Isozyme diversity in *Vigna vexillata* (L.) A. Rich (Fabaceae) complex

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Received 31 July 1997; revised 8 December 1997

An electrophoretic comparison of variation at 32 isozyme loci was performed for 122 *Vigna vexillata* and 3 *V. lobatifolia* accessions. The results do not fit the present delimitation of taxa. According to the results obtained during this study, *Vigna lobatifolia* could be included within *V. vexillata*, and both Asian and West African taxa could deserve a subspecific rank within *V. vexillata*. Considering only Africa, some similarities between the organization of the gene pools of *V. vexillata* and *V. unguiculata* have been found.

Keywords: Fabaceae, *Vigna vexillata*, isozyme polymorphism, Nei's distance.

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Introduction

Vigna Savi (Fabaceae: Phaseoleae) is a pantropical genus which comprises 82 species according to Maréchal *et al.* (1978). One species, *V. unguiculata* (L.) Walp (cowpea), is of major economic importance in Africa. Africa is the only continent where wild forms of cowpea are encountered, and cowpea was also domesticated in Africa (Pasquet 1996b).

The *Vigna unguiculata* gene pool includes several perennial subspecies which are genetically distant and an annual subspecies (subsp. *unguiculata*) which includes both cultivated forms (var. *unguiculata*) and wild forms [var. *spontanea* (Schweinf.) Pasquet] (Pasquet 1996a). However, there are introgressions between wild and cultivated cowpeas, and also a crop-weed complex, at least in the dry savannas of West Africa (Pasquet unpublished data).

In this context, it is interesting to study the gene pool of species closely related to *V. unguiculata,* as they do not include cultivated forms in Africa. *Vigna vexillata* (L.) A.Rich. is one of these species, and is the most closely related to *V. unguiculata* (Vaillancourt & Weeden 1993; Vaillancourt *et al.* 1993a). Flowers and pods are similar, but there are differences in stipule and keel shape. Keel shape and position of anthers and stigma induce nototribic pollination in *V. vexillata* and sternotribic pollination in *V. unguiculata* (Hedström & Thulin 1986).

Vigna vexillata (L.) A.Rich. is a twining vine or prostrate herb that is widely distributed in tropical Africa, Asia and Australia. It produces large, thickened, edible tubers. In addition to its role in food production, *V. vexillata* is also used as a pasture cover crop, a green manure plant, and an erosion control plant.

Vigna vexillata seeds are resistant to cowpea weevil, one of the main pests of cowpea (Birch *et al.*, 1985; Birch *et al.*, 1986). Therefore, the main focus in research on *V. vexillata* is to transfer, by hybridization or genetic transformation, the cowpea weevil resistance genes of *V. vexillata* to *V. unguiculata,* even if the successful hybridisation between both species has not yet been achieved (Fatokun 1991; Barone *et al.*, 1992).

Unfortunately, both gene pools are poorly characterized. Until recent works with living cowpea accessions (Panella & Gepts 1992; Vaillancourt & Weeden 1992; Panella *et al.* 1993; Pasquet 1993a, 1993b; Vaillancourt *et al.* 1993b), knowledge of the gene pool of *V. unguiculata* came mainly from herbarium studies (Verdcourt 1970; Pienaar & Van Wyk 1992; Mithen & Kibblewhite 1993). In the same way, the classification of *V. vexillata* is still primarily that of Verdcourt (1970) (Maréchal *et al.* 1978; Babu *et al.* 1987; Pienaar & Kok 1991).

Verdcourt (1970) recognized six species in subgenus *Plectrotropis* (Schum.) Verdc. which are characterized by large purple flowers and a keel with a pocket and a long beak: *V. longissima* Hutch. (morphologically clearly distinct from the other species), *V. hundtii* Rossberg, *V. nuda* N.E.Br., *V. lobatifolia* Bak., *V. davyi* Bolus, and *V. vexillata* (the last five species being more closely related).

The specific and infra-specific delimitation of taxa in the *V. vexillata* complex is much more confused than that of *V. unguiculata.* Nearly forty taxa relevant to *V. vexillata* were described by botanists. It can be explained by the wider geographic distribution of *V. vexillata,* as many taxa were described from Asian or American material. Verdcourt (1970) recognized three infra-specific taxa i.e. var. *angustifolia* (Schumach. & Thonn.) Bak. (forms with narrow leaflets), var. *dolichonema* (Harms) Verdc. (forms with very long calyx lobes) and var. *vexillata.* In addition, Maréchal *et al.* (1978) added *V. vexillata* var. *yunnanensis* Franch. and var. *pluriflora* Franch. (Himalayan forms with small leaflets, close to the earlier var. *stocksii* Benth. ex Bak.), and var. *macrosperma* Maréchal, Mascherpa and Stainier (cultivated forms with large pods and seeds). Later, Babu *et al.* (1987) added var. *wightii* (Berth. ex Bak.) Babu and Sharma (South Indian forms with thick leaflets). Finally, Pienaar and Kok (1991) added var. *ovata* (E. Mey.) Pienaar (coastal forms from South Africa) while they reduced *V. davyi* to a varietal rank, i.e. var. *davyi* (Bolus) Pienaar (unifoliolate forms from Swaziland and Transvaal).

For the purpose of this study, we consider *V. vexillata* var. *linearis* Craib. (coastal forms from South East Asia and Australia, morphologically close to var. *angustifolia* from West Africa) and the *V. vexillata* var. *vexillata* group located East of Himalaya. Herbarium specimens belonging to this latter group show markedly lower ovule number and often larger seeds and pods (Pasquet, unpublished data).

In order to understand *V. vexillata* gene pool organization, we undertook an isozymatic study of available *V. vexillata* and *V. lobatifolia* accessions.

Material and methods

Plant material

The 125 accessions used in this study are presented in Table 1. The accessions NI were from the IPGRI base collection of Phaseoleae maintained at the National Botanic Garden of Belgium, Meise. AC accessions were from the CSIRO (Santa Lucia, Australia) collection. IL accessions were from the ILRI (Addis Ababa, Ethiopia) collection. The MT accessions were collected by Mithen and are now

Table 1 Accessions studied. V 273, V 274, NI 546 are *V. labatifolia* accessions. AUS: Australia; BRA: Braxil; CIV: Ivory Coast; CMR: Cameroon; COL: Colombia; CRI: Costa Rica; EUC: Ecuador; ETH: Ethiopia; HVO: Burkina Faso; IND: India; KEN: Kenya; MDG: Madagascar; MOZ: Mozambique; MWI; Malawi; NAM: Namibia; NER: Niger; NGA: Nigeria; Pan: Panama; PHL: Phillipines; RWA: Rwanda; SDN: Sudan; SEN: Senegal; SUR: Suriname; SWZ: Swaziland; TGO: Togo; TZA: Tanzania; VEN: Venezuela; ZAF: South Africa; ZAR: Zaire; ZMB: Zambia; ZWE: Zimbabwe

