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USING AFLP TO IDENTIFYGENETIC RELATIONSHIPS IN *CASSIA* SPECIES FROM THAILAND

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Abstract

Several species of *Cassia* are used in Thai folk medicine as a laxative and a treatment for skin infections. However, the taxonomy of the Genus*Cassia* is quite complex and intriguing. Thus, the correct identification of the species of this genus is necessary for efficacy and safety. The phylogenetic relationships among the 16 species of *Cassia*genus existing in Thailand were evaluated using Amplified Fragment Length Polymorphism (AFLP) technique. Combinations of 70 primers were screened and eleven primer combinations produced a total of 849 distinct and reproducible bands ranging from 60 to 100 bands with an average of 77.18 bands per primer combination. The genetic distances were calculated based on the AFLP bands that had been amplified using the eleven primer combinations. The similarity indices (SI) ranged from 0.25 to 0.78. The dendrogram was created using the Unweighted Pair Group Method of the Arithmetic Average (UPGMA) and the genotypes were divided into two major groups. The results indicate that the phylogenetic relationships are associated with the morphological characterization. In conclusion, an AFLP marker could be an efficient and reliable tool for the identification of a *Cassia* species.

Key words: *Cassia* species, Amplified fragment length polymorphism, Phylogenetic relationships.

Introduction

The genus *Cassia*, belonging to the family Fabaceae, subfamily Caesalpinioideae (Monkheang *et al.*, 2011), is grown in many tropical countries, including Thailand, for use as ornamentals, food and medicines. In addition, *Cassia fistula* is Thailand's national flower. Thirty-three*Cassia* species have been recorded in the Thai Forest Bulletin (Pooma & Suddee, 2014), and pharmacologically studied for their use in the treatment of skin infections and for various biological activities, such as laxative, antimicrobial, antipyretic, antioxidant, antihyperglycemic, antimalarial and anti-inflammatory activities (Pooviboonsuk *et al.*, 2000; Sakulpanich & Gritsanapan, 2009; Anjali *et al.*, 2009; Dolui *et al.*, 2012; Choudhary & Nagori, 2014). In particular, *Cassia fistula* L., *Cassia siamea*Lam. and *Cassia alata*L. have been noted as Thai herbal medicines for their laxative and purgative properties, as well as the ability to treat skin diseases. However, the taxonomy of the plants of the genus *Cassia* is quite complicated due to their synonymous vernacular names and similar morphological features. Quality control for the efficacy and safety of herbal products and medicinal plants is necessary because misidentification may lead to ineffective treatment. Therefore, genetic assessment by a convenient method for the identification of the *Cassia*species is important. Several DNA-based marker techniques, such as polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP), random amplified polymorphic DNA (RAPD), DNA sequencing, sequence characterized amplified region (SCAR) and amplified fragment length polymorphism (AFLP), have recently been applied for the identification and standardization of medicinal plants. Among these, as a reliable, stable, and highly reproducible method, the AFLP marker is a DNA fingerprinting technique that approaches ideally advances toward a marker system that can resolve genetic diversity (Muller & Wolfenbarger, 1999). Moreover, the marker can be used to survey a whole genome without any prior sequence knowledge (Keeratinijakal *et al.*, 2010). AFLP markers have been used favorably to identify herbal plants and evaluated genetic diversity in various species, such as *Rhododendron* spp., *Amomum*spp. and *Swertia* spp. (Kaewsri *et al.*, 2007; Misra *et al.*, 2010; Zhao *et al.*, 2012), but not for the *Cassia* species in Thailand. The present study aims to investigate the genetic diversity and phylogenetic relationships of the *Cassia* species existing in Thailand regarding the use of AFLP marker.

Materials and Methods

Plant materials: The fresh young leaves of the 16 *Cassia* species (*C. bakeriana, C. fistula, C. grandis, C. javanica, C. alata, C. angustifolia, C. garrettiana, C. hirsuta, C. occidentalis, C. spectabilis, C. siamea, C. sophera, C. surattensis, C. sulfurea, C. timoriensis,* and *C. tora*) and one out-group plant (*Andrographis paniculata*, Family Acanthaceae) were collected from different locations throughout Thailand (each sample was collected from 3 different locations, n=48). The plant samples were authenticated by an expert (N.R.) and compared with the herbarium specimens at The Botanical Garden Organization, Ministry of Natural Resource and Environment, Bangkok, Thailand. The voucher specimens were deposited at the College of Public Health Sciences, Chulalongkorn University, Thailand.

