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Evaluation of a Natural Hybrid of *Dumasia* DC. (Fabaceae) from Taiwan Based on the Isozymes and RAPD Studies

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ABSTACT: This paper describes a natural hybrid, derived from the putative parent of *D. miaoliensis* and *D. villosa* subsp. *bicolor*. This hybrid is not only morphologically intermediate between its putative parents but also at three loci (*Aat-1, Aat-2, Mdh-3*) from isozymes data, and twenty-four RAPD bands represent a combination nature in its putative parental species. The nearly complete lack of novel alleles suggest that it is of recent origin and that insufficient time has elapsed for the accumulation of mutation at isozyme loci, but it has little mutations at the DNA level.

KEY WORDS: ***Dumasia*, Fabaceae, Hybrid, Isozyme, RAPD, Taiwan.**

INTRODUCTION

The genus *Dumasia* DC. belongs to the tribe Phaseoleae of the subfamily Faboideae of Fabaceae consists of about 8 species (Lackey, 1981) and is mainly distributed in Asia and Africa particularly in southwest of China.

D. miaoliensis is distributed only in Erpenshong of Tahu, Miaoli County, location at altitudes between 1000 and 1500 m. *D. villosa* subsp. *bicolor*, an endemic subspecies in Taiwan is distributed throughout the mountains around altitudes between 500-2500 m. There are several individuals which also found near Erpenshong of Tahu, Miaoli County, are morphologically intermediate between *D. miaoliensis* Liu & Lu and *D. villosa* DC. Subsp. *bicolor* (Hayata) Ohashi & Tateishi (Huang and Ohashi, 1993).

Molecular markers such as isozymes and random amplified polymorphic DNA (RAPD) have been extensively used for genetic and plant hybridization studies (Arnord *et al.*, 1991; Waugh *et al.*, 1992; Crawford *et al.*, 1993; Kurihara *et al.*, 1996; Padgett *et al.*, 1998). Gallez and Gottlieb (1982) studied allozymes in the three species as an additional test of hybrid origin of *Stephanomeria diegensis*. Based on alleles at four loci in *S. diegensis* which represent a combination of its putative parental species, they considered it as a hybrid origin. Crawford *et al.* (1993) using RAPDs data to document the origin of an intergeneric hybrid (*Margyracaena skottsbergii*), and concluded that RAPDs were very attractive for the study of hybridization in rare species because they can provide numerous genetic markers while only requiring minimal amounts of DNA. Arnord *et al.* (1991) used RAPDs to analyze the hybridization and introgression in Louisiana irises. They reported intermediate frequencies for RAPD markers in putative hybrid population. Waugh *et al.* (1992) used six RAPD primers for detection of introgression in potato, and concluded that RAPDs would be useful for the detection for gene introgression in both natural and cultivated plant population.

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The natural hybridization in other flowering plants has been reported scarcely in Taiwan. Most evidences are from morphological and cytological data (Hu and Chang, 1975; Kuo 1988, 1990; Peng, 1983, 1990; Lin and Wang, 1991, Wang and Huang, 1992; Peng and Sue, 2000). The objective of this study is to use the isozymes and RAPD techniques to verify the potential occurrence of natural hybridization of Taiwan's *Dumasia* species.

MATERIALS AND METHODS

Plant materials

Samples from sixteen populations (Table 1, numbers 1-16) were used for isozyme study, and six additional populations (Table 1, numbers 17-22) were used for RAPD study. The sample sites and vouchers are given in Table 1. The locations of the investigated populations are presented on a map in Figure 1.

Leaf tissues were obtained from 30 individuals of each populations, except for the populations 3 and 19, only 5 individuals per population were sampled.

Table 1. Locality of *Dumasia* included in this study; samples used for isozyme (1-16) and RAPD (17-22) are from different population.

Taxa	Population	Collection site	Sample size	Vouchers
D. miaoliensis	1	CHINGCHIENG	30	*Liu 835*
	2	ERPENSHONG	30	*Liu 821*
The putative hybrid	3	ERPENSHONG	5	*Liu 822*
D. villosa subsp. *bicolor*	4	WULAI	30	*Liu 825*
	5	BALIN	30	*Liu 830*
	6	CHINGCHIENG	30	*Liu 834*
	7	ERPENSHONG	30	*Liu 823*
	8	KUNWU	30	*Liu 824*
	9	LISHAN	30	*Liu 826*
	10	WUSHE	30	*Liu 827*
	11	TENGTEIN	30	*Liu 829*
	12	CHUYUNSHAN	30	*Liu 828*
	13	MEISHAN	30	*Liu 830*
	14	TAIWU	30	*Liu 832*
	15	HUITOUWAN	30	*Liu 833*
	16	YENHALLINTAO	30	*Liu 836*
D. miaoliensis	17	CHINGCHIENG	30	*Liu 861*
	18	ERPENSHONG	30	*Liu 863*
The putative hybrid	19	ERPENSHONG	5	*Liu 866*
D. villosa subsp. *bicolor*	20	WULAI	30	*Liu 855*
	21	CHINGCHIENG	30	*Liu 862*
	22	ERPENSHONG	30	*Liu 864*

Isozyme electrophoresis

Young fresh leaf tissues were grounded with extraction buffer (Feret, 1971), followed by electrophoresis and staining procedures based on Soltis *et al.* (1983) and Cheliak and Pitel (1984). Nine enzyme systems, namely *AAT* (L-aspartate aminotransferase, E.C.2.6.1.1), *DIA* (diaphorase, E.C.1.8.1.4), *HK* (hexokinase, E.C.2.7.1.1), *IDH* (isocitrate dehydrogenase, E.C.1.1.1.42), *MDH* (malate dehydrogenase, E.C.1.1.1.37), *ME* (malic enzyme, E.C.1.1.1.40), *6PGDH* (6-phosphogluconate dehydrogenase, E.C.1.1.1.44), *PGM* (phosphoglucomutase, E.C.5.4.2.2) and *SKDH* (shikimate, 5-dehydrogenase, E.C.1.1.1.25) were examined for isozyme study.

