JNSF 25-2-73

J. Natn. Sci. Coun. Sri Lanka 1997 25(2): 73-82

PHYLOGENETIC RELATIONSHIPS OF THE SUBGENUS *CERATOTROPIS* BASED ON RANDOM AMPLIFIED POLYMORPHIC DNA

V.A. SUMANASINGHE[1,2], N. TOMOOKA[2] and S. FUKUOKA[2]
[1] *Department of Agricultural Biology, Faculty of Agriculture, University of Peradeniya, Peradeniya.*
[2]*National Institute of Agrobiological Resources, Tsukuba - shi, Ibaraki - 305, Japan.*

(Received: 14 March 1996; accepted: 07 March 1997)

Abstract: Random Amplified DNA Polymorphism (RAPD) was detected for 24 different accessions of 12 different species in the subgenus *Ceratotropis* of the genus *Vigna*. Of the 52 RAPDs generated with 8 decamer primers only one was monomorphic and 51 were polymorphic. Each accession could be distinguished from each other by at least a single RAPD fragment. The cluster analysis based on UPGMA identified 2 major clusters at 70% dissimilarity level representing the azuki and mungbean groups. The 4 accessions of the 3 species in the mungbean group were more divergent than the other 20 accessions of 9 species of the azuki group. *V. reflexo-pilosa* and *V. glabrescens* exhibited the closest affinity among the species. The results were in agreement with the reported analyses based on RFLPs and isozymes.

Key words: *Ceratotropis,*PCR, phylogeny, RAPD, *Vigna.*

INTRODUCTION

The subgenus *Ceratotropis* (Asian *Vigna*) is a morphologically homogeneous and specialized group of Asian origin. The taxonomic treatment of this group is confusing. Verdcourt,[1] transferred the group from the genus *Phaseolus* to the genus *Vigna*. Maréchal *et al.*[2] followed Verdcourt and presented a monograph on the *Phaseolus-Vigna* complex and this has now become the most widely accepted taxonomic treatment on the *Phaseolus-Vigna* complex.

According to Maréchal *et al.*[2], the subgenus *Ceratotropis* consists of 16 species which includes five cultivated crop species, i.e., mungbean (*Vigna radiata*), blackgram (*V. mungo*), moth bean (*V. aconitifolia*), rice bean (*V. umbellata*) and azuki bean (*V. angularis*). Maekawa[3] suggested that *Ceratotropis* should be divided into two separate genera (*Azukia* and *Rudua*) on the basis of differences in seedling characters. Species within the genus *Rudua* show epigeal germination having a primary leaf with a very short petiole, while species belonging to the genus *Azukia* show hypogeal germination having a primary leaf with a long petiole. Although this concept has not been supported by further studies, it is convenient to use this concept in this study. The genus *Rudua* includes mungbean, blackgram and moth bean, while the genus *Azukia* contains rice bean and azuki bean as cultivated species. Therefore, the genus *Rudua* and the genus *Azukia* are hereafter referred to as "mungbean group" and "azuki group", respectively, in the present paper. The species of the "mungbean group" are distributed mainly in the Indian Subcontinent, while the species of the "azuki group" are found principally in East and Southeast Asia. Since the available number of specimens for the wild species in "azuki group" is so limited, suggestion was made[2] that the taxonomic treatment of the wild species in the "azuki group" should be rearranged by further examination of the newly collected specimens. Recently, several wild species belonging to the "azuki group" were collected in Thailand, Japan and Malaysia by exploration.[4-7]

The objective of the present study was to apply RAPD (Random Amplified Polymorphic DNA) analysis in the species in the subgenus *Ceratotropis* to clarify the phylogenetic relationships among the species.