Accession	Country	Latitude and longitude	Locality	Other number
V 7	CMR	10°14' N 14°12' E	Moutouroua -> Laf	
V 31	CMR	10°41' N 13°36' E	Tourou	
V 52	CMR	8°20' N 14°50' E	km 10 Mayo Djarendi -> Massi	
V 117	CMR	3°55' N 11°45' E	Nkolbisson	NI 1360
V 118	CMR	3°52' N 11°25' E	km 7 Ongot -> Yaoundé	
V 134	CMR	6°12' N 10°23' E	Anyajua	
V 137	CMR	3°49' N 12°00' E	Mengang -> Nyodo	
V190	CIV	7°46' N 8°05' W	Glanlé	
V 216	CMR	9°16' N 12°57' E	Tchirkolchi	
V 222	CMR	5°34' N 14°06' E	km 7 Ndokayo -> Bétaré Oya	
V 237	CMR	9°21' N 12°58' E	Barndaké	
V 252	MWI	16°21' S 34°56' E	Shire river, 2 ml N Alimenda	
V 255	ZMB	12°48' S 28°14' E	Kitwe	
V 260	TZA	5°13' S 38°35' E	Kisaralle nr Mnyuzy Railway Sta.	
V 261	ETH		1 km E of Jimma	
V 262	MOZ		Monte Domué	
V263	TGO	6°12' N 1°12' E	Lomé-Cacaveli	
V 264	TGO		Dapaong, barrage Tantikou	
V 265	SDN		Kop 2 km SW Nyani camp	
V 266	SEN		Cap Skirring	
V267	ZAR		Kivu, Masisi	
V 268	NER	12°2'2 N 3°25' E	5 km W Tounouga (c. Sabongari)	
V 269	HVO		Djibo	
V271	NER	11°58' N 3°34' E	Sud de Bengou	
V 272	MDG			
V 273	NAM		Gobabis, 10 miles N Eiseb Omuramba	
V 274	NAM		55 miles S Tsunkwe Pat	
V 276	IND		Pulney hills	
V 277	ZAF	25°54' S 28°19' E	Pretoria, Rietvlei Dam	
V 278	ZAF	25°49' S 29°21' E	km 12 Witbank -> Middelburg	
V 279	ZAF		Kosi Bay coas. for., Matando channel	
V 280	ZAF	25°08' S 30°45' E	Sabie, km 52 Nelspruit -> Lydenberg	
V 286	ZAF	25°46' S 30°26' E	near Barberton, Transvaal	
V 287	ZAF	26°07' S 27°59' E	Johannesburg, N edge of Delta Park	
V 288	ZAF	25°38' S 28°20' E	Roodeplaat Nature Reserve	
V289	ZAF	33°03' S 27°52' E	Gonubie mouth	
V290	TZA	2°20' S 31°30' E	Kabanga, near Burundi border	
V291	SWZ	26°03' S 31°16' E	near Nkomati	
V292	SWZ	26°04' S 31°12' E	Nkomati, old bridge	
AC 301	AUS	13°47' S 131°12' E	Oolloo station	

Table 1 Continued

AC 303	AUS	16°35' S 145°29' E	20 km S Port Douglas	
AC 304	AUS	16°50' S 145° 38' E	Barron River Falls	
AC 305	AUS	21°10' S 149°10' E	Slade Point, Mackay	NI 1450
AC 308	AUS	29°44' S 152°58' E	Clarenza, few km SE Grafton	
AC 317	AUS	27°28' S 152°58' E	Simpson's road, Mt Cootha	
AC 318	AUS	27°28' S 152°58' E	Crest of Mt Cootha	
AC 322	AUS	20°59' S 149°05' E	The Causeway, Habana, Wharf Rd	
AC 330	AUS	19°33' S 147°03' E	km 27 Woodstock -> Giru	NI 1457
AC 347	AUS	12°32' S 131°03' E	8 km N Kakadu turn-off	
AC 349	AUS	17°09' S 125°28' E	30 km before Mt. House station	
IL 1103	ZAR	0°53' S 29°22' E	2.5 km SE Rwindi -> Rutshuru	
IL 7310	KEN		Msabaha	
IL 7311				
IL 7901	ETH	10°47' N 39°50' E	Iseye Gola	
IL 8430	ETH	8°04' N 36°28' E		
IL 8440	ETH	6°51' N 36°37' E		
IL13264	KEN	0°01' S 34°44' E	13 km Kisumu -> Nandi hills	
IL 15362	ETH	9°00' N 38°45' E	Addis Abeba	
MT 7	ZWE	17°45' S 31°05' E	5 km N Harare	NI 1451
MT 10	ZWE	18°35' S 32°15' E	5 km W Rusape	
MT 11	ZWE	18°30' S 32°18' E	5 km W Rusape	
MT 12	ZWE	18°22' S 32°40' E	1 km E Mont Clair Hotel	NI 1452
MT 22	ZWE	19°16' S 32°42' E	Udu Dam	
MT 27	ZWE	17°33' S 31°03' E	Balkiza, 29 km N Harare	NI 1458
MT 51	ZWE	19°05' S 32°44' E	15 km SE Mutare	
MT 52	ZWE	19°12' S 32°45' E	20 km SE Mutare	NI 1454
MT 59	ZWE	20°10' S 32°55' E	3 km E Chimanimani	
MT 69	ZWE	20°05' S 32°44' E	27 km SW Chimani.-Chipeaz rd	
MT 276	ZWE	17°45' S 31°08' E	12 km NW Harare	
MT 327	ZWE	17°41' S 31°01' E	15 km N Harare	
MT 331	ZMB	15°10' S 28°30' E	15 km NE Lusaka	NI 1455
MT 350	MWI	15°11' S 31°20' E	290 km SE Lilongwe	
MT 353	MWI	15°22' S 35°18' E	9 km NE Zo	
MT 469	MWI	15°40' S 34°50' E	28 km from Chileka airport	
MT 477	MWI	15°47' S 35°04' E	54 W junction with M1	
NI 111				
NI 180	SUR			
NI 224	ZAR	2°19' S 28°45' E	Mulungu, 35 km NW Bukavu	
NI 244	ZAR	2°19' S 28°45' E	Mulungu	
NI 245	ZAR	2°19' S 28°45' E	Mulungu	
NI 246	ZAR	2°19' S 28°45' E	Mulungu	
NI 331	CRI			
NI 336	CRI			
NI 339				

Table 1 Continued

NI 379	CRI			
NI 388	RWA	2°15' S 30°15' E	Bugesera, lac Kilimbi	
NI 391	BRA		Sao Paulo	
NI 412	ZAR	11°45' S 27°40' E	Lumumbashi	
NI 422	RWA			
NI 438	ZAR	6°45' S 23°57' E	INEAC Gandajika	
NI 458	ZAR	11°34' S 27°24' E	Kipopo, 25 km NW Lumumbashi	
NI 460	ZAR	11°34' S 27°24' E	Kipopo, 25 km NW Lumumbashi	
NI 522	SEN			
NI 546	NAM		Nama Pan	
NI 557	ZAF		Dap Mande Dam	
NI 620	AUS	11°00' S 142°30' E	Queensland, cape York	FLG 4140
NI 740	COL		Valle de Cauca, Palmira	
NI 821	PAN	7°26' N 80°11' W	Los Santos, 35 km E Tonosi	
NI 827	BRA	15°18' S 39°05' W	Bahia, Una	
NI 828	COL		Boyaca	
NI 854	ECU		Pichilingue	
NI 857	COL		Magdalena, 4 km W Palomino	
NI 862	COL		Pichilingue	
NI 863	COL		Meta	
NI 880	COL		Magdalena	
NI 920	KEN	0°34' S 34°25' E	10 km S Homa Bay -> M'Bita	
NI 930	TZA	5°08' S 38°40' E	25 km W Mlingano	
NI 931	NGA		Ibadan, St Patrick High School	
NI 932	PAN	8°38' N 79°53' W	Panama City	
NI 934	NGA		25 km W P. Harcourt	
NI 935	TZA	4°53' S 38°17' E	Mombo, 35 km N Korogwe	
NI 936	COL	11°17' N 73°53' W	km 35 E Santa Marta	
NI 938	NGA		Motunde, 30 km N Ibadan	
NI 940	KEN	3°53' S 39°37' E	km 7 S Kaloleni -> Mayera	
NI 941	VEN	10°04' N 66°56' E	km 10 N San Casimiro	
NI 942	TZA	4°53' S 38°17' E	Mombo, 35 km N Korogwe	
NI 943	KEN	0°41' S 35°04' E	km 5 W Sotik -> Kisii	
NI 946	TZA	4°53' S 38°17' E	Mombo, 35 km N Korogwe	
NI 948	TZA	4°53' S 38°17' E	Mombo, 35 km N Korogwe	
NI 950	NGA	7°28' N 8°56' E	km 14 N Yandev -> Makurdi	
NI 1014	IND		Kasauli	
NI 1020	IND		km 8 Galadur -> Seaforth	
NI 1180	PHL		Laguna	
NI 1227	BRA	6°27' S 39°09' W	4 km SE Caico	
NI 1300	VEN	10°11 N 66°27' W	32 km E Santa Teresa	