Genomic DNA extraction: Five grams of fresh young leaves of each sample were pulverized in liquid nitrogen to obtain a fine powder. The genomic DNA was isolated by CTAB extraction method as described by Doyle and Doyle (1987) with some modifications. DNA quantification was performed using a NanoDrop Spectrophotometer ND-1000 (NanoDrop Technologies, Inc., Wilmington, DE, USA). The extracted genomic DNA was stored at -20°C for AFLP analysis.

AFLP analysis: Regarding the AFLP procedure provided by Vos *et al.* (1995) with some modifications: "The genomic DNA approximately 100 ng/µl was digested using two restriction enzymes, *Eco*RI and *Mse*I (New England Biolabs, USA), in 10x buffer A (Promega) and incubated for 1 h at 37°C. After digestion, the restricted DNA fragments were ligated to an *Eco*RI-adapter and *Mse*I-adapter using T4 DNA ligase (New England Biolabs, USA) for at least 3 h at 37°C in order to generate a DNA template for PCR amplification. The completeness of the ligation process was detected by loading 5 µl of ligation reaction and 1 µl of 6x loading dye in 1% agarose gel electrophoresis in a 0.5x TBE buffer. Each ligation reaction was diluted as 10-folded with sterilized distilled water and the aliquots were stored at -20ºC. Five microliters of the 1:10 diluted DNA template was first pre-amplified (Px2 Thermal Cycler; Thermo Electron Corporation, USA) using *Eco*RI+A and *Mse*I+C primers with 1 selective nucleotide at the 3' end. The pre-amplification was conducted using the following cycling parameters: 94˚C for 5 min, 20 cycles of 30 s denaturing at 95˚C, 60 s annealing at 56˚C and 60 s extension at 72˚C, ending with 10 min at 72˚C to complete extension. Then, the pre-amplified DNA was diluted to 1: 9 with sterilized distilled water and 3 µl of the pre-amplified product was used for selective amplification in a reaction tube containing 20 µl of selective amplification using *Eco*RIand *Mse*Iprimers with 3 selective nucleotides at the 3' end. Seventy primer combinations were screened for the selective amplification. The selective PCR amplification reaction was performed using the following cycling parameters: 95˚C for 2 min, 36 cycles of 30 s denaturing at 95˚C, 30 s annealing and 60 s extension at 72˚C. Annealing was initiated at a temperature of 65˚C, which was then reduced by 0.7˚C for the next 12 cycles and maintained at 56˚C for 23 subsequent cycles. The final PCR products were run on a 4.5% denaturing polyacrylamide gel electrophoresis in a 1x TBE buffer in a Sequi-Gen GT Sequencing Cell (Bio-Rad, USA)." DNA fragments on the gels were stained with silver nitrate (Bassam *et al.,* 1991). The gels were purged with distilled water and air-dried on mirror plates, and then the AFLP fragments were analyzed.

Data analysis: The AFLP fragments were visually scored as present (1) or absent (0) in order to create a binary data set as discrete variables for genetic similarity analysis. Jaccard's coefficient of similarity (Jaccard, 1908) was calculated for all pair-wise comparisons among the *Cassia* species as follows: "Jaccard = $N_{AB}/(N_{AB}+N_A+N_B)$, where N_{AB} is the number of fragments shared by two cultivars (*A* and *B*), $N_A$represents amplified fragments in cultivar *A*, and N_B represents fragments in cultivar *B*." The Unweighted Pair Group Method of the Arithmetic Average (UPGMA) was selected to construct a dendrogram, clustering by FreeTree software (Hampl *et al.,* 2001). To evaluate the strength of the resulting branches, bootstrap probabilities were calculated by FreeTree software using 1,000 bootstrap resampling pieces of data.

Results

AFLP analysis: A total of 70 primer combinations were initially screened among these 11 primer combinations which produced visible and clear bands in all plant samples (Table 1). Each species was collected from 3 different localities, but showed the same patterns of AFLP profiles, so an individual representative sample of each species was selected. The results demonstrated that different primers generate different fragment numbers and lengths. A total of 849 amplified fragments, ranging from 80 to 700 base pairs in size, were generated from 11 primer combinations (Table 1). The bands that were produced from the 11 primer combinations ranged from 60 to 100 bands with an average of 77.18 polymorphic bands per primer combinations and generated a high percentage (100%) of polymorphic bands. The highest number of the amplified fragments was obtained from the primer pair E+AAC/M+CAA (100 bands) (Fig. 1), while the lowest number was obtained from the primer pair E+AAC/M+CCC (60 bands).