METHODS AND MATERIALS

Materials: All the plants analyzed were grown in a greenhouse at the National Institute of Agrobiological Resources, Tsukuba, Japan. Forty five accessions involving at least two accessions each from 20 species both from azuki and mungbean groups were included at the DNA isolation stage and 24 selected accessions from 12 species at the RAPD detection. The young tender leaves harvested from mainly 1-2 week old seedlings or from somewhat mature plants were used. The species, accessions and their place of origin are given in Table 1.

Extraction of DNA: Two methods were adapted in DNA isolation. At the inception, isolation of DNA in larger quantities was attempted based on Murray & Thompson.[8] Tender leaves (about 8g of fresh weight) were collected in an ice container, cut into small pieces, frozen in liquid nitrogen and stored at -80^{0}C. They were freeze dried for 48 h and ground to a fine powder using Cyclotec sample mill grinder.

The powdered leaf sample was transferred into a 50 ml vial and 15 ml of hot 1x CTAB buffer (1% cetyltrimethyl ammonium bromide; 0.1% Tris-HCl,pH 8.0; 1.4M sodium chloride) was added into it and then placed in a water bath at 57^{0}C for 30 min. Proteins were separated into a heavier lower phase by centrifugation at 2500 rpm for 15 min after mixing 10 ml of 1 isoamyl alcohol : 24 chloroform v/v solution. DNA was precipitated with an equivalent volume of precipitation buffer (1% CTAB; 50mM Tris-HCl, pH 8.0; 10mM EDTA) and recovered by centrifugation at 2500 rpm for 15 min. The pellet was completely dissolved in 10 ml of high salt-TE buffer (1M NaCl; 10mM Tris-HCl,pH 8.0; 1mM EDTA) at 57^{0}C in a water bath for 3-4 h. DNA was re-precipitated with an equal volume of isopropanol after centrifugation again at 2500 rpm for 10 min to remove undissolved contaminants and transferred carefully with a blunt-tipped disposable pipette into a 1.5 ml effendorp vial. The pellet was washed with 2-3 changes of 70% ethanol and stored at 4^{0}C in a refrigerator in 0.1x TE + 100 x RNase (100µg/ml RNase).

Table 1: Accessions used in the experiment.

ID No.	Species Name	Accession Name	Origin
C1-1	*V. angularis* var. *angularis*	Kyoto Dainagon	Japan
C1-2	"	102	"
C2-1	*V. angularis* var. *nipponensis*	Sendai	"
C2-2	"	VA0001	"
C3-1	*V. umbellata* var. *umbellata*	89-531	Nepal
C3-2	"	NT9152	Thailand
Chan	"	Chanta Buri	"
C4-1	*V. umbellata* var. *gracilis*	6-1-1	"
C4-2	"	DKL	"
C5-1	*V. riukiuensis*	Irio-5	Japan
C5-2	"	TNH 25277	Taiwan
C6-1	*V. nakashimae*	Kankoku	Korea
C6-2	"	Ukushima	Japan
C7-1	*V. minima* var. *minima*	Fujieda	Taiwan
C7-2	*V. minima* subsp. *gracilis*	M27	Malaysia
C8-1	"	Species C	Thailand
C8-2	"	Somyot 23	"
C9-1	unidentified species	Species D	"
C9-2	"	Somyot 20	"
C10	*V. nepalensis*	Nepalen	Nepal
C11-1	*V. trinervia*	M18	Malaysia
C11-2	"	NT9144	Thailand
M3	"	M3	Malaysia
M12	"	M12	"
M36	"	M36	"
C12-1	*V reflexo-pilosa*	M23	"
C12-2	"	Yona 5	Japan
C13	*V. glabrescens*	V1160	Philippines
C14-1	*V. radiata* var. *radiata*	Tanegashima	Japan
C14-2	"	250007	Iran
C15-1	*V. radiata* var. *sublobata*	TC1965	India
C15-2	"	TC1966	Madagascar
C16-1	*V. mungo* var. *mungo*	Subsomotod	Thailand
C16-4	"	BC48	"
C17-1	*V. mungo* var. *silvestris*	TC2211	India
C17-2	"	Somyot 5	Thailand
C17-3	"	TC2210	India
C18	*V. grandiflora*	Species A	Thailand
C19-1	*V. aconitifolia*	Nilgiri	India
C19-2	"	2752.5	Pakistan
C19-3	"	2754.3	"
C19-4	"	2762.2	"
C20-1	*V. trilobata*	NI251	India
C20-2	"	NI1030	"
C21	*V. radiata* var. *setulosa*	NI11135	"