duplicated in Meise. The V accessions were from the ORSTOM collection which is now being duplicated in Meise. Each accession was made of one to three autogamous lines, and maintained as such, with each of these lines coming from one seed of the original stock.

Procedure

The extracts were taken from seeds soaked for 24 hours in water. The gels were prepared according to the protocol described by Second and Trouslot (1980). The histidine/citrate system at pH 6.0 with a starch concentration of 14% was used for all the enzyme systems. The enzyme systems and the staining procedures which were used are indicated in Table 2. AMP was stained with leucine-β-naphthylamide (Amp2) or alanine-β-naphthylamide (Amp2 and Amp4) (Wendel & Weeden 1989), and FLE was stained with 4methyl-umbelliferyl-acetate (Harris & Hopkinson 1978).

For each enzymatic system, the presumed loci were numbered by increasing distance from the anode, and the numeration was the same as in *V. unguiculata* (Pasquet 1993b). For each isozyme, the most common allele was designated as 100 and the other allozymes were measured in millimeters in relation to that standard. This procedure is the same one utilized by Koenig and Gepts (1989) with *Phaseolus vulgaris* L.

The data from the enzymatic analysis allowed the calculation of Nei's distances (Nei 1972). The unweighted pair group method with arithmetic averaging (UPGMA) (Sneath & Sokal 1973) was computed using the BIOSYS software version 1.7. Principal Component Analysis (PCA) was computed using ADDAD 89.1.

Results

The 19 enzyme systems enabled the scoring of 32 loci (Table 2). Fdh, Me, Gdh, Gr, Enp and Mpi appeared as single bands. Sdh and G6pd were expressed as a double band.

Diaphorase (Dia) yielded numerous bands, most of which stained poorly. Only the fastest band (Dia1) was scored. Dia1 also showed a menadione reductase activity. Seed extracts mainly revealed one single βEst band (Est3).

A few slow αEst bands were observed but not scored due to the fact that they were very faint.

Alcohol dehydrogenase (Adh) appeared in the form of a heterodimer and revealed two loci, Adh1, which was more darkly stained, and Adh2, which was lighter. Pgd and Pgm also revealed two isozymes, with the fast Pgm isozyme having the strongest activity. Fle produced two bands (Fle1 and Fle3), the faster being more lightly stained. For Amp, two isozymes were observed with the seed extracts: Amp2, which cleaved both alanine- and leucine-β-naphthylamide and showed a much stronger activity, and Amp4 which reacted with alanine-β-naphthylamide.

Superoxyde dismutase (Sod) was expressed as three bands: a fast band (Sod1), which stained poorly and was not scored, a strong medium band (Sod2), and a slow thin band (Sod3). Idh revealed three isozymes, the faster (Idh1) showing a stronger activity.

Phosphoglucose isomerase (Gpi) revealed also three isozymes. Gpi1 was assumed to be chloroplastic, as it is in cowpea (Vaillancourt *et al.* 1993b) and other species (Gottlieb & Weeden 1981). Gpi2 and Gpi3 usually formed a heterodimer and two homodimers, Gpi2 product being more darkly stained. However, in one fourth of the accessions studied, Gpi2 appeared as a single strongly stained band. In this latter case, Gpi3 was considered as a null allele. Although an overlap of Gpi2 and Gpi3 is not impossible, gene silencing is more likely. On the one hand, Gpi silencing is reported in *Strophostyles*, a closely related *Phasolinae* genus (Weeden *et al.* 1989). On the other hand, if overlaps

Table 2 Enzyme systems studied and applicable staining procedures

Enzyme system, abbreviation and code	Number of loci scored	Staining procedure
Alcohol dehydrogenase (Adh) 1.1.1.1	2	Second & Trouslot (1980)
Aminopeptidase (Amp) 3.4.11.1	2	Second & Trouslot (1980)
NADH Diaphorase (Dia) 1.6.2.2	1	Harris & Hopkinson (1978)
Endopeptidase (Enp) 3.4.-.-	1	Wendel & Weeden (1989)
Esterase (Est) 3.1.1.1	1	Second & Trouslot (1980)
Formate dehydrogenase (Fdh) 1.2.1.2	1	Wendel & Weeden (1989)
Fluorescent esterase (Fle) 3.1.1.2	2	Harris & Hopkinson (1978)
Glutamate dehydrogenase (Gdh) 1.4.1.2	1	Second & Trouslot (1980)
Glucose-6-phosphate dehydrogenase (G6pd) 1.1.1.49	1	Vallejos (1983)
Phosphoglucose isomerase (Gpi) 5.3.1.9	3	Second & Trouslot (1980)
Glutathione reductase (Gr) 1.6.4.2	1	Harris & Hopkinson (1978)
Isocitrate dehydrogenase (Idh) 1.1.1.42	3	Second & Trouslot (1980)
Malate dehydrogenase (Mdh) 1.1.1.37	4	Second & Trouslot (1980)
Malic enzyme (Me) 1.1.1.40	1	Wendel & Weeden (1989)
Mannose phosphate isomerase (Mpi) 5.3.1.8	1	Harris & Hopkinson (1978)
Phosphogluconate dehydrogenase (Pgd) 1.1.1.43	2	Second & Trouslot (1980)
Phosphoglucomutase (Pgm) 2.7.5.1	2	Second & Trouslot (1980)
Shikimate dehydrogenase (Sdh) 1.1.1.25	1	Second & Trouslot (1980)
Superoxyde dismutase (Sod) 1.15.1.1	2	Wendel & Weeden (1989)

Table 3 Allelic frequencies, mean gene diversity index (H), proportion of polymorphic loci (L) and number of alleles at polymorphic loci (A) of the different groups from the cluster analysis (Figure 1). Numbers of accessions are in brackets