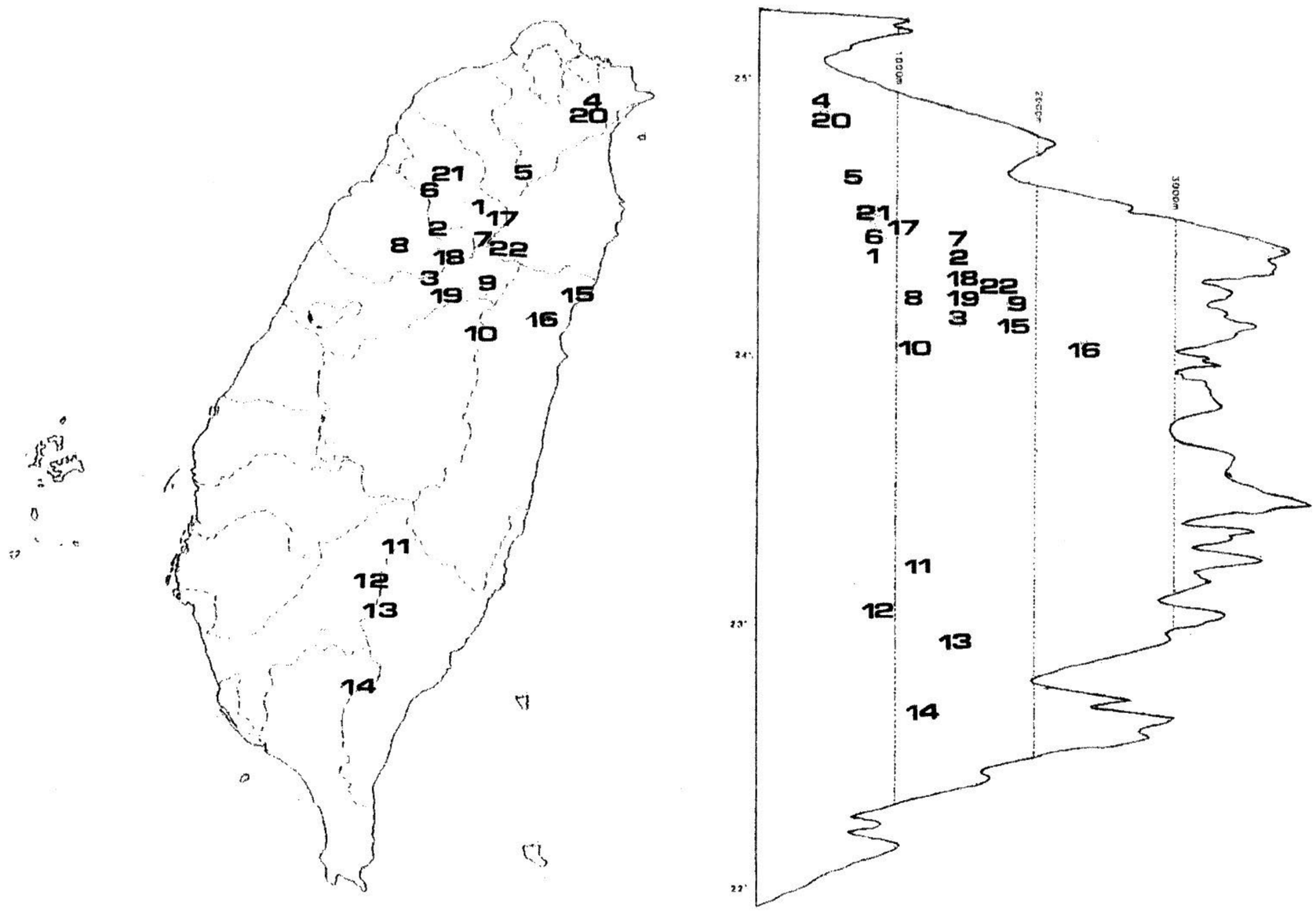

Fig. 1. Map distribution of the samples investigated in this study. *D. miaoliensis* (1, 2, 17 and 18), putative hybrid (3 and 19) and *D. villosa* subsp. *bicolor* (3-16 and 20-22).

DNA preparation

Total DNA was prepared from 0.05 g of young leaf tissue using a modified mini-CTAB method (Doyle and Doyle, 1990). Leaves were frozen in liquid nitrogen and grounded to fine powder in the 1.5 ml eppendoff tube. After adding 600 μL 2% CTAB-PVP and heating at 65℃ for 30 min, and samples were centrifuged in a Sigma 202-MC centrifuge (Sigma) at 12000 rpm for 15 min. They were extracted with 500 μL chloroform:isoaml alcohol (24:1) and centrifuged at 12000 rpm for 10 min. The DNA was precipitated by adding 1 ml 100% absolute ethanol and 150 μL 5M NaCl and pelleted by centrigugation at 13000 rpm for 10 min, then washed with 70% ethanol and dried with 100% absolute ethanol. The pellet was dissolved in 100 μL TE buffer and stored at -20℃.