Table 1. The list of 11 primer combinations and the number of AFLP bands, size ranges and percentages of polymorphic bands.

Primer combination	Number of AFLP band	Size range (bps)	Percentage of polymorphic band
E+AAC/M+CCA	61	80-700	100
E+AAC/M+CAA	100	80-700	100
E+AAC/M+CGT	64	80-700	100
E+AAC/M+CCC	60	80-700	100
E+ACC/M+CAA	78	80-700	100
E+ACC/M+CCA	67	80-700	100
E+AAG/M+CCA	99	80-700	100
E+AAG/M+CAT	90	80-700	100
E+AAG/M+CAA	79	80-700	100
E+AGC/M+CCA	89	80-700	100
E+AGC/M+CAA	62	80-700	100
Total	**849**	**80-700**	**100**

Genetic relationships: The dendrogram was generated by Jaccard's similarity matrix and the UPGMA method. Figure 2 shows the genetic relationships among the sixteen *Cassia* species and outgroup plants.

According to the dendrogram, two major groups were classified as having bootstrap values higher than 80%. The bootstrap values of the different clusters and subclusters are displayed in the bootstrap tree (Fig. 2). The first group is composed of *C. bakeriana, C. fistula, C. grandis*and *C. javanica* with the similarity index 0.54-0.61 and 100% bootstrap support. The second group can be divided into 3 subgroups (98% bootstrap) with the first subgroup being composed of *C. garrettiana, C. siamea, C. timoriensis, C. alata*and *C.spectabilis* with a 0.45-0.64 similarity index. *C. tora, C. surattensis* and *C. sulfurea* were clustered into a second subgroup with a 0.47-0.78 similarity index. The last subgroup belongs to *C. hirsuta, C. occidentalis, C. sophera* and *C. angustifolia* with the similarity index 0.39-0.63. According to the dendrogram, the outgroup plant, *A. paniculata,* was clearly separated from sixteen *Cassia* species with 100% bootstrap support.