		Total (125)	India (3)	America (20)	Guinean West Africa (8)	*V. lobatifolia* (3)	IL 15362 (1)	var. *macrosperma* (4)	var. *angustifolia* (12)	var. *linearis* (12)
Adh 1	100	0.984	1	1	1	1	1	1	1	1
	95	0.008	0	0	0	0	0	0	0	0
	90	0.008	0	0	0	0	0	0	0	0
Adh2	105	0.008	0	0	0	0	0	0	0	0
	100	0.992	1	1	1	1	1	1	1	1
Amp2	100	0.912	0	1	1	0	0	0.25	1	1
	97	0.088	1	0	0	1	1	0.75	0	0
Amp4	105	0.008	0	0	0	0	0	0	0.04	0
	102	0.264	0	1	0.25	0	0	0	0	0.08
	100	0.384	1	0	0	1	1	0.12	0	0.92
	95	0.152	0	0	0.75	0	0	0	0.12	0
	93	0.004	0	0	0	0	0	0	0	0
	83	0.148	0	0	0	0	0	0	0.80	0
	80	0.040	0	0	0	0	0	0.87	0.04	0
Dia	100	0.968	0	1	1	1	1	1	1	1
	96	0.008	0	0	0	0	0	0	0	0
	88	0.024	1	0	0	0	0	0	0	0
Enp	108	0.044	0.83	0	0	0	0	0	0	0
	102	0.008	0.16	0	0	0	0	0	0.04	0
	100	0.844	0	1	1	1	1	0.25	0.92	0.21
	96	0.028	0	0	0	0	0	0.75	0.04	0
	92	0.076	0	0	0	0	0	0	0	0.79
Est3	110	0.008	0	0	0.12	0	0	0	0	0
	102	0.064	0	0	0.19	0	0	0	0.04	0
	100	0.860	0	1	0.69	1	1	0.25	0.87	1
	98	0.008	0	0	0	0	0	0	0.08	0
	92	0.060	1	0	0	0	0	0.75	0	0
Fdh	103	0.076	0	0	0	0.33	0	0	0.25	0
	100	0.640	1	0.15	0.18	0.66	0	0.87	0.62	1
	97	0.184	0	0.40	0.56	0	0	0.12	0.12	0
	95	0.100	0	0.45	0.25	0	1	0	0	0
Fle1	103	0.051	1	0	0.25	0	0	0	0	0
	100	0.924	0	1	0.50	1	1	1	1	1
	97	0.024	0	0	0.25	0	0	0	0	0
Fle3	100	0.824	1	0.97	1	1	1	0	0.33	0.75
	92	0.168	0	0.02	0	0	0	1	0.66	0.25
	84	0.008	0	0	0	0	0	0	0	

Table 3 Continued

Gdh	110	0.016	0	0	0	0	0	0	0	0
	105	0.012	0	0	0	0	0	0	0	0
	100	0.908	1	1	1	1	0	1	1	0.42
	95	0.056	0	0	0	0	0	0	0	0.58
	90	0.008	0	0	0	0	1	0	0	0
G6pd	103	0.032	1	0	0	0	0	0	0	0
	100	0.928	0	1	1	1	1	1	0.75	1
	97	0.040	0	0	0	0	0	0	0.25	0
Gpi1	106	0.048	0	0.05	0	1	0	0	0.08	0
	103	0.372	1	0.90	1	0	0	0.50	0.25	0.08
	100	0.580	0	0.05	0	0	1	0.50	0.66	0.92
Gpi2	104	0.016	0	0	0	0	0	0	0	0
	100	0.684	0	0.95	0.25	1	1	1	0.96	0.96
	98	0.008	0	0	0	0	0	0	0	0
	95	0.036	1	0.05	0	0	0	0	0.04	0.04
	93	0.156	0	0	0.75	0	0	0	0	0
	88	0.016	0	0	0	0	0	0	0	0
	86	0.084	0	0	0	0	0	0	0	0
Gpi3	100	0.444	0	0.05	0.12	1	0	0	0.58	0.87
	97	0.008	0.33	0	0	0	0	0	0	0
	94	0.284	0.17	0.95	0.25	0	0	1	0.41	0.08
	0	0.264	0.50	0	0.62	0	1	0	0	0.04
Gr	103	0.008	0	0	0	0	0	0	0	0
	100	0.976	1	1	1	1	1	1	1	1
	97	0.008	0	0	0	0	0	0	0	0
	94	0.008	0	0	0	0	0	0	0	0
Idh1	100	1	1	1	1	1	1	1	1	1
Idh2	112	0.016	0	0	0.25	0	0	0	0	0
	106	0.212	0	0	0.50	0	0	0.75	0.96	0.08
	100	0.720	1	1	0.25	1	1	0.25	0.04	0.83
	94	0.052	0	0	0	0	0	0	0	0.08
Idh3	109	0.024	0	0	0	0	0	0	0	0
	103	0.048	0	0	0	0	0	0	0.08	0
	100	0.928	1	1	1	1	1	1	0.92	1
Mdh1	100	1	1	1	1	1	1	1	1	1
Mdh2	105	0.228	0	1	1	0	0	0	0	0
	100	0.764	1	0	0	1	1	1	1	1
	95	0.008	0	0	0	0	0	0	0	0
Mdh3	100	0.228	0	1	1	0	0	0	0	0
	95	0.764	1	0	0	1	1	1	1	1
	90	0.008	0	0	0	0	0	0	0	0
Mdh4	110	0.032	0	0	0	0	0	0	0.08	0
	100	0.968	1	1	1	1	1	1	0.92	1

Table 3 Continued

Me	103	0.120	0	0.05	0	0.67	0	0.25	0.17	0.25
	100	0.632	0.5	0.25	0.44	0.33	1	0.75	0.83	0.75
	97	0.248	0.5	0.70	0.56	0	0	0	0	0
Mpi	111	0.324	0	0.90	0.62	0.67	0	0.12	0.96	0
	108	0.036	0	0	0.12	0.33	0	0	0	0
	105	0.020	0	0	0	0	1	0	0	0
	100	0.612	1	0.10	0.25	0	0	0.87	0.04	1
	96	0.008	0	0	0	0	0	0	0	0
Pgd1	105	0.040	0.67	0	0	0	0	0	0	0
	100	0.924	0.16	0.95	0.87	0.67	1	1	1	1
	95	0.020	0.16	0	0	0.33	0	0	0	0
	90	0.016	0	0.05	0.12	0	0	0	0	0
Pgd2	105	0.060	0	0.05	0.12	0	0	0	0	0
	100	0.940	1	0.95	0.87	1	1	1	1	1
Pgm1	102	0.028	0.17	0	0	0	0	0	0	0
	100	0.928	0.83	0.95	1	1	1	0.87	0.96	0.92
	95	0.044	0	0.05	0	0	0	0.12	0.04	0.08
Pgm2	103	0.008	0	0	0	0	0	0	0	0
	100	0.920	0.17	1	1	1	1	1	0.92	0.58
	97	0.036	0.66	0	0	0	0	0	0	0.08
	95	0.032	0	0	0	0	0	0	0.08	0.33
	92	0.004	0.17	0	0	0	0	0	0	0
Sdh	113	0.004	0	0	0	0	0	0	0	0.04
	105	0.072	0	0	0	0	0	0	0	0.75
	103	0.036	0	0	0	0	0	0	0	0
	100	0.820	0.5	1	1	0	0	1	1	0.21
	97	0.024	0	0	0	1	0	0	0	0
	95	0.040	0.5	0	0	0	1	0	0	0
Sod2	100	1	1	1	1	1	1	1	1	1
Sod3	100	0.928	1	1	0.87	1	1	1	1	1
	96	0.008	0	0	0	0	0	0	0	0
	92	0.064	0	0	0.12	0	0	0	0	0
H		0.248	0.099	0.061	0.165	0.056	-	0.101	0.128	0.111
L		0.906	0.219	0.312	0.375	0.125	-	0.312	0.500	0.375
A		3.760	2.430	2.300	2.500	2.000	-	2.000	2.375	2.333

between Gpi2-86 and Gpi3-94 and Gpi2-93 and Gpi3-100 are possible, overlaps involving Gpi2-95 and Gpi2-100 would require Gpi3-102 and Gpi3-107 which were not observed.