Polymerase chain reaction

PCR conditions for RAPD reaction with the Perkin-Elmer GENEAMP PCR SYSTEM 9700 Thermal Cycler are described as follows. Each sample comprises 50 mM Tris-HCl (pH 8.5) buffer containing 20 mM KCl, 2 mM $MgCl_2$, 0.5 mg/mL BSA, 200 μM each of dATP, dCTP, dGTP, dTTP, 0.4 μM primer, 10 ng of template DNA and 0.6 units of Taq DNA polymerase (Lifetech) in a final volume of 10 μL. The first two cycles of PCR starts with denaturation at 94℃ for 60 sec, primer annealing at 36℃ for 30 sec, and primer extension at 72℃ for 90 sec. The time for template denaturation and annealing was then reduced to 3 sec and 20sec for 43 cycles. Reactions were further incubated at 72℃ for 4 min and the samples were stored at 4℃ before gel electrophoresis. One hundred random primers (Operon kits A, B, H, O and P; Operon Technologies) were used to screen genomic DNA. Nine primers (Table 2) produced clear hybridization bands. PCR products were separated using 2.0% NuSieve 3:1 agarose (FMC BioProducts) gels by electrophoresis in 0.5 X TBE buffer, and visualized by a ultraviolet light transilluminator after stained with ethidium bromide.

Table 2. Nine primers used for this study.

Primers	Sequence
OPA-04	5′-AATCGGGCTG-3′
OPA-07	5′-GAAACGGGTG-3′
OPA-08	5′-GTGACGTAGG-3′
OPA-12	5′-TCGGCGATAG-3′
OPA-14	5′-TCTGTGCTGG-3′
OPA-18	5′-AGGTGACCGT-3′
OPA-20	5′-GTTGCGATCC-3′
OPB-01	5′-GTTTCGCTCC-3′
OPH-18	5′-GAATCGGCCA-3′

Data analysis

Data matrices based on the presence and absence of loci were analyzed using Jaccard's similarity coefficients. The relationship between populations was then displayed as a dendrogram using the UPGMA (unweighted pair group method arithmetic average) routine in the NTSYS-pc 1.8 program (Rohlf, 1993).

RESULTS

Electrophoretic analysis of nine enzyme systems has generated a total of 11 scored loci and identified three useful loci (*Aat-1, Aat-2* and *Mdh-3*) for discriminating putative hybridization taxa. The isozyme phenotype of *Aat-1, Aat-2* and *Mdh-3* gives strong evidence of hybridization, as the alleles fixed in populations of *D. miaoliensis* (*Aat-1a, Aat-2b and Mdh-3b*) is different from the allele fixed in populations of *D. villosa* subsp. *bicolor* (*Aat-1b, Aat-2a and Mdh-3a*) and both alleles are present in the putative hybrid (Table 3). The average of genetic identities among the 2 populations of *D. miaoliensis* was 0.982, and among the 13 populations of *D. villosa* subsp. *bicolor* was 0.932. The mean value of genetic identities between *D. miaoliensis* and the putative hybrid was 0.895, between *D. villosa* subsp. *bicolor* and the putative hybrid was 0.845. The UPGMA dendrogram produced clusters of populations separating *D. miaoliensis*, the putative hybrid and *D. villosa* subsp. *bicolor* into three distinct groups (Fig. 2).

One hundred random 10-mer primers (Operon Technologies) were used for RAPD analysis. Nine primers (OPA-04, OPA-07, OPA-08, OPA-12, OPA-14, OPA-18, OPA-20, OPB-01, and OPH-18) used in this survey generated a total of 36 scored polymorphic bands out of 44 total bands (82%) in the six populations. *D. miaoliensis* has 13 species-specific bands (OPA-04$_{1250}$, OPA-07$_{1610}$, OPA-08$_{1150}$, OPA-12$_{590}$, OPA-14$_{600,460}$, OPA-18$_{750,520}$, OPA-20$_{1000}$, OPB-01$_{2500,510}$, OPH-18$_{570,520}$), and *D. villosa* subsp. *bicolor* has 11 species-specific bands (OPA-04$_{1600}$, OPA-07$_{540}$, OPA-08$_{1000}$, OPA-12$_{1300}$, OPA-14$_{1150}$, OPA-18$_{610}$, OPA-20$_{750}$, OPB-01$_{1300,680}$, OPH-18$_{1050,470}$). All of the twenty-four bands are present in the putative hybrid populations (Table 4). Three of the RAPD gel patterns are presented in Figures 3 to 5. In Fig. 3 (primer OPA-20), a molecular weight 1000 bp band is present in *D. miaoliensis* (lanes 2-6) and the hybrid (lanes 7-11), but not in *D. villosa* subsp. *bicolor* (lanes 12-16). The 750 bp band is present in *D. villosa* subsp. *bicolor* (lanes 12-16) and the hybrid (lanes 7-11), but not in *D. miaoliensis* (lanes 2-6) (Fig. 3). In Fig. 4 (primer OPB-01), the bands 2500 bp. and 510 bp. are common in *D. miaoliensis* (lanes 2-6) and hybrid (lanes 7-11), but not found in *D. villosa* subsp. *bicolor* (lanes 12-16); the bands 1300 bp. and

Table 3. Genotype frequencies at eleven polymorphic loci in the sixteen populations of *Dumasia*. *Hk*, *Mdh-1*, *Mdh-2*, *Me*, *Pgdh-3* and *Skdh-1* are monomorphic loci.

