Genome Relationship among Nine Species of Millettieae (Leguminosae: Papilionoideae) Based on Random Amplified Polymorphic DNA (RAPD)

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Random amplified polymorphic DNA (RAPD) marker was used to establish intergeneric classification and phylogeny of the tribe Millettieae sensu Geesink (1984) (Leguminosae: Papilionoideae) and to assess genetic relationship between 9 constituent species belonging to 5 traditionally recognized genera under the tribe. DNA from pooled leaf samples was isolated and RAPD analysis performed using 25 decamer primers. The genetic similarities were derived from the dendrogram constructed by the pooled RAPD data using a similarity index, which supported clear grouping of species under their respective genera, inter- and intra-generic classification and phylogeny and also merger of *Pongamia* with *Millettia.* Elevation of *Tephrosia purpurea* var. *pumila* to the rank of a species (*T. pumila*) based on morphological characteristics is also supported through this study of molecular markers.

Key words: Genome Relationship, RAPD, Millettieae

Introduction

Leguminosae (Fabaceae) is one of the largest families of flowering plants, comprising over 650 genera and 18,000 species (Polhill, 1981). The family is economically very important being the major source of food and forage and its great diversity (the third largest family in flowering plants) also has attracted much interest in ecological as well as systematic studies. The predominantly tropical tribe Millettieae, consisting of over 40 genera and nearly 1,000 species, is generally thought to have given rise to many temperate herbaceous groups and several tropical tribes of papilionoid legumes, such as Phaseoleae, Indigofereae, Galegeae and their allies (Polhill, 1981; Geesink, 1984) and is considered to be one of the most problematic groups in legume systematics. This tribe is generally defined as those tropical woody papilionoids with some derived flower features (fused keel petals) and seeds containing non-protein amino acids but there are many exceptions (Evans *et al.*, 1985). The paraphyly of Millettieae has been suggested in many studies based on morphological characters (Geesink, 1984; Zandee and Geesink, 1987), biochemical (Evans *et al.*, 1985), molecular (Bruneau *et al.*, 1994; Lavin *et al.*, 1998; Hu *et al.*, 2000) and cytological data (Goldblatt, 1981).

The tribe is traditionally divided into three subgroups, with *Tephrosia*, *Millettia* and *Derris* as the major components in each (Geesink, 1984). *Derris* and allies (*e.g.*, *Lonchocarpus*) have been placed in the tribe Dalbergieae because of indehiscent pods (Bentham, 1860). *Millettia* and *Tephrosia*, with dehiscent pods, were separated from *Derris* and *Lonchocarpus* and placed within the broadly circumscribed tribe Galegeae (Bentham, 1865) or in the more narrowly circumscribed tribe Tephrosieae (Gillett 1971). As suggested by Geesink (1981), the structural differences between an indehiscent and dehiscent pod is not morphologically based, transitions do occur, and a classification based upon pod shapes is certainly more complicated with the present state of knowledge of this group than a simple division into dehiscent and indehiscent. He established Millettieae (formerly Tephrosieae s.l.) and included all the genera mentioned above.

Several molecular markers have been widely used to assess the genetic diversity and study of phylogenies in a number of legume taxa like *Acacia* (Casiva *et al.*, 2002), *Afgekia* (Prathepha and Baimai, 2003), *Astragalus* (Sanderson and Liston, 1995), *Atylosia-Cajanus* complex (Parani *et al.*, 2000), *Lathyrus* (Asmussen and Liston, 1998), *Lotus* (Campos *et al.*, 1994), *Medicago* (Bena *et al.*,

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1998), Phaseoleae (Bruneau *et al.*, 1994) and *Wisteria* and *Callerya* (Liston, 1995). The molecular phylogeny of Millettieae has been reviewed and analyzed by Hu *et al.* (2000).

In order to authenticate the existing classification and phylogeny of the Indian members of the tribe Millettieae based on taxonomic evidences, to circumscribe certain problematic genera and to establish the taxonomic status of some infra-specific categories in *Tephrosia*, an attempt was made to characterize 9 representative species of the tribe belonging to 5 traditionally recognized genera by RAPD marker.