However, since some accessions, at the dilution stage tend to produce smears and unclear bands, a small-scale modified method was attempted at DNA isolation.[9] Mercapto ethanol 0.3% was added to 1ml of 2x CTAB buffer in order to remove phenolic contaminants. After isoamyl alcohol 1 : chloroform 24 extraction, DNA was precipitated using ammonium acetate : 6 ethanol v/v solution. Re-purification of DNA isolates was accomplished using phenol-chloroform method.[10] DNA concentration was estimated by the mini-gel method in comparison with λ -DNA of known concentrations.

PCR Runs: Polymerase chain reaction (PCR)[11] was performed in a polycarbonate microplate (Techne Hi-Temp 96) of 12 x 8 wells but using only the centermost 48 wells per run. 1ng of template DNA was first placed at the bottom of the well and subsequently 8 μl of premix consisting of 5.4 μl of sterilized, demineralized distilled water, 1.0 μl of 10x buffer(10mM Tris-HCl, pH 8.3; 50mM KCl, 2mM $MgCl_2$, 0.001% (w/v) gelatin, 0.2mM each of dATP, dCTP, dGTP, dTTP, 0.2 μl of 25mM magnesium chloride, 2μM of random primer and 0.2 units of Taq DNA polymerase (Toyo, Japan) added and finally overlaid with a drop of mineral oil. The PCR apparatus (Techne PHC-Thermal Cycler) was programmed for 45 cycles each consisting of 93°C for 1 min, 35°C for 2 min and 72°C for 3 min and the holding cycle with 72°C for 7 min. The amplified DNA was electrophoresed on a submerged 1.6 or 2.0 % agarose gel at 50 v for 1.5 h, stained with ethidium bromide and photographed under UV light.

The importance of the concentration of the DNA template used for the amplification was evident. Some failed to produce amplified products with 5ng/ μl templates. When dilutions of 1.0, 0.5 and 0.25 ng/μ l of the same sample were tested, they produced consistent RAPDs and consequently 0.5 ng templates were used in this study. The effect of concentration on AP-PCR (i.e. RAPD) has been reported by Welsh & McClelland.[12]

Data analysis: Twelve random primers (P1,P2,P3,P4,P16,P21,P22,P25,P28,P29, P31 & P34 maintained at the Plant Germplasm Introduction Laboratory, NIAR) each ten bases in length were selected from a pool of primers that gave reasonable numbers of amplification under the PCR reaction conditions described above. Each cultivar was scored for the presence or absence of reliable amplification product and the data were entered into a symmetrical data matrix. Coefficient of dissimilarity as the % of mismatched RAPDs were calculated on the basis of the formula[13]:

$$D = 1 -[2n_{xy}/(n_x + n_y)]$$

where D equals the percentage of mismatched fragments between the pair of accession x and y and n_{xy} represents the matched fragments for both accessions. Cluster analysis using UPGMA (unweighted pair group method with averages)

Cluster analysis using UPGMA (unweighted pair group method with averages) was performed and a dendrogram was constructed.[13]

RESULTS

In isolating DNA, the modified method served to be more cost and time effective than the mass method. Even though the yield of DNA was a little lower, it did not require freeze drying of material nor larger volumes of samples and reagents. The samples were directly crushed with a small volume of liquid nitrogen into a powder and DNA could be extracted the same day saving 2-3 days of time usually spent on isolation with the original method.