Locus/allele	Population															
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
Aat-1																
a	1.00	1.00														
ab			1.00													
b				1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Aat-2																
a				1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
ab			1.00													
b	1.00	1.00														
Dia-1																
a		0.22	0.60	1.00	1.00	0.88	1.00	1.00	1.00	1.00	0.90	1.00	0.90	0.50	1.00	1.00
ab			0.40			0.12										
b	1.00	0.78									0.10		0.10	0.50		
Dia-2																
a	1.00	1.00	0.60			0.07	1.00									0.43
b			0.40	1.00	1.00	0.93		1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	0.57
Idh-1																
a	1.00	1.00	0.40	1.00	1.00	0.97	1.00	0.70	1.00	1.00	1.00	1.00	1.00	0.80	1.00	1.00
b			0.60			0.03		0.30						0.20		
Idh-2																
a	1.00	1.00	1.00													
b				1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Mdh-3																
a				1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
ab			1.00													
b	1.00	1.00														
Pgdh-1																
a	1.00	1.00	1.00													
b				1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Pgdh-2																
a				1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
ab	0.40	0.27	1.00													
b	0.60	0.73														
Pgm																
a			0.40	0.40	0.13	0.37	0.03	0.10	0.07	1.00		0.67	0.53	0.30	0.17	0.20
b	1.00	1.00	0.60	0.60	0.87	0.63	0.97	0.90	0.93		1.00	0.33	0.47	0.70	0.83	0.80
Skdh-2																
a	0.83	0.32	0.60		0.07	0.13	0.90	0.13	0.00	0.54	0.08	0.03	0.03			0.66
ab								0.87	1.00	0.12		0.09				0.08
b	0.17	0.68	0.40	1.00	0.93	0.87	0.10			0.34	0.92	0.88	0.97	1.00	1.00	0.26

680 bp. are common in *D. villosa* subsp. *bicolor* (lanes 12-16) and hybrid (lanes 2-6) but not in *D. miaoliensis* (lanes 7-11)(Fig. 4). In Fig. 5 (primer OPH-18), the bands 570 bp. and 520bp. are common in *D. miaoliensis* (lanes 2-7) and hybrid (lanes 8-12) but not in *D. villosa* subsp. *bicolor* (lanes 13-19); the bands 1050bp. and 470bp. are common in *D. villosa* subsp. *bicolor* (lanes 13-19) and hybrid (lanes 8-12) but not in *D. miaoliensis* (lanes 2-7). In comparison, there is a 590 bp. band only present in hybrid population, but not detected in their putative parents (Fig. 5). The average of identities among the 2 populations of *D. miaoliensis* was 0.947, and among the 3 populations of *D. villosa* subsp. *bicolor* was 0.962. The mean value of genetic identities between *D. miaoliensis* and the putative hybrid was

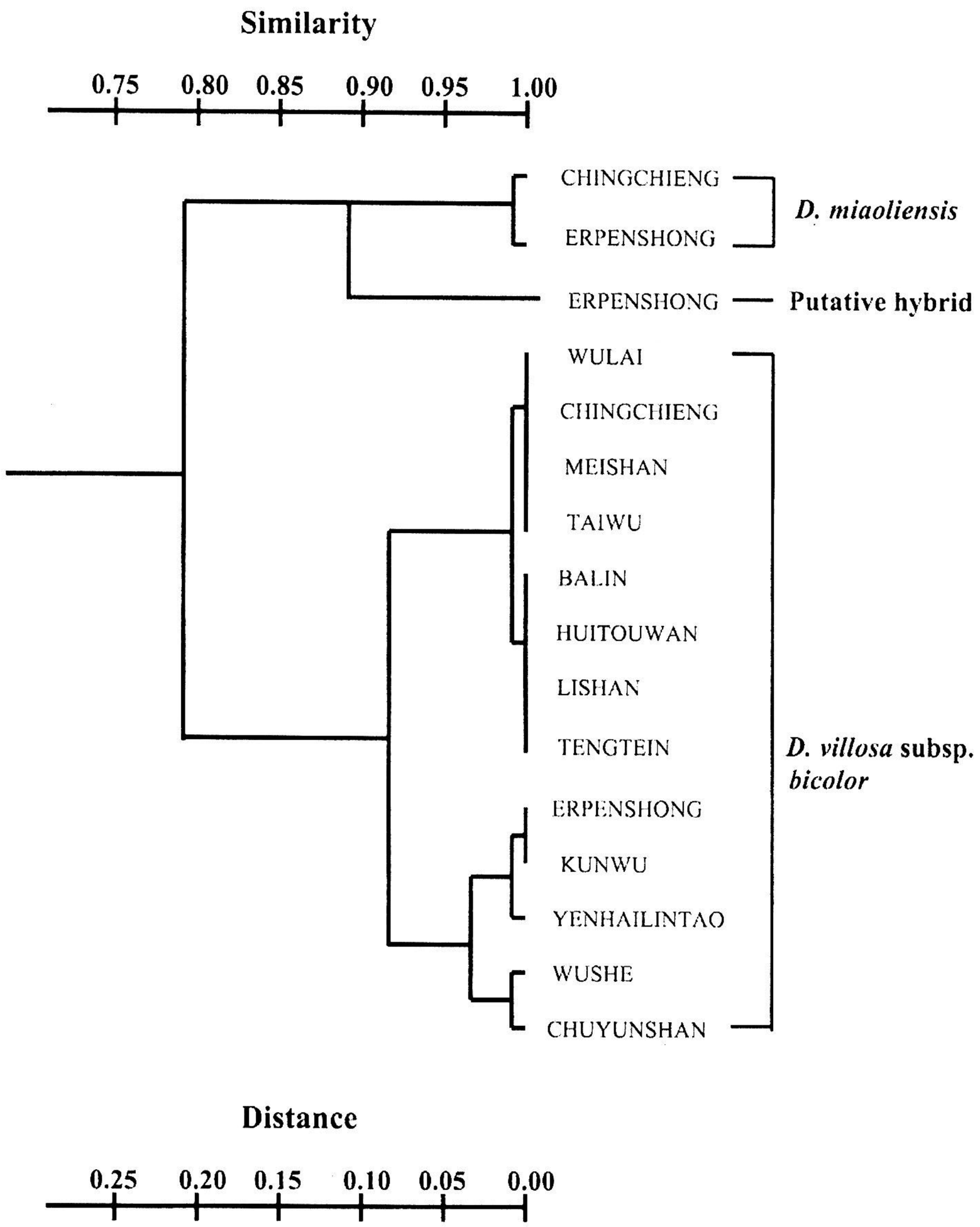

Fig. 2. UPGMA dendrogram of *D. miaoliensis*, the putative hybrid and *D. villosa* subsp. *bicolor* populations based on isozymes data.