Materials and Methods

Plant material

Seeds of three species of *Tephrosia*, two species of *Derris*, two species of *Milletia* and one species each of *Pongamia* and *Piscidia* were collected from the garden of Regional Plant Resource Centre, Bhubaneswar and different forest areas of Orissa, India and plants were raised in the nursery for collection of samples for the present study. The correct botanical names with author citation, synonym(s), habit, flowering and fruiting time, collection locality with field numbers etc. are given in Table I. The voucher herbarium specimens are deposited in the Herbarium of Regional Plant Resource Centre, Bhubaneswar, Orissa, India. Very tender and healthy leaves were taken for isolation of genomic DNA.

Genomic DNA isolation

DNA was isolated from young and freshly collected leaves using the CTAB method as described by Saghai-Maroof *et al.* (1984). RNA was removed by giving RNaseA treatment (@ 60 μg for 1 ml of crude DNA solution at 37 °C) followed by two washes with phenol/chloroform/isoamylalcohol (25:24:1) and subsequently two washes with chloroform/isoamylalcohol (24:1). After centrifugation, the upper aqueous phase was separated, 1/10 volume 3 M sodium acetate (pH 4.8) was added and DNA was precipitated with 2.5 volume of prechilled absolute ethanol. DNA was dried and dissolved in $T_{10}E_1$ buffer (Tris-HCl 10 mM, EDTA 1 mM, pH 8). Quantification was made by running the dissolved DNA in 0.8% agarose gel along side uncut λ DNA of known concentration. The DNA was diluted to 25 ng per μl for RAPD analysis.

Table I. Botanical names, synonym(s), habit, flowering/fruiting time and collection locality and field numbers of 9 species of the tribe Millettieae.

Name of the species	Habit	Locality with field collection number
Tephrosia pumila (Lam.) Pers. [*T. purpurea* (L.) Pers. var. *pumila* (Lam.) Baker]	Undershrub	Utkal Univ. campus, Bhubaneswar, Orissa, LKA –2795
Tephrosia purpurea (L.) Pers.	Undershrub	Regional Plant Resource Centre, Bhubaneswar, Orissa, LKA – 13.
Tephrosia villosa (L.) Pers. (*T. hirta* Buch.-Ham.)	Undershrub	Regional Plant Resource Centre, Bhubaneswar, Orissa, LKA – 2531
Derris trifoliata Lour. [*D. uliginosa* (Willd.) Benth.]	Climber	Bhitarkanika Mangrove Forests, Orissa, LKA – 2523
Derris scandens (Roxb.) Benth.	Climber	Khandagiri hills, Bhubaneswar, Orissa, LKA – 6176
Millettia peguensis Ali (*M. ovalifolia* Kurz)	Small tree	Regional Plant Resource Centre, Bhubaneswar, Orissa, LKA – 6178
Millettia racemosa (Roxb.) Benth.	Woody climber	Khandagiri hills, Bhubaneswar, Orissa, LKA – 830
Millettia pinnata (L.) Geesink [*Pongamia pinnata* (L.) Pierre.] [*Derris indica* (Lam.) Bennet]	Small tree	Regional Plant Resource Centre, Bhubaneswar, Orissa, LKA – 3440
Piscidia piscipula (L.) Sargent [*P. erythrina* (L.)]	Tree	Regional Plant Resource Centre, Bhubaneswar, Orissa, LKA – 6177