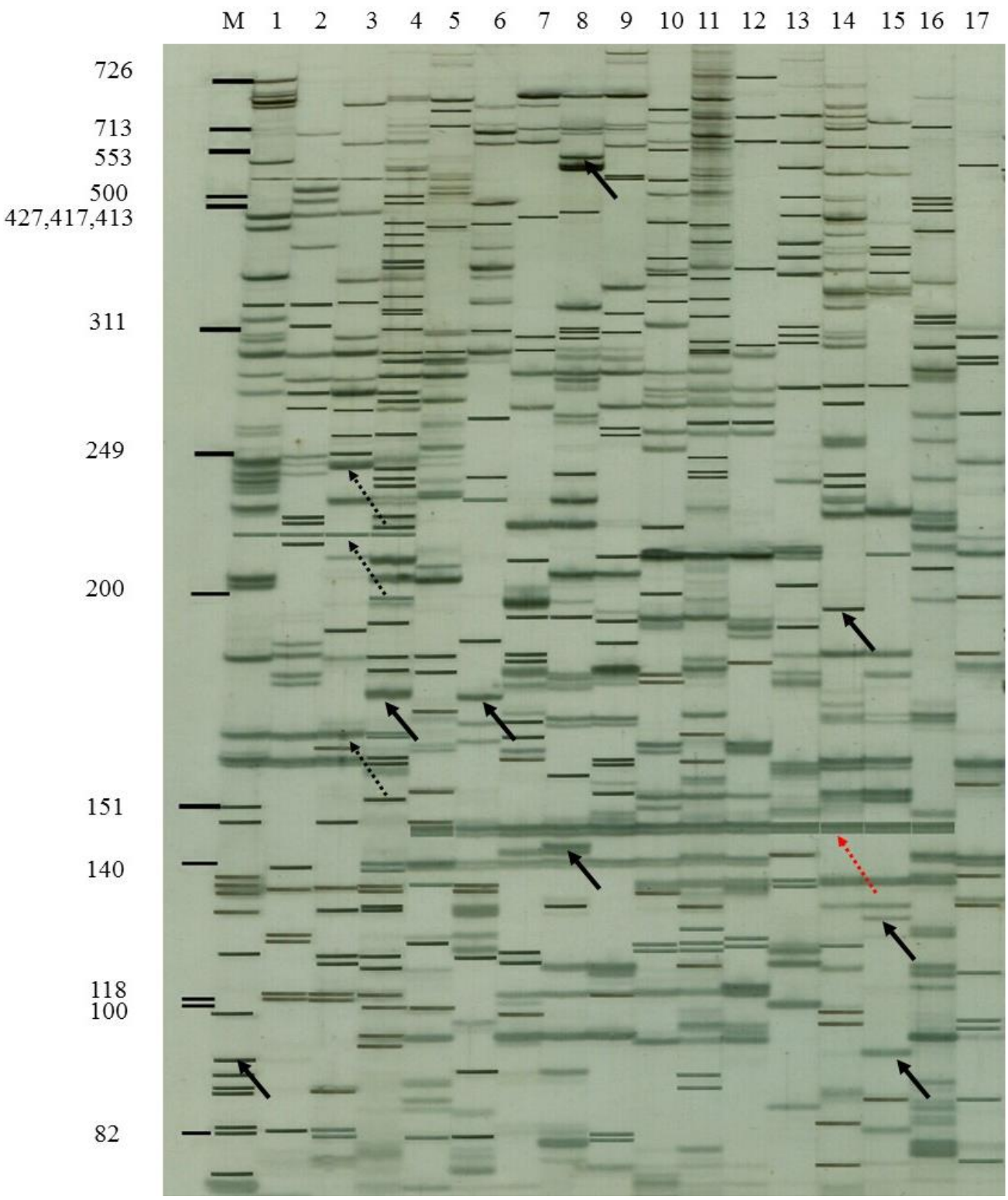

Fig. 1. AFLP fingerprint of sixteen *Cassia* species and *A. paniculata* (outgroup plants) obtained from E+AAC/M+CAA primer combinations.

→ Indicates unique bands of *Cassia* species.
- - -▶ Indicates monomorphic bands of *Cassia* species.
- - -▶ Indicates monomorphic bands of *Senna* species.

Lane designations with accession number are as follows:

Lane M: phiX174 DNA/*Hinf*I Marker Lane 1: *C. fistula*, Lane 2: *C. grandis*, Lane 3: *C. bakeriana*, Lane 4: *C. javanica*, Lane 5: *C. alata*, Lane 6: *C. spectabilis*, Lane 7: *C. siamea*, Lane 8: *C. timoriensis*, Lane 9: *C. garrettiana*, Lane 10: *C. hirsuta*, Lane 11: *C. occidentalis*, Lane 12: *C. sophera*, Lane 13: *C. tora*, Lane 14: *C. surattensis*, Lane 15: *C. sulfurea*, Lane 16: *C. angustifolia* , Lane 17: *A. panicula*

