Dinucleotide oligos $(CT)_{10}$ was used for hybridization into the genomic DNA of Indian neem and Thai neem. The enriched fragments were ligated into pGEM-T easy vector and transformed to *E. coli* strain DH5α. After blue-white colony selection, a total of 68 colonies were obtained.

Sequencing of the colonies resulted 47 (69.1%) sequences containing at least one microsatellites. Out of 47 sequences, 26 (55%) were suitable for the primer design. These 26 primers were tested in 24 populations of neem. Finally, 8 primer pairs showed polymorphism and was used for assessment of genetic variation in both Indian neem and Thai neem. To estimate the genetic structure and variation samples were collected from 24 neem populations from FAO International Provenance Trials established in Kanchanaburi, Thailand in August, 1997.

The total number of alleles of Indian neem and Thai neem were 53 and 49, respectively. Locus NpCT_5 showed the highest (9) number of alleles in both populations. Allele frequencies in all populations showed some alleles at loci NpCT_6 and NpCT_11 were found only in Indian neem or Thai neem.

The observed heterozygosity in both Indian neem and Thai neem were lower than expected heterozygosity. But Indian neem showed the higher deviation of the observed from the expected heterozygosity than Thai neem.

The cluster analysis using UPGMA revealed that the Indian neem and Thai neem were separated into 2 groups based on the taxon clearly. The result from testing of Hardy-Weinberg equilibrium showed that the locus NpCT_14 deviated significantly from the equilibrium in both neem populations. Testing of F-coefficient in both populations showed the F_{st} value of Indian neem was higher than Thai neem which indicated that Indian neem has more differentiation among populations than among Thai neem population.

In conclusion, the microsatellite markers developed in this study were useful to determine the genetic diversity and differentiation of neem both species and population level. Furthermore, since the microsatellites show high level of polymorphisms (up to 9 alleles per locus) they are also useful for estimation mating system, gene flow as well as clone and hybrid identification in the future.