Malate dehydrogenase (Mdh) presented four isozymes. The most anodal, Mdh1, appeared as a weak band. Next was the three bands pattern made by homodimers and heterodimer from Mdh2 and Mdh3 products. Then Mdh4 appeared as a very slow strong band.

Allelic frequencies, mean gene diversity index (H), proportion of polymorphic loci (L), and number of alleles at polymorphic loci (A) are represented in Table 3. Total genetic diversity (H_T) was 0.248 over all the accessions. Within accession diversity (H_S) was 0.012, therefore between accession diversity (D_{ST}) was responsible for all the genetic diversity present and the coefficient of gene differentiation ($G_{ST} = D_{ST}/H_T$) approached 1.

The UPGMA cluster analysis based on Nei's distances is shown in Figure 1. It showed that the three Indian accessions (cluster A) were clearly separated, as was a large group (cluster B) that included all the Americans accessions and a few accessions from West Guinean Africa (Togo, Nigeria, Cameroon). NI 946 from Tanzania, characterized by several rare alleles (Amp4-93, Fdh-103, Idh3-103, and Pgd2-105) fell into this B

Table 4 Distribution of Nei's genetic distances between individuals within or between taxa: upper line is minimum distance, middle line is mean distance (in bold), lower line is maximum distance. For each column, the number of accessions studied is given in brackets. NI 946 and V 261 are excluded from the American group

	India (3)	Guinean West Africa America (28)	*V. loabtifolia* (3)	IL 15362 (1)	Other accessions (90)
	0.025				
India	**0.078**				
	0.115				
	0.492	0.000			
Guinean West Africa America	**0.642**	**0.140**			
	0.811	0.421			
	0.497	0.288	0.032		
V. lobatifolia	**0.549**	**0.439**	**0.088**		
	0.617	0.693	0.134		
	0.505	0.330	0.247		
IL 15362	**0.512**	**0.419**	**0.288**		
	0.524	0.575	0.330		
	0.293	0.135	0.134	0.170	0.000
Other accessions	**0.520**	**0.347**	**0.332**	**0.305**	**0.224**
	0.778	0.750	0.575	0.462	0.540

cluster because this accession was intermediate between some of the American accessions (it showed Gpi1-103, Gpi3-94, Me-97, and Mpi-111) and some of the East African accessions (especially NI 940 and NI 943 from Kenya). In the same way, V 261 from Ethiopia was intermediate between some of the accessions from Cameroon (0.154–0.187), some of the accessions from America (it showed Fdh-97, Mdh2-105, and Mdh3-105), and some of the accessions from Ethiopia (0.144–0.280). On the other hand, NI 1180 from the Philippines clearly belonged to the American group. Distances between NI 1180 and American accessions (0.040–0.208) were shorter than between NI 1180 and other accessions (0.208–0.638).

Furthermore, Nei's distances UPGMA cluster C included the three *V. lobatifolia* accessions and IL 15362, a very peculiar *V. vexillata* accession from Ethiopia characterized by arillate seeds, which is unusual within *V. vexillata.* IL 15362 was close to other accessions from Ethiopia (0.208 with IL 8440 and 0.231 with IL 7901) and accessions from Southern Africa (0.170 with V 279 and V 292). IL15362 is also characterized by a few rare alleles: Sdh-95, Gdh-90 (nowhere else encountered), and Mpi-105.

These groups (clusters A, B, and C) were well separated by high Nei's genetic distances (Table 4) and were well characterized by several loci (Table 3).

Nei's distances UPGMA cluster D included the three var. *macrosperma* accessions, var. *angustifolia* and a few accessions characterized by association of rare alleles:

— V 252 from Malawi, close to MT 12 (0.203) and NI 953 (0.170), with Adh1-95, Est3-92, Fle3-84, and Pgm1-95;

— MT 59 and MT 69, close to several accessions from the Southern African cluster (0.146–0.192), with Amp4-105, Fdh-103, Gdh-105, G6pd-97, Idh3-109, and Pgd2-105;

— V 277 and V 287 from South Africa, close to other accessions from South Africa (0.118–0.170) and accessions NI 935 (0.162) and NI 942 (0.170) from Tanzania, with Adh1-90, Adh1-95, Gdh-110, Gr-103, Gpi2-105, Mpi-96, and Sdh-1 03;

— AC 347 from Australia, close to AC 301 (0.205) and some accessions from var. *angustifolia* (0.173–0.192), with Idh2-94 and Pgm1-95.

The rest of the accessions (clusters E, F, and G), including var. *linearis* accessions and var. *ovata* accession (V 289), were poorly separated. A few accessions from very remote origins appeared to be very close genetically. For example, between V 222 from Cameroon, IL 7901, IL 8330, IL 8440 from Ethiopia, V 262 from Mozambique, NI 930 from Tanzania, and MT 52 from Zimbabwe, average Nei's distance is 0.084, where the minimum is 0.040 and the maximum is only 0.130.

However, a few groups of accessions from the same origin clustered together. These included accessions from Rwanda and Zaire, var. *linearis*, and the cluster G which mainly included accessions from Southern Africa, i.e. Southern Zaire, Zambia, Malawi, Zimbabwe, Swaziland, and South Africa.

All these latter groups of accessions, with the exception of the Southern African group (cluster G), were characterized by allele combinations:

— var. *linearis* with Gdh-95, Enp-92, Sdh-105;

— the first thirteen accessions from cluster E (V 222, IL 8440...) with Gpi2-93, Gpi3-0, Mpi-111;

— within cluster E, the nine accessions from Zaire and Rwanda (V 267, NI 930...) with Gpi2-86, Gpi3-0, Sod3-92;

— var. *angustifolia* with Amp4-83, Fle3-92, Idh2-106;

— var. *macrosperma* with Amp2-97, Amp4-80, Enp-96, Est3-92, Fle3-92, and Idh2-106.

Principal Component Analysis based on Nei's distances (Figure 2) gave a different picture of these latter groups (accessions from clusters D, E, F, G, and accessions IL 15362, NI 946, and V 261). Accession AC 347 appeared close to var. *angustifolia.* This accession displayed Fle3-92 but neither Idh2-106 nor Amp4-83. With the exception of accession AC 347, var. *angustifolia* and var. *linearis* were clearly separated and var. *linearis* was close to the Southern African group on the right side of Figure 2. On the