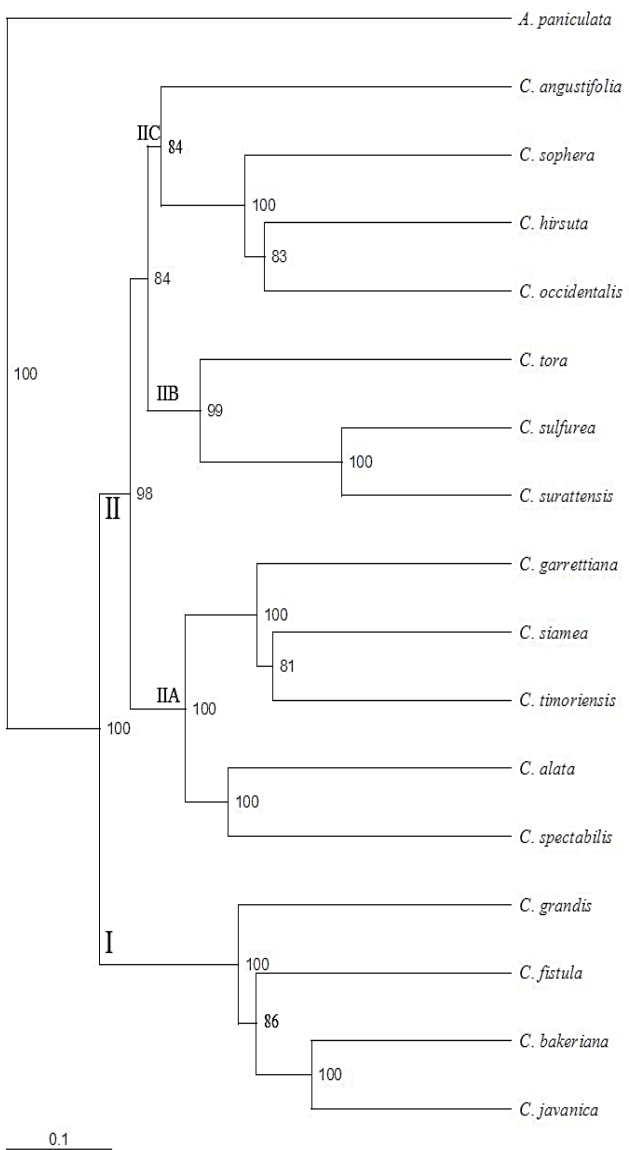

Fig. 2. UPGMA dendrogram based on Jaccard's similarity coefficient among *Cassia* species and outgroup plants.

The pair-wise comparisons of the AFLP profiles were based on both of the shared and unique amplification bands, and were used to generate a similarity index. Among the 48 accessions of 16 species, the genetic similarity ranges from 0.25 to 0.78 (Table 2). *C. surattensis* and *C. sulfurea* showed the highest genetic similarity value (0.78), whereas *C. fistula*and *C. hirsuta*showed the lowest genetic similarity value (0.25).

Discussion

The AFLP technique is commonly applied for plant classification, genetic relationships and genetic diversity in many plant species, such as *Curcumacomosa*, *Punica granatum* and *Panax notoginseng* (Kwon *et al.*, 2009; Keeratinijakal *et al.*, 2010; Moslemi *et al.*, 2010). Comparative studies using PCR- RFLP, RAPD and AFLP techniques have revealed that AFLP techniques are the most efficient and effective due to their high reproducibility, high quantity of information throughout multiple loci in the genome, and high resolution. In the current study, 11 primer combinations produced clear and reproducible amplified bands. A total of 849 amplified fragments were detected. The high percentage (100%) of polymorphism indicates that there is a high level of genetic diversity among the 16 *Cassia* species. The dendrogram was created based on the genetic similarity index and showed that all of the *Cassia* species could be clustered into two main groups that have bootstrap values higher than 80%. Bootstrap analysis revealed that the branching in the tree was stable and robust. The first group consists of *C. bakeriana, C. javanica, C. grandis*and *C. fistula*. This result is similar to those previously finding that *C. javanica*, and *C. fistula* had been clustered into the same group on the basis of RAPD fingerprints (Tripathi & Goswami, 2011). Based on SSR and ISSR fingerprints, *C. fistula*, *C. grandis* and *C. javanica* were also clustered together (Mohanty *et al.*, 2010). The second group can be divided into 3 subgroups with the first subgroup being composed of *C.garrettiana, C. siamea, C. timoriensis*, *C. alata* and *C. spectabilis*. The second subgroup is composed of *C. tora*, *C. surattensis* and *C. sulfurea*. The last subgroup belongs to *C. hirsuta, C.occidentalis*, *C. sophera* and *C. angustifolia*. The result is consistent with previous report regarding to RAPD fingerprints that clustered *C. tora*, *C. surattensis* and *C. sulfurea* together (Tripathi & Goswami, 2011). Moreover, *C. hirsuta, C. occidentalis* were clustered together based on the SSR and RAPD fingerprints, whereas *C. siamea* and *C. spectabilis* were clustered into the same group based on the SSR, ISSR and RAPD fingerprints (Mohanty *et al.*, 2010; Tripathi & Goswami, 2011). The outgroup taxon, *A. paniculata*, was clearly separated from the *Cassia* species. The taxonomic relationships between *Cassia* and other genera in the *Cassiinae* subtribe have been discussed for a long time. Several taxonomists have classified *Cassia* genus into different systems based on various morphological characteristics. According to the classification of Irwin and Barneby (1981), the *Casiinae* subtribe was first classified into three genera, *Cassia*, *Senna* and *Chamaecrista*, using the characteristics of filaments and the presence or absence of bracteoles. The revised classification is widely accepted in many countries, including Thailand. Thai plant names Tem Smitinand revised edition 2014 (Pooma & Suddee, 2014) revealsthat seventeen species out of the thirty-three species of *Cassia* distributed throughout Thailand had been moved into the genus *Senna*, which is supported by the results of the AFLP done in this study. The monomorphic banding patterns derived by AFLP fingerprinting were clearly separated between the genus *Cassia* (group I) and *Senna* (group II) (Fig. 1). In addition, the AFLP data was used as molecular characters for phylogenetic analyses to reveal the evolutionary relationships among the *Cassia* and *Senna* species. Moreover, the genetic relationships through the AFLP markers were also correlated with the morphological characteristics. The members of the first group have similar morphological characteristics whenconsidering their curved filaments. Two bracteoles under the peduncles and pods are terete, whereas all members of the second group have similar short and straight filaments with no bracteole under the peduncles and pods being flat to terete. This was corroborated by the findings of Irwin and Barneby (1982), and Kidyue (2003). The results of the AFLP phylogenetic analysis could be an important basis for further taxonomic, evolutionary, breeding and pharmacological studies of the genus *Cassia*.