RAPD analysis

For RAPD analysis, PCR amplification of 25 ng of genomic DNA was carried out using standard 18 decamer oligonucleotide primers, *i.e.* OPA02, OPA03, OPA10, OPD02, OPD03, OPD07, OPD08, OPD18, OPD20, OPN04, OPN05, OPN06, OPN07, OPN08, OPN10, OPN11, OPN15 and OPN16 (Operon Technologies, Alameda, USA). The RAPD analysis was performed as per the standard methods of Williams *et al.* (1990). Each amplification reaction mixture of 25 μl contained 20 ng of template DNA, 2.5 μl of 10X assay buffer (100 mM Tris-HCl, pH 8.3, 0.5 M KCl and 0.01% gelatin), 1.5 mM $MgCl_2$, 200 μM each of dNTPs, 20 ng of primer and 0.5 U Taq DNA polymerase (Bangalore Genei Pvt. Ltd., Bangalore, India). The amplification was carried out in a thermal cycler (Perkin Elmer, Model 2400, USA). The first cycle consisted of denaturation of template DNA at 94 °C for 5 min, primer annealing at 37 °C for 1 min and primer extension at 72 °C for 2 min. In the subsequent 42 cycles the period of denaturation was reduced to 1 min while the primer annealing and primer extension time was maintained same as in the first cycle. The last cycle consisted of only primer extension at 72 °C for 7 min. PCR products were separated on a 1.5% agarose gel containing ethidium bromide solution (@ 0.5 μg/ml of gel solution). The size of the amplicons was determined using size standards (100 bp ladder plus; MBI Fermentas, Lithuania). DNA fragments were visualized under UV light and photographed.

Data analysis

The presence/absence of bands in RAPD analysis was recorded in binary (0, 1) form. All the bands (polymorphic and monomorphic) were taken into account for calculation of similarity with a view to avoid over/underestimation of the distance (Gherardi *et al.*, 1998). Jaccard's coefficient of similarity (Jaccard, 1908) was measured and a dendrogram based on similarity coefficients generated by the un-weighted pair group method using arithmetic averages (UPGMA) (Sneath and Sokal, 1973) and SHAN clustering. All the analyses were done by using the computer package NTSYS-PC (Rohlf, 1997).

Results

Nine species of the tribe Millettieae were fingerprinted with 25 RAPD primers out of which only 18 produced reproducible and scorable bands. The details of the primers producing scorable bands, their nucleotide sequence, number of amplicons, number of polymorphic/monomorphic bands,

Table II. RAPD data of nine species of Millettieae using 16 random primers.

Name of the primer	Primer sequence	Total bands amplified	No of polymorphic bands	No of monomorphic bands	No of unique bands
OPA02	TGCCGAGCTG	22	22	0	6
OPA03	AGTCAGCCAC	24	24	0	6
OPA10	GTGATCGCAG	21	21	0	0
OPD02	GGACCCAACC	12	11	1	6
OPD03	GTCGCCGTCA	16	16	0	4
OPD07	TTGGCACGGG	25	25	0	5
OPD08	GTGTGCCCCA	22	22	0	6
OPD18	GAGAGCCAAC	14	14	0	0
OPD20	ACCCGGTCAC	14	14	0	0
OPN04	GACCGACCCA	15	13	2	9
OPN05	ACTGAACGCC	30	30	0	6
OPN06	GAGACGCACA	27	27	0	5
OPN07	CAGCCCAGAG	16	16	0	0
OPN08	ACCTCAGCTC	16	16	0	10
OPN10	ACAACTGGGG	15	15	0	0
OPN11	TCGCCGCAAA	16	16	0	7
OPN15	CAGCGACTCG	26	19	0	7
OPN16	AAGCGACCTG	16	16	0	3
Total		*347*	*344*	*3*	*80*

Fig. 1. RAPD pattern of 9 species of Millettieae using OPN04, OPN05, OPN06. M = 100 bp ladder plus (MBI Fermantus, Lithuania); lane 1 to 9 are *Tephrosia pumila*, *Tephrosia purpurea*, *Tephrosia villosa*, *Derris trifoliata*, *Derris scandens*, *Millettia peguensis*, *Millettia racemosa*, *Pongamia pinnata*, *Piscidia piscipula*, respectively.

number of unique bands and the range of amplified fragments are presented in Table II. Out of the total number of 347 bands amplified, 344 were polymorphic and 3 were monomorphic bands. The maximum number of amplicons was observed in primer OPN05 (30) and least in case of primer OPD02 (12). In OPD02 and OPN04, one and three monomorphic bands were detected, respectively. The RAPD banding pattern in 9 species of the tribe Millettieae using primer OPN04, OPN05 and OPN06 is presented in Fig. 1.