Of the studied species of sub-genus *Ceratotropis*, isolation was easier with accessions of azuki bean group but accessions from the mungbean subgroup were not amicable with isolation. They either yielded no DNA at all or only minute amounts of fragmentized DNA. The modified isolation method improved the situation and it yielded DNA even from the above difficult species. However, many accessions of them produced poor results at amplification and as a result all the accessions could not be included in RAPD analysis. Thus differences between azuki and mungbean groups were indicated even at the stages of DNA isolation and amplification. Since some of the accessions yielded pigmented DNA, necessity of further purification is suggested.

Altogether 12 random primers were attempted and data on 8 repeatable and persistent primed events were taken into consideration; two of the primers, P4 & P29, produced no amplification. The screening of 8 decamer primers against 24 accessions of *Vigna* resulted in the amplification of discernible DNA fragments ranging from 2500 to 100 bp. The bands beyond this range which were neither prominent nor consistent were not considered in the statistical analysis. This resulted in an average of 6.5 fragments per primer. The size of amplified DNA was measured in comparison with Lambda-DNA digested with *Hind-III* and *Pst-I* restricion enzymes.

An example of a typical analysis is illustrated in Fig. 1. Oligonuleotide P1 primed the amplification of 6 fragments of which 5 were polymorphic and 1 is shared by all the accessions.

DISCUSSION

RAPDs have become useful in determining relationships among various taxa and have been successfully deployed in studying polymorphism in wheat,[14] elucidating phylogenetics of cereals,[15] identifying accessions of rice,[16] determining parentage of maize[12] and tracing resistant gene markers in *Phaseolus*.[17]