Table 2. Pair-wise genetic similarity index (SI) of sixteen *Cassia* plants and outgroup plants according to the index of Jaccard.

	C. fistula	*C. grandis*	*C. bakeriana*	*C. javanica*	*C. alata*	*C. spectabilis*	*C. siamea*	*C. timoriensis*	*C. garrettiana*	*C. hirsuta*	*C. occidentalis*	*C. sophera*	*C. angustifolia*	*C. tora*	*C. surattensis*	*C. sulfurea*	*A. paniculata*
C. fistula	1.0000																
C. grandis	0.6069	1.0000															
C. bakeriana	0.6083	0.5866	1.0000														
C. javanica	0.6136	0.5354	0.7165	1.0000													
C. alata	0.3587	0.3586	0.3765	0.4116	1.0000												
C. spectabilis	0.3138	0.3225	0.3405	0.3707	0.5568	1.0000											
C. siamea	0.3402	0.3511	0.3288	0.3313	0.4900	0.4497	1.0000										
C. timoriensis	0.3070	0.3275	0.3191	0.3314	0.4783	0.4921	0.6421	1.0000									
C. garrettiana	0.3215	0.3511	0.3211	0.3338	0.4744	0.4656	0.6126	0.6121	1.0000								
C. hirsuta	0.2537	0.2766	0.2831	0.2838	0.3667	0.3899	0.4092	0.4149	0.4326	1.0000							
C. occidentalis	0.2928	0.3008	0.2884	0.3097	0.4004	0.3809	0.4072	0.4206	0.4236	0.6267	1.0000						
C. sophera	0.2596	0.2715	0.2786	0.2794	0.3467	0.3548	0.3732	0.3832	0.4095	0.5810	0.5961	1.0000					
C. angustifolia	0.2741	0.3050	0.2921	0.2620	0.3925	0.3321	0.3525	0.3518	0.3556	0.3946	0.4383	0.4512	1.0000				
C. tora	0.2784	0.2773	0.2766	0.3000	0.3495	0.3389	0.3259	0.3424	0.3354	0.4433	0.4838	0.4220	0.3712	1.0000			
C. surattensis	0.2955	0.3053	0.3023	0.3251	0.3570	0.3233	0.3624	0.3780	0.3552	0.3871	0.4647	0.3939	0.3650	0.5342	1.0000		
C. sulfurea	0.2909	0.3066	0.3008	0.3184	0.3331	0.3342	0.3408	0.3656	0.3588	0.3668	0.4061	0.3636	0.3580	0.4726	0.7750	1.0000	
A. paniculata	0.0267	0.0325	0.0492	0.0475	0.0202	0.0192	0.0301	0.0342	0.0395	0.0306	0.0332	0.0211	0.0165	0.0417	0.0359	0.0357	1.0000

SI value range from 0 to 1.0000 according to the increasing similarity index

Conclusion

In conclusion, AFLP fingerprinting is found to be a useful technique for plant identification and confirmation of the phylogenetic relationships of selected *Cassia* species. For further research, a SCAR marker should be developed for the identification of plants in the genus *Cassia*.

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