D. villosa subsp. *bicolor* and the putative hybrid was 0.402. The UPGMA dendrogram also produced clusters of populations separating *D. miaoliensis*, the putative hybrid and *D. villosa* subsp. *bicolor* into three distinct groups based on RAPD data (Fig. 6).

DISCUSSION

The hybrid of *Dumasia miaoliensis* and *D. villosa* subsp. *bicolor* is morphologically intermediate between its putative parents (Liu and Huang, 2001). Our results from the alleles at three loci in isozyme data and twenty-four RAPDs data support such hybridization event does occur and is congruent with results of the past study. The genotypes of putative hybrid appear to be a composite of alleles contributed by *D. miaoliensis* and *D. villosa* subsp. *bicolor*. The strongest supporting evidence for the origin hybrid is found at the loci *Aat-1*, *Aat-2 and*

Table 4. Frequencies at nine random primers in the six populations of *Dumasia*.

Primer/bp.	Population					
	17	18	19	20	21	22
OPA-04						
2100	0.12	0.00	0.00	0.88	0.88	1.00
1600	0.00	0.00	1.00	1.00	1.00	1.00
1250	1.00	1.00	1.00	0.00	0.00	0.00
450	0.00	0.04	0.00	1.00	1.00	0.88
OPA-07						
1610	1.00	1.00	1.00	0.00	0.00	0.00
1300	0.88	1.00	0.20	0.00	0.00	0.12
540	0.00	0.00	1.00	1.00	1.00	1.00
OPA-08						
2300	0.76	0.88	0.88	0.00	0.00	0.12
1150	1.00	1.00	1.00	0.00	0.00	0.00
1000	0.00	0.00	1.00	1.00	1.00	1.00
510	1.00	1.00	0.80	0.00	0.00	0.12
420	0.75	0.58	1.00	1.00	1.00	0.88
OPA-12						
1300	0.00	0.00	1.00	1.00	1.00	1.00
980	0.04	0.12	0.40	1.00	1.00	0.84
590	1.00	1.00	1.00	0.00	0.00	0.00
480	1.00	1.00	1.00	0.88	0.88	0.76
OPA-14						
1150	0.00	0.00	1.00	1.00	1.00	1.00
680	0.12	0.08	0.20	1.00	1.00	1.00
600	1.00	1.00	1.00	0.00	0.00	0.00
460	1.00	1.00	1.00	0.00	0.00	0.00
OPA-18						
950	1.00	0.88	1.00	1.00	1.00	0.76
750	1.00	1.00	1.00	0.00	0.00	0.00
610	0.00	0.00	1.00	1.00	1.00	1.00
520	1.00	1.00	1.00	0.00	0.00	0.00
OPA-20						
1000	1.00	1.00	1.00	0.00	0.00	0.00
750	0.00	0.00	1.00	1.00	1.00	1.00
OPB-01						
2500	1.00	1.00	1.00	0.00	0.00	0.00
1300	0.00	0.00	1.00	1.00	1.00	1.00
680	0.00	0.00	1.00	1.00	1.00	1.00
510	1.00	1.00	1.00	0.00	0.00	0.00
OPH-18						
1050	0.00	0.00	1.00	1.00	1.00	1.00
650	0.00	0.24	0.00	1.00	1.00	1.00
590	0.00	0.00	1.00	0.00	0.00	0.00
570	1.00	1.00	1.00	0.00	0.00	0.00
520	1.00	1.00	1.00	0.00	0.00	0.00
470	0.00	0.00	1.00	1.00	1.00	1.00

Mdh-3, where one of the two fixed alleles at each locus is present in *D. miaoliensis* and the other in *D. villosa* subsp. *bicolor* (Table 3).

The value of genetic identities of isozyme and RAPD markers between the hybrid and *D. miaoliensis* was 0.895 and 0.462, higher than 0.845 and 0.402 obtained from the hybrid and *D. villosa* subsp. *bicolor* which both revealed that the hybrid phylogenetically is closed to *D.*