The dendrogram constructed on the basis of RAPD data showed two major clusters in the tribe (Fig. 2). The genus *Tephrosia* with three species namely *Tephrosia pumila*, *T. purpurea* and *T. villosa* formed a distinct clad and the other members of the remaining genera *Derris*, *Millettia*, *Pongamia* and *Piscidia* were grouped under the second cluster. There were two nodes in the second clad

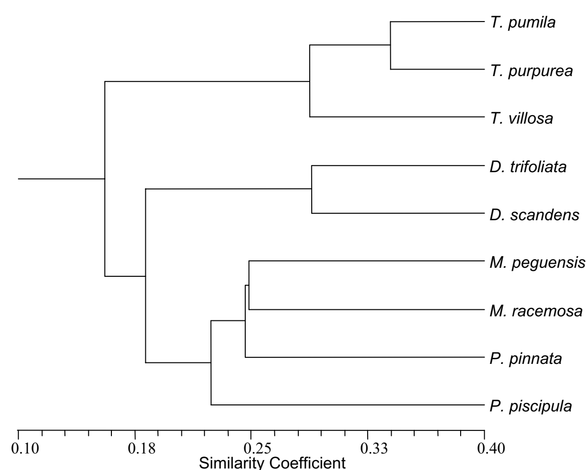

Fig. 2. Dendrogram showing the genomic relationship among 9 species of Millettieae using RAPD marker.

formed by the above 4 genera. While the first node is shared by the two species of *Derris* (*D. scandens* and *D. trifoliata*), the second node is comprised of two species of *Millettia* (*M. peguensis* and *M. racemosa*) and the lone species of *Pongamia* (*P. pinnata*). *Piscidia piscipula* also shared a node with the *Millettia-Pongamia* group at an approximately 22.6% level of similarity. Interestingly, the monotypic genus *Pongamia* shared a node with the species of *Millettia* at a 24.83% level of similarity exhibiting close genetic similarity with it.

Discussion

Hu *et al.* (2000) reconstructed phylogenetic relationships in the tribe Millettieae and allies from chloroplast *trnK/matK* sequences and recognized a well-supported "core Millettieae" clade comprising the 4 large genera *Millettia*, *Lonchocarpus*, *Derris* and *Tephrosia.* Among the core-Millettieae taxa, the genus *Tephrosia* formed a separate clade distantly placed from the second clade comprising of genera *Piscidia*, *Millettia*, *Lonchocarpus*, *Derris* and others. In the latter group, Piscidia was also singled out leaving *Millettia*, *Lonchocarpus*, *Derris* etc. to form a cluster. Our findings based on RAPD analysis are in conformity with the observations of Hu *et al.* (2000).

The genus *Pongamia* exhibited close genetic similarity with *Millettia* and shared a node at a 24.83% level of similarity. This justifies merger of the genus *Pongamia* with *Millettia* on morphological grounds as suggested by Geesink (1984). The merger of the genus *Pongamia* with *Derris* and subsequent transfer of *Pongamia pinnata* to *Derris* (Bennet, 1972) are not tenable and even not supported by taxonomic evidences.

Baker (1876) reduced *Tephrosia pumila* (Lam.) Pers. (= *Galega pumila* Lam.) to a variety under

the widespread *T. purpurea* (L.) Pers., and named it as *T. purpurea* (L.) Pers. var. *pumila* (Lam.) Baker. *T. pumila* differs from *T. purpurea* in its procumbent habit, villous stems, 1–3 flowered racemes, larger red flowers, sub-apical strophiole on the seeds and several other morphological characters. With so much morphological variations, the former taxon deserves the status of a species and not merely an intra-specific category under *T. purpurea* as has been conceived by many subsequent workers. In conjunction, molecular data obtained during the present study on the basis of RAPD analysis also revealed that the 2 taxa exhibit only 38% genetic similarity and are thus distantly related. It appears, therefore, logical to treat *T. pumila* as a distinct species distinguishable from *T. purpurea* by a number of well-defined and consistent characters.

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