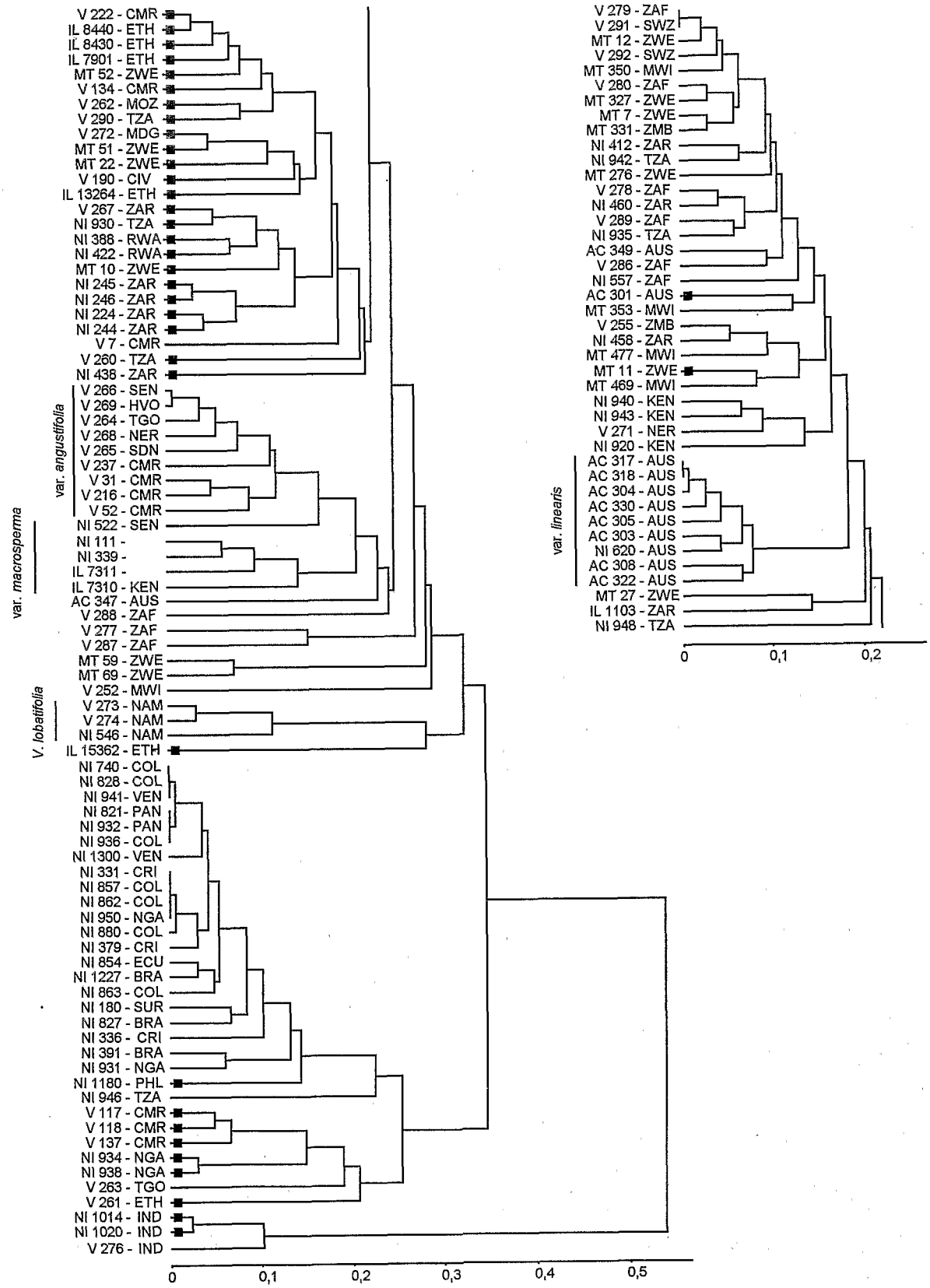

Figure 1 Nei's distances UPGMA. Accessions marked with a solid square display Gpi3 silencing.

other hand, var. *macrosperma* and var. *angustifolia* appeared closer than shown previously in the cluster analysis. The accession of var. *angustifolia* from Tanzania (V 260) was far from each of these groups (0.231–0.375 with var. *angustifolia*, 0.239–0.330 with var. *linearis*, and 0.375–0.446 with var. *macrosperma*) and did not cluster with any of them.

Beside the Southern Africa group, accessions from Eastern Africa were close and accessions from Central Africa (Cameroon and Western Zaire) and Ethiopia more remote, respectively on the left and on the bottom left of Figure 2.

Figure 2 shows an opposition between var. *linearis* - var. *angustifolia* - var. *macrosperma* (top right) and accessions from West Guinean Africa, Central Africa and Ethiopia (bottom left). This PCA diagram illustrates the morphological cline between glabrous *V. vexillata* with narrow leaflet (var. *angustifolia sensu* Verdc.) and pubescent *V. vexillata* with larger leaflet (var. *vexillata sensu* Verdc.). All accessions showing Gpi3-0 (with the exception of NI 946) appeared in the bottom left of Figure 2.

Discussion

Isozyme patterns

Isozyme patterns are very close to those observed in *V. unguiculata* (Panella & Gepts 1992; Pasquet 1993b; Vaillancourt *et al.* 1993b) and *V. reticulata* (Garba & Pasquet, unpublished data). Differences are few.

In the ADH pattern, the fast monomer presents a stronger activity, as in *V. reticulata*, and this is contrary to what is observed in *V. unguiculata*. In the Mdh pattern, Mdh4 does not migrate very far, as in *V. unguiculata* and *V. reticulata*, and this is contrary to what appears in blue- and yellow-flowered *Vigna* species (Pasquet & Vanderborght, unpublished data). In the Idh pattern, the strongest activity (Idh1) is that of the faster isozyme, contrary to what happens in *V. unguiculata* and *V. reticulata*. In addition, Idh3 is scored in the present study, but Idh3 is not observed in *V. unguiculata* nor in *V. reticulata*. Regarding diaphorase, Dia yields many more bands than in *V. unguiculata*, just as in *V. reticulata*. In the Est pattern, the βEst band (Est3) is faster than the αEst bands in *V. vexillata*, contrary to what happens in *V. unguiculata* and *V. reticulata*. In the Gpi system, the strongest activity is that of the fast isozyme (Gpi2), as in *V. unguiculata* and *V. reticulata*, and opposite to what is encountered in most blue and yellow-flowered *Vigna* species (Pasquet & Vanderborght, unpublished data).

As in cowpea (Pasquet, 1994; Vaillancourt, pers. comm.), variability (in mobility, not in staining intensity) in Adh is only encountered in Southern Africa. With the exception of Gdh, which is monomorphic in cowpea, all the isozymes with the most variability are the same in both species (*V. unguiculata* and *V. vexillata*), i.e. Sdh, Est3, Enp, Amp4, Gpi2 and Mpi.

The absence of a Gpi3 band is mainly encountered in Ivory Coast, Nigeria, Cameroon, Zaire, Rwanda, Ethiopia, and India. These accessions are marked with a solid square in the cluster analysis (Figure 1) and they appear in the bottom left corner of the principal component analysis (Figure 2). Most of the accessions from Eastern and Southern Africa have a Gpi3 band as accessions from America and accessions from var. *angustifolia*, var. *macrosperma*, and var. *linearis*.

Gpi3 silencing was already reported within the Phaseolinae (Weeden *et al.*, 1989), but this is the first report of the silencing of this locus within genus *Vigna*.

Diversity

Diversity indexes (H, L, A) are slightly lower than those observed in wild *V. unguiculata* with 164 accessions and 35 loci (Pasquet 1994), but higher than those observed in *V. reticulata* with 40 accessions and 35 loci (Garba & Pasquet, unpublished data). This is related to the fact that Nei's distances UPGMA (Figure 1) shows a gene pool in *V. vexillata* that is less hierarchically organized and structured than in the wild *V. unguiculata*, or one that is as hierarchized but with fewer groups. With the exception of the Indian accessions and the accessions from America and West Guinean Africa which are markedly separated from the other accessions, the genetic distances between *V. vexillata* accessions are much smaller than those encountered in *V. unguiculata*. However, it must be emphasized that an important group was not included in the study, *V. nuda* which is morphologically more distant from *V. vexillata* than is *V. lobatifolia*.