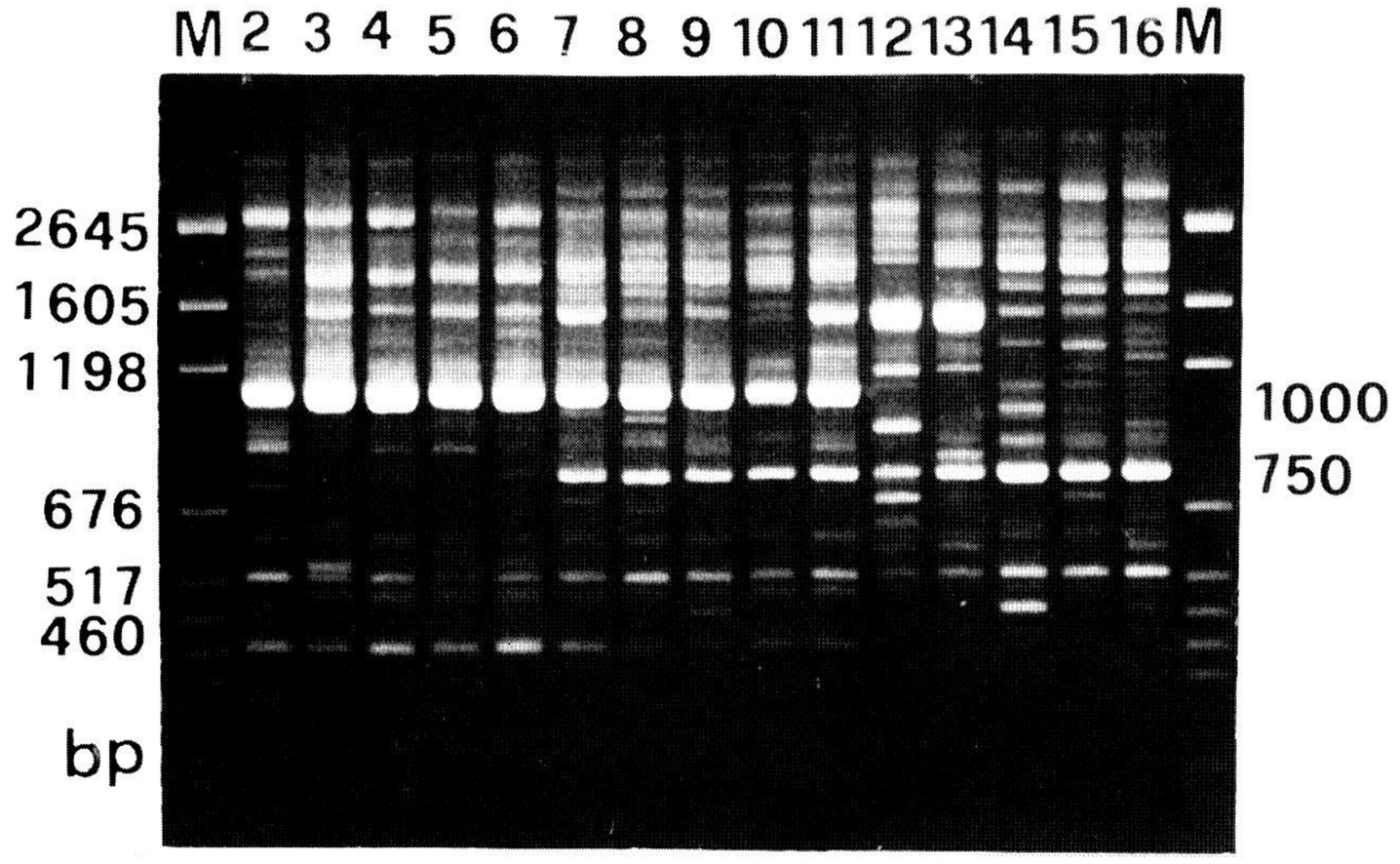

Fig. 3. RAPD banding patterns using primer OPA-20. Lanes 2-6 from the *D. miaoliensis*; lanes 7-11 the putative hybrid; lanes 12-16 *D. villosa* subsp. *bicolor*. M represents pGEM DNA size marker.