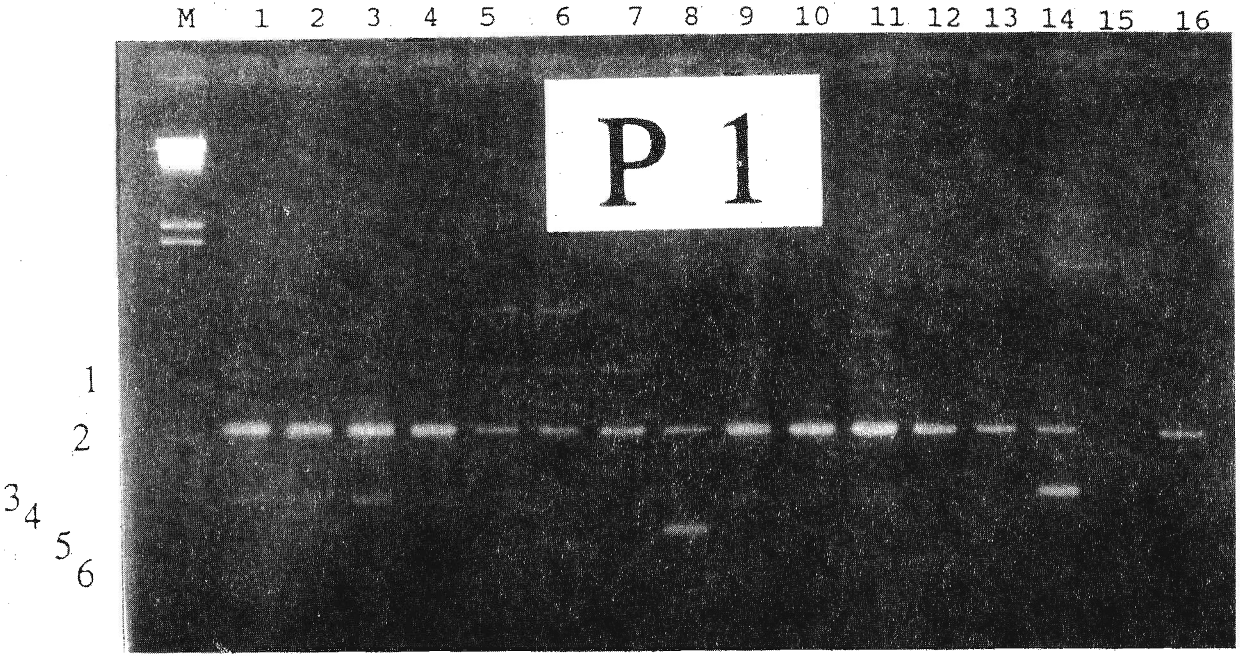

Figure 1: Random Amplified Polymorphic DNAs (RAPD) generated by the decanucleotide primer P1 with base sequence 5'-GTCTGACGGT. Lanes: M=-DNA digested with *Hind-III*, 1=C1-1, 2=C1-2, 3=C2-1, 4=C2-2, 5=C3-1, 6=C3-2, 7=C4-1, 8=C4-2, 9=C5-1, 10=C5-2, 11=C6-1, 12=C6-2, 13=C7-1, 14=C7-2, 15=C8-1, 16=C8-2.
Fragment 2 was monomorphic while 1,3,4,5 and 6 were polymorphic.

In the present study, a total of 52 RAPDs were recorded from the PCR experiments. Only one fragment of the P1 primer was monomorphic among all the accessions and, therefore almost all the observed amplification fragments were phylogenetically important and could be deployed in generating a cladogram.

The cluster analysis produced two major clusters at 70% dissimilarity level representing the azuki and mungbean groups (Fig. 2). The larger cluster with less divergence at 62% level consists of most of the studied species which belong to the hypogeal germination type. The separation of the 2 groups has been reported by RFLP analysis[18] and differences in carbohydrate composition.[19]