This lack of structure in *V. vexillata* gene pool organization could be related to the existence of a weedy group as in cowpea. In cowpea, wild annuals, *V. unguiculata* var. *spontanea* occupy most African savannas and often colonize disturbed areas like roadsides or fallows. In *V. vexillata*, we observe a similar situation but in wetter habitats like the rain forest areas of South Cameroon. This could explain why some accessions from very remote origins seem so closely related. As the supposed recent extension of the annual and often weedy *V. unguiculata* var. *spontanea* (which hybridizes with all perennial taxa) made the different perennial taxa evolution scheme less clear, so could the extension of possibly weedy *V. vexillata* have blurred the organization of the *V. vexillata* complex.

Similarities with *V. unguiculata* gene pool organization

If we do not consider the Asian *V. vexillata*, we can observe a striking convergence between both *V. unguiculata* and *V. vexillata* gene pools. The most obvious is the convergence regarding the pyrophytic taxa from the Zaire-Zambezi watershed, from the Huilla Hills in Angola to northern Zimbabwe: *V. unguiculata* subsp. *dekindtiana* (Harms) Verdc. (*sensu stricto*) and *V. nuda* display exactly the same crescent shaped geographic distribution (Pasquet, unpublished data), and the same habit. Unfortunatly, both taxa cannot be studied through living accessions.

In both *V. unguiculata* and *V. vexillata* studies, the first African group separated by the Nei's distances UPGMA is a group from West Guinean Africa i.e. *V. unguiculata* subsp. *baoulensis* (A. Chev.) Pasquet and the group of accessions from America and West Guinean Africa in *V. vexillata*. This latter group could be considered as only West African because variability of the American part is much lower and almost devoid of original alleles. Therefore, American *V. vexillata* may be an introduction from the Gulf of Guinea Coast.

Accessions with unique isozyme patterns come from highland areas where perennial *V. unguiculata* are encountered: IL 15362 from Ethiopia (*V. unguiculata* subsp. *aduensis*), MT 59 and MT 69 from eastern Zimbabwe (*V. unguiculata* subsp. *pawekiae*). Some poorly separated *V. vexillata* groups could be linked with other perennial *V. unguiculata* subspecies. Some examples of this would be linking *V. vexillata* from southern Zaire with *V. unguiculata* subsp. *letouzeyi* Pasquet, or linking the *V. vexillata* from Kivu (Zaire) and Rwanda with *V. unguiculata* subsp. *burundiensis* Pasquet.

Taxonomic inferences

Isozyme polymorphism results do not fit actual nomenclature, as in *V. luteola* (Jacq.) Benth. - *V. marina* (Burm.) Merrill - *V. oblongifolia* A. Rich. (Pasquet & Vanderborght, unpublished data), and *V. membranacea* A.Rich. - *V. frutescens* A. Rich. (Pasquet & Vanderborght, unpublished data). While waiting for morphological and cross-compatibility data, isozyme data suggest some taxonomic changes in the future.

Asian *V. vexillata* could be considered as a distinct subspecies, even if our three accessions are poorly representative of the whole

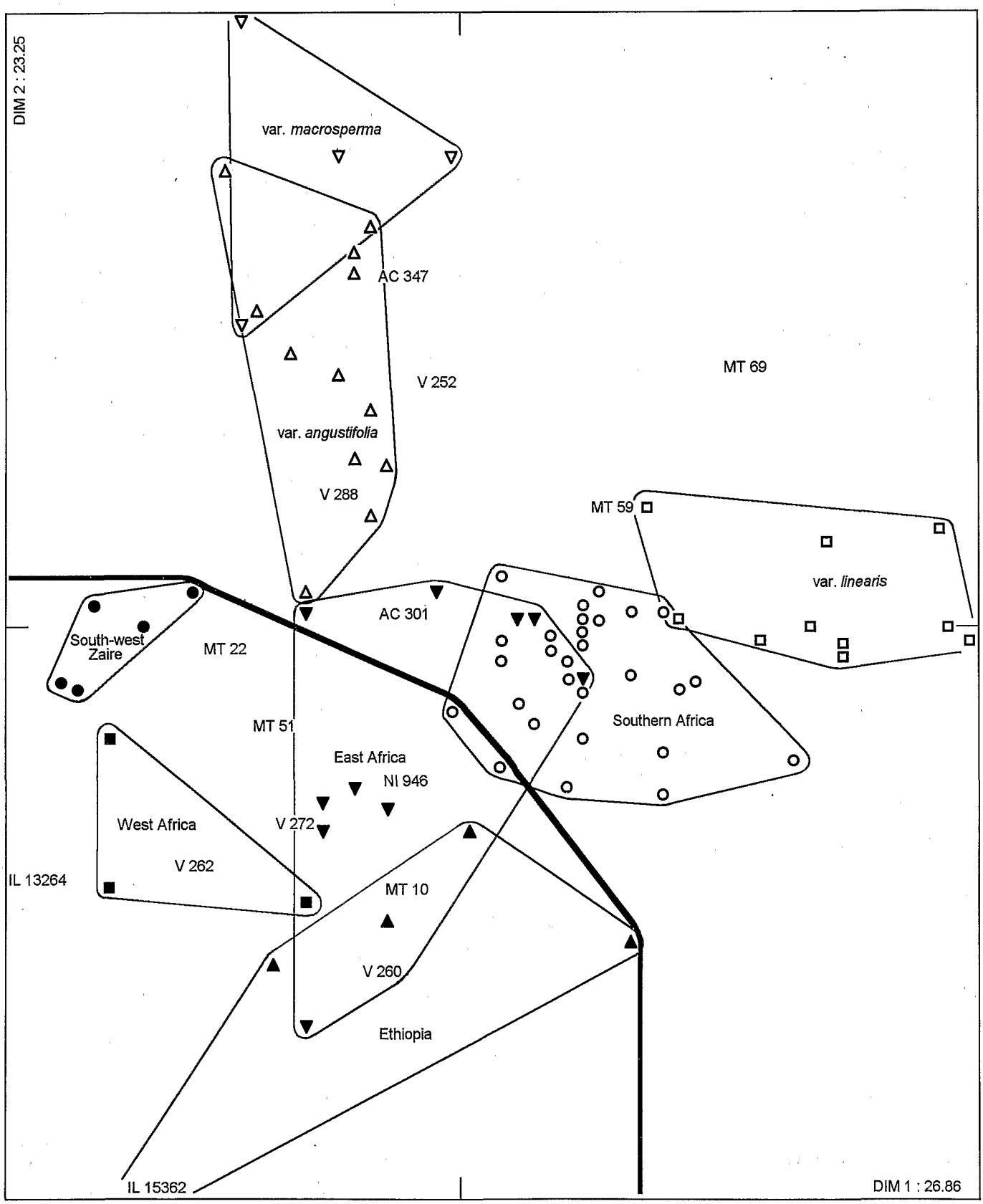

Figure 2 Nei's distance Principal Component Analysis. Accessions from clusters A and B are not included in the analysis. Accessions are represented either by a symbol or by their number. Accessions in the bottom left area within the large line display Gpi3 silencing (with the exception of Nl 946).

Asian *V. vexillata*, as the three accessions from India show an ovule number intermediate between African *V. vexillata* and *V. vexillata* specimens from China. American and especially West African rain forest accessions could be also considered as a different subspecies.