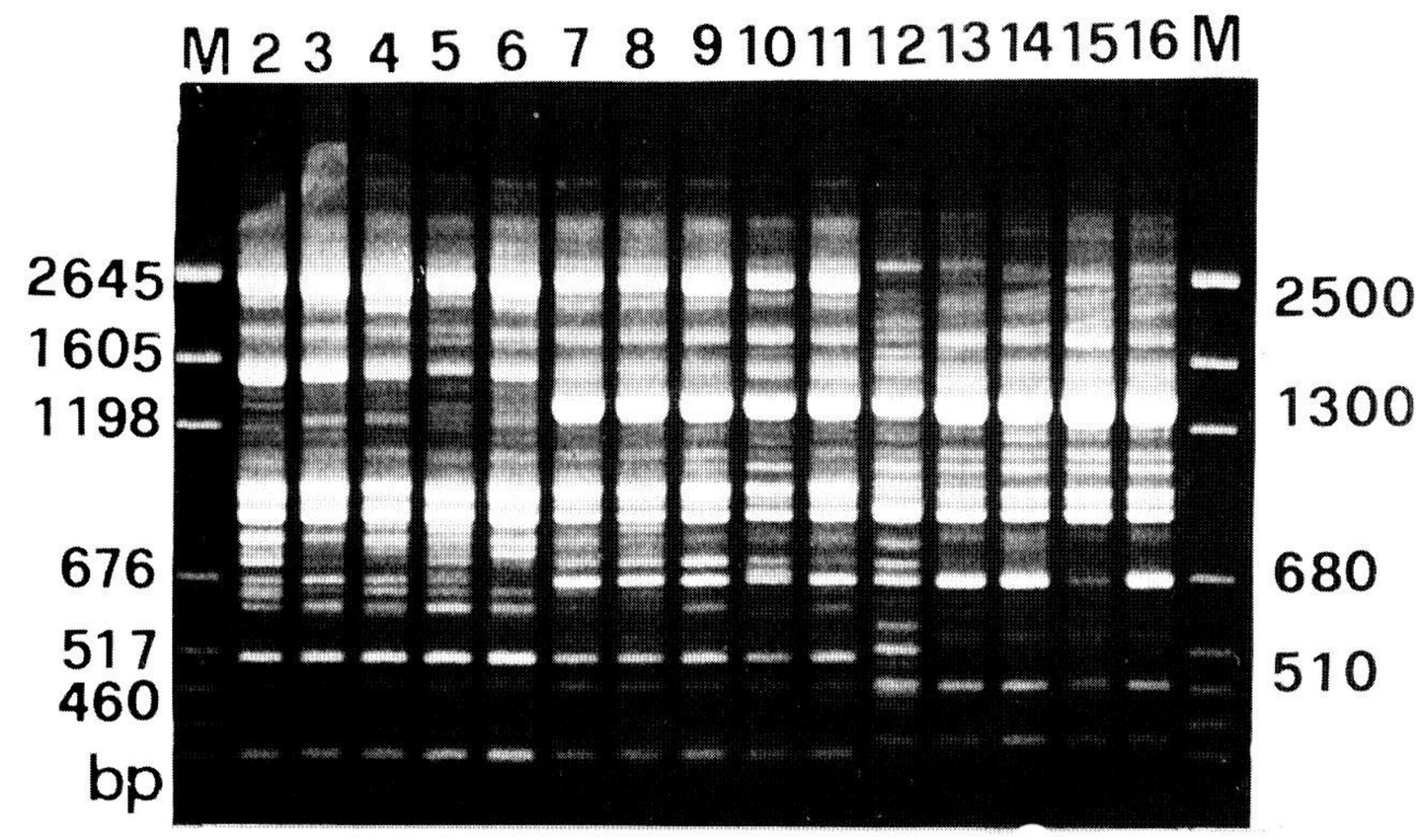

Fig. 4. RAPD banding patterns using primer OPB-01. Lanes 2-6 from the *D. miaoliensis*; lanes 7-11 the putative hybrid; lanes 12-16 *D. villosa* subsp. *bicolor*. M represents pGEM DNA size marker.

miaoliensis than to *D. villosa* subsp. *bicolor* (Figs. 5 and 6). It is ascribable to the hybrid and *D. miaoliensis* were fixed for genotypes *Idh-2a* and *Pgdh-1a*, *D. villosa* subsp. *bicolor* was fixed for genotypes *Idh-2b*, *Pgdh-1b* and *Pdgh-2a* (Table 3), and the appearance of hybrid may be resulted from the hybrid backcrossing to *D. miaoliensis*. This introgression from *D. villosa* subsp. *bicolor* was evident of alleles other than *Idh-2, Pgdh-1 and Pgdh-2*.

Of 11 alleles detected at polymorphic loci in isozyme, there is no unique alleles present in the hybrid derivative species. In comparison, there is one unique band present in the hybrids from RAPD data, which is not found in their putative parents (Fig. 5, Arrow). The nearly complete lack of novel alleles suggest that it has recent origin and that insufficient time has elapsed for the accumulation of mutation at isozyme loci. But it has a mutation in the DNA level, it reveal that the RAPD techniques is more powerful than isozyme in the detection of the hybridization in this case.

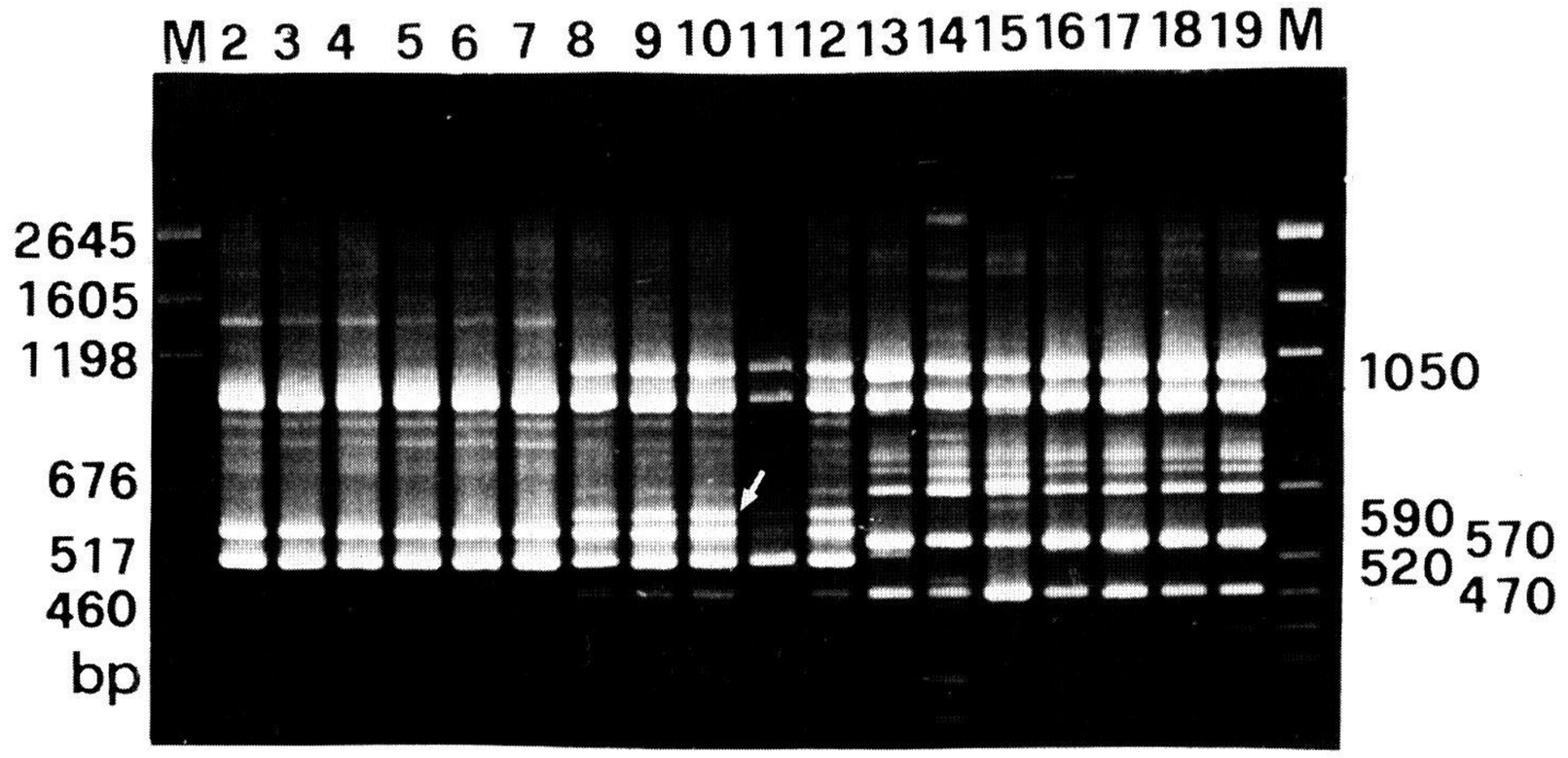

Fig. 5. RAPD banding patterns using primer OPH-18. Lanes 2-7 from the *D. miaoliensis*; lanes 8-12 the putative hybrid; lanes 13-19 *D. villosa* subsp. *bicolor*. M represents pGEM DNA size marker; arrow showing the novel band.