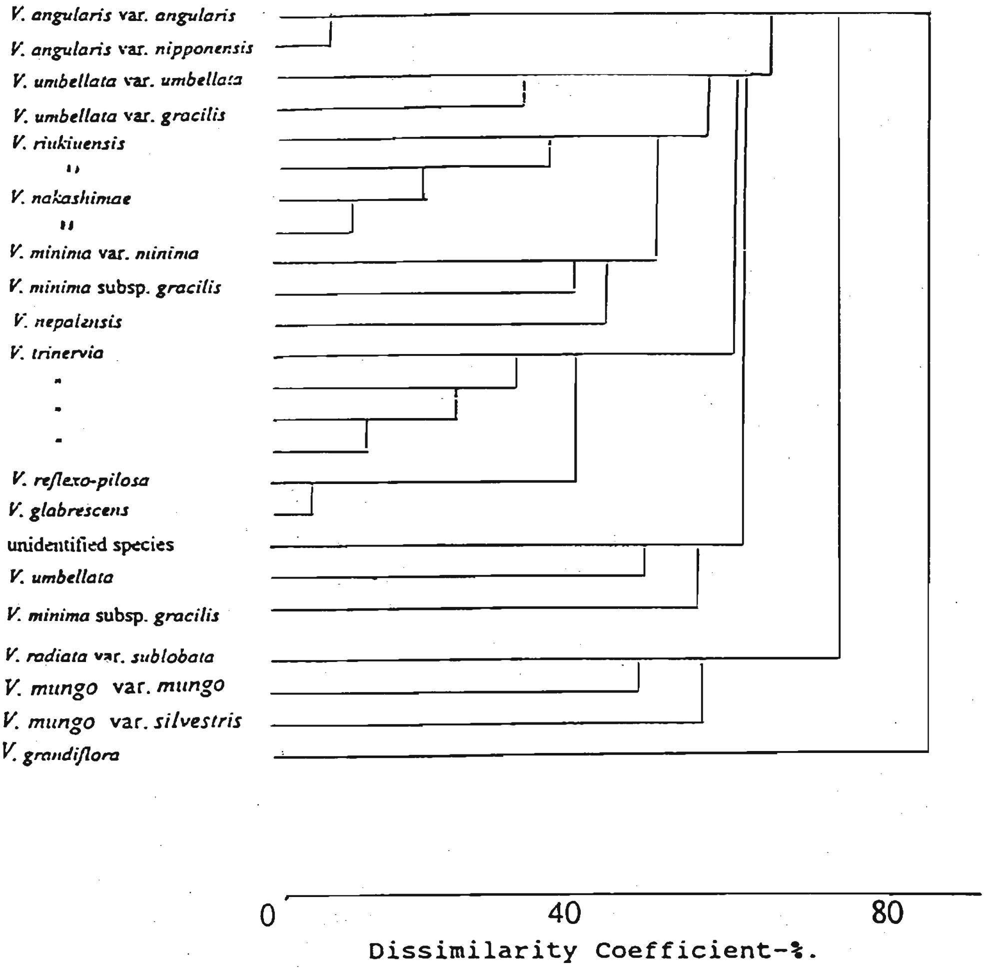

Figure 2: **Dendrogram depicting the phylogenetic relationships of the 24 accessions of the *Ceratotropis* sub-genus.**

Of the accessions, the closest pair was *V. reflexo-pilosa* and *V. glabrescens* which show more than 96% of similarity. This supports the hypothesis that *V. reflexo-pilosa* is the putative ancestor of *V. glabrescens*. In the group, both of them are unique being the only tetraploids with 2n=4x=44, and successful crosses have been obtained between the two species implying the homogeneity of the two genomes. The next most closely related pair was the two varieties *angularis* and *nipponensis* of *V. angularis* as is expected. The pair forms a mini cluster with the two varieties of *V. umbellata* at a higher diverging point. The relative position of *V. angularis* and *V. umbellata* in azuki cluster and of *V. mungo* and *V. radiata* in the mungbean cluster is in good agreement with RFLP results.[18] The isolation of the four accessions of *V.trinervia* into a common cluster at 31% level indicates their closeness to each other although three are from Malaysia and the fourth is from Thailand. This mini cluster identifies *V. reflexo-pilosa* and *V. glabrescens* pair as their closest relatives. However, placement of *V. trinervia* in the middle of the major cluster contradicts the belief that *V.trinervia* is intermediary between mungbean and azuki groups. The unidentified species, C9-1, exhibits congruity to wild *V. umbellata*, but the distant separation of wild *V. umbellata* from its cultivated counterparts and of C8-2 from other *V. trinervia* relatives is inexplicable.

The four members of the mungbean group show more divergence among themselves than their counterparts in the azuki bean group. Similar findings have been reported.[5] *V. grandiflora* was the most divergent indivdual of all. Since it failed to produce AP-PCR with several primers, its position in relation to the others is not reliable.

In the present study only four accessions of the mungbean group could be included due to the inability of many others to produce RAPDs. The failure may have been caused by two factors. Either they require conditions and time periods for PCR different from the conditions which were suitable with azuki members. Or it may be due to some contaminants still remaining in the template even after attempts of re-purification of DNA which inhibt DNA from amplifying. Therefore different means of DNA isolation and more conducive conditions in PCR is suggested for the mungbean group.

RAPD is a much faster and more convenient method in the detection of polymorphism than the traditional RFLP analysis. RAPD data can be obtained within 2 h after PCR as opposed to the Southern Blotting and DNA hybridization required in RFLP. Further it does not require specific base sequences identified by restriction endonucleases and thus any arbitrary sequences can be utilized. The linkage between RAPDs with RFLP and isozyme markers has been established in legumes[20] and consequently RAPD in association with the latter would provide faster methods in gene mapping. Therefore the results presented here show promise first in employing them in biodiversity and germplasm evaluation and then in breeding programmes of *Vigna* species.

Acknowledgement

I thank the Japan International Cooperation Agency for providing me with the financial assistance to conduct this research at the National Institute of Agrobiological Resources in Japan.

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