Vigna lobatifolia does not deserve a specific rank but instead a subspecific rank, as the *V. lobatifolia* accessions are clearly within the confines of genetic diversity observed in *V. vexillata*. On the other hand, var. *angustifolia* and var. *ovata*, the latter one not displaying any unique isozyme pattern and clustering with other South African accessions, do not merit more than their varietal rank, according to these isozyme results.

Conclusion

Unfortunately, this study emphasizes the lack of available accessions from important parts of the *V. vexillata* gene pool: *V. nuda*, Himalayan var. *stocksii*, and Asian var. *vexillata* (from China, Korea and Japan). Several taxa like var. *wightii* and var. *davyi* are also missing.

However, this study provides a first glimpse of the organization of the *V. vexillata* gene pool. It shows many similarities between *V. vexillata* and *V. unguiculata* gene pool organizations, while also showing interesting Asian variability of *V. vexillata*.

In the end, this study suggests some taxonomic modifications in the future: on the one hand, Asian and West African taxa will deserve a subspecific rank, on the other hand, *V. lobatifolia* will not deserve a specific rank.

Ackowledgements

This study was granted by the International Foundation for Science, Stockholm. We thank K. Braun (Swaziland National Trust Commission, Mbabane, Swaziland), M. Jooste and M. Erasmus (Range and Forage Institute, Lynn East, South Africa), J. Hanson (ILRI, Addis Ababa, Ethiopia), R.J. Lawn (CSIRO, Bt Lucia, Australia), H. Moss (IBPGR, Harare, Zimbabwe), S.P. Paramanathen (St Joseph's College, Tiruchirapalli, India), and T. Vanderborght (Jardin Botanique National, Meise, Belgium) for providing some of the seed samples used in this study.

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Effect of pyridine nucleotides on the catalytic activity of a carthamin-synthesizing enzyme from dyer's saffron (*Carthamus tinctorius* L.) seedlings

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Received 22 September 1997; revised 6 January 1998

The effect of NADPH, $NADP^+$, NADH and NAD^+ on carthamin formation was investigated with a partially purified enzyme extract from dyer's saffron (*Carthamus tinctorius* L.) seedlings. In the presence of 10 pmol Mn^{2+}, 10 pmol NADPH enhanced carthamin formation, catalyzed by a carthamin-synthesizing enzyme most prominently (13.8% increase). In the presence of Mn^{2+}, NADH gave the second best result (7.2% increase). The promotive carthamin formation activities of NADPH and NADH were strongly dependent upon the presence of Mn^{2+}. Under the same experimental conditions, neither $NADP^+$ (2.9%) nor NAD^+ (4.0%) showed such positive effects as did NADPH or NADH. A conceivable participation of pyridine nucleotides on the catalytic activity of a still unnamed carthamin-synthesizing enzyme is proposed in view of the experimental findings.

Keywords: Carthamin formation, carthamin-synthesizing enzyme, pyridine nucleotide, dyer's saffron (*Carthamus tinctorius* L.) seedling.

Introduction

Carthamin is known to be formed from a precursor, precarthamin, via enzymatic (Saito *et al.* 1983; Saito 1992) and non-enzymatic processes (Saito & Takahashi 1985; Saito & Utsumi 1996), the former of which can be further sub-divided into two catalytic branches, namely the direct (Saito *et al.* 1983; 1985) and indirect (Saito 1992; 1993) enzymatic branches. The direct control of carthamin formation is initiated by an enzyme preparation of dyer's saffron (*Carthamus tinctorius* L.) tentatively named carthamin-synthesizing enzyme (Saito *et al.* 1983). The enzyme requires O_2 for the reaction to proceed and is also dependent on the presence of Mn^{2+}. To the contrary, other metal cations such as Fe^{2+}, Fe^{3+} and Cu^{2+} as well as phosphorus substances inhibit enzyme activity (Saito *et al.* 1985). These facts led us to believe that the carthamin-synthesizing enzyme differs from ferruginous peroxidase and coppery polyphenolase. Indeed, no cartham -in-synthesizing reaction in the presence of guaiacol or 3,4-dihydroxyphenylalanine is catalyzed by the enzyme (Homma *et al.* 1985 and unpublished results). Although a co-factor requirement for the enzyme action has long been expected, no studies have been carried out to date.

In this study, we tested the effect of four co-factors, namely NADPH, $NADP^+$, NADH and NAD^+, on the carthamin-synthesizing enzyme activity. The main aim was to obtain more information on the enzyme in order to distinguish its properties more clearly from those of peroxidase and polyphenolase. It was also envisaged that this could lead to insight into the enzyme mechanism of carthamin formation from precarthamin in dyer's saffron tissues.

Materials and Methods

The precarthamin sample was from our laboratory stock. NADPH, $NADP^+$, NADH and NAD^+ were purchased from Wako Pure Chemical (Osaka, Japan). Sephadex G-25 and G-100 were obtained from Pharmacia Fine Chemicals (Uppsala, Sweden). Plant materials were prepared from seeds of Mogami-Benibana, a cultivar of dyer's saffron (*Carthamus tinctorius* L.), which were sown on damp Vermiculite and cultured at 23 ± 2°C in the light for 2 weeks.

Preparation of carthamin-synthesizing enzyme extract

Green seedlings (14.3 g fresh weight) were dipped in 70% (v/v) ethanol for 3 min to sterilize the plant surface, then washed 4–5 times with sufficient amounts of distilled water until the smell of alcohol could no longer be detected. Seedlings were macerated in 50 mM citrate/phosphate buffer, pH 7.0, containing 0.1 mM 2-mercaptoethanol and 20 mM D-iso-ascorbic acid, and squeezed through a nylon cloth. The filtrate was centrifuged at 13000 × g for 20 min. The supernatant was fractioned with solid $(NH_4)_2SO_4$ and the fraction precipitated between 0–80% $(NH_4)_2SO_4$ saturation which was collected by means of centrifugation (13000 × g, 5 min). Desalting of the protein pellet was obtained by dissolving it in 50 mM citrate/phosphate buffer, pH 7.0, applying it onto a Sephadex G-25 column (2.5 × 20 cm), equilibrated and eluted with the same buffer. The column eluate was fractioned again with $(NH_4)_2SO_4$ and the protein which precipitated between 0–80% saturation was dissolved in 2 ml of 50 mM citrate/phosphate buffer, pH 7.0. The protein solution was transferred to a Sephadex G-100 column (0.9 × 90 cm) and developed with 50 mM citrate/phosphate buffer, pH 7.0. Fractions of 4.4 ml were collected at a flow rate of 0.12 ml/min and retained at 3–4°C until the following enzyme assay procedure could be carried out.

Enzyme assay and activity determination

The standard reaction mixtures contained, if not stated otherwise, 3 ml of 50 mM acetate buffer, pH 4.8; 0.1 ml protein solution (8.5–9.4 μg protein), 0.116 μmol precarthamin. The mixtures were incubated at 30°C for 5 min. Enzyme activity was monitored automatically with a Hitachi, model U-3210 spectrophotometer. The data from the spectrophotometer readings at A_{521} were used for the enzyme activity determination. The blank test was carried out under the same conditions as described above, except that no precarthamin was added to the reaction mixtures. Protein contents were estimated by the method of Lowry *et al.* (1951) using bovine serum albumin as standard.

Results and Discussion

Evidence is presented here for the influence of pyridine nucleotides on the carthamin formation *in vitro*. On incubating protein extracts from green seedlings of dyer's saffron together with precarthamin under the standard assay condition, a considerable amount of carthamin was formed. In the presence of 10 pmol Mn^{2+}, both NADPH and NADH enhance the enzyme activity