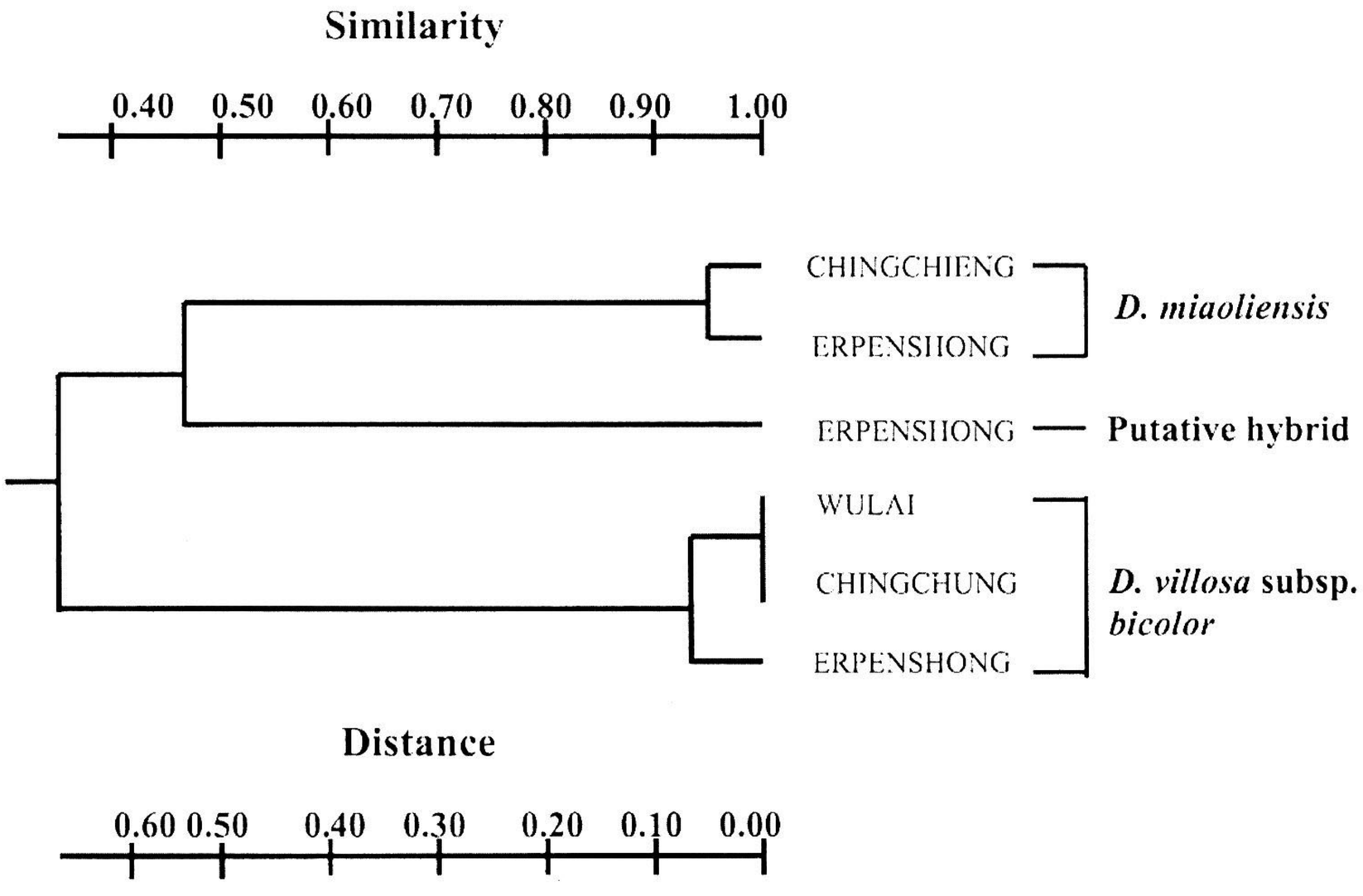

Fig. 6. UPGMA dendrogram of *D. miaoliensis*, the putative hybrid and *D. villosa* subsp. *bicolor* populations based on RAPD data.

It could not find additivity at every locus in every plant because the hybrid was probably not simple F1 hybrids but rather different stabilized recombinant types (Crawford, 1990). Such conclusion can not be drawn in confident since our study for the hybrid was based on only three loci in the isozymes and 24 bands in RAPD.

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利用同功酶和逢機放大多型性 DNA 評估台灣產山黑扁豆屬之一雜交種

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摘　　要

本文利用同功酶和逢機放大多型性DNA研究苗栗野豇豆和台灣山黑扁豆之間的雜交種。雜交種不僅在形態上和同功酶三個基因座 (*Aat-1*, *Aat-2*, *Mdh-3*) 的等位基因條帶介於兩親本之間，且共有疑似親本二十四條逢機放大多型性 DNA 條帶。在同功酶上幾乎沒有新的等位基因產生，此雜交種可能是最近才產生而沒有足夠時間累積突變，但在 DNA 層次上發生少許突變。

關鍵詞：山黑扁豆屬、豆科、雜交種、同功酶、逢機放大多型性 DNA、台灣。

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