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Enzyme polymorphism in *Encephalartos* Lehmann (Zamiaceae)

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Riassunto. Centoventuno esemplari di 34 specie di *Encephalartos* (Zamiaceae, Cycadales) coltivati nell'Orto Botanico di Napoli sono stati esaminati per valutare la presenza di polimorfismi negli enzimi fosfoglucomutasi, 6-fosfogluconato deidrogenasi e isocitrico deidrogenasi. I risultati ottenuti indicano un elevato livello di polimorfismo genetico in contrasto con quanto precedentemente osservato in altri generi di Cycadales.

Abstract. One hundred and twentyone specimens belonging to 34 species of *Encephalartos* (Zamiaceae, Cycadales) cultivated at the Botanical Garden of Naples, Italy, have been examined to evaluate the occurrence of polymorphism in the enzymes phosphoglucomutase, 6-phosphogluconate dehydrogenase and isocitric dehydrogenase. Data indicate a high level of genetic polymorphism in contrast with previous evidence available for other genera of Cycadales.

Key words: *Encephalartos*, Enzyme polymorphisms, Zamiaceae.

INTRODUCTION

Cycadales (cycads) are a group of gymnosperms whose fossil record dates back to the Lower Permian (CRANE, 1988). They are presently represented by eleven genera, distributed in the whole intertropical belt (STEVENSON, 1990).

In recent year, biologists have become interested in cycad genetics and molecular biology. Despite the potential interest of such investigations, however, no study is available dealing with the genetic structure of a significant number of species in a genus. At present, only three species have been studied by

using enzyme polymorphisms, i. e., *Cycas rumphii* Miq. (LEIBENGUTH, 1984; 1985), *Zamia pumila* L. (WALTERS & DECKER-WALTERS, 1991), and *Macrozamia communis* L.A.S. Johnson (ELLSTRAND et al., 1990).

In the latter two studies, the authors found an unusual genetic structure with relatively low level of polymorphism and generally rare alternate alleles. These results strongly contrast with similar findings on other gymnosperms (HAMRICK & GODT, 1990).

To verify the level of polymorphism in other cycad taxa, we analysed the variation of phosphoglucomutase, isocitric dehydrogenase and 6-phosphogluconate dehydrogenase within the African genus *Encephalartos*.

Encephalartos is the second largest genus in the Cycadales and consists of over 50 species, plus a number of yet undescribed taxa (GOODE, 1993; STEVENSON & OSBORNE, 1993). Its distribution is presently restricted to the African continent, with more than half of the species occurring in South Africa. While some species of *Encephalartos* have a fairly wide distribution (i.e. *E. natalensis*), others have a narrow range or even a point distribution (GOODE, 1993; MORETTI et al., 1989).

MATERIAL AND METHODS

A total of 121 specimens belonging to 34 *Encephalartos* species were investigated; all specimens are cultivated at the Botanical Garden of Naples (Tab. 1). The specimens were either field collected or supplied by other botanical institutions. It is extremely unlikely, therefore, that any of the specimens sampled was clonal propagation of another.

For phosphoglucomutase (PGM - E.C. 2.7.5.1), 121 specimens were examined; for 6-phosphogluconate dehydrogenase (6-PGD - E.C. 1.1.1.44), 101 specimens; for isocitric dehydrogenase (IDH - E.C. 1.1.1.42), 107 specimens (Tab. 1).

Young and healthy leaflets (3 g) were collected from each specimen and washed several times in sterile distilled water. The leaflets were then minced and homogenized in a Waring Blendor (3 pulses of 5 sec each). Homogenization buffer contained 100 mM

Tab. 1 – *Encephalartos* species in study with their geographic origins and number of examined specimens.

Species	Geographic origin	No. of specimens examined for each enzyme		
		PGM	6PGD	IDH
E. altensteinii Lehmann	Rep. South Africa (RSA)	4	3	3
E. arenarius R.A. Dyer	RSA	5	4	5
E. bubalinus Melville	Tanzania, Kenya	1	1	1
E. caffer (Thunberg) Lehmann	RSA	2	2	2
E. cycadifolius (Jacquin) Lehmann	RSA	3	1	2
E. eugene-maraisii Verdoorn	RSA	8	6	6
E. ferox Bertolini fil.	RSA, Mozambique	5	5	4
E. friderici-guilielmi Lehmann	RSA	2	2	1
E. gratus Prain	Malawi, Mozambique	2	1	2
E. heenanii R.A. Dyer	RSA, Swaziland	1	1	1
E. hildebrandtii A. Braun & Bouche	Kenya, Tanzania	2	2	2
E. horridus (Jacquin) Lehmann	RSA	6	5	6
E. inopinus R.A. Dyer	RSA	4	4	4
E. kisambo Faden & Beentje	Kenya	1	1	1
E. lanatus Stapf & Burtt Davy	RSA	4	4	4
E. latifrons Lehmann	RSA	1	1	1
E. laurentianus De Wildeman	Zaire, Angola	1	1	1
E. lebomboensis Verdoorn	RSA, Swaziland, Mozambique	4	3	4
E. lehmannii Lehmann	RSA	3	3	3
E. longifolius (Jacquin) Lehmann	RSA	10	7	10
E. manikensis Gilliland (Gilliland)	Zimbabwe, Mozambique	3	3	2
E. marunguensis Devred	Zaire	2	1	2
E. natalensis R.A. Dyer & Verdoorn	RSA	11	10	10
E. ngoyanus Verdoorn	RSA, Swaziland	1	1	1
E. paucidentatus Stapf & Burtt Davy	RSA, Swaziland	5	5	5
E. poggei Ascherson	Zaire	6	6	3
E. sclavoi De Luca, Stevenson & Moretti	Tanzania	1	1	1
E. transvenosus Stapf & Burtt Davy	RSA	2	1	2
E. trispinosus (Hooker) R.A. Dyer	RSA	3	3	3
E. umbeluziensis R.A. Dyer	Swaziland, Mozambique	5	3	4
E. villosus Lemaire	RSA, Swaziland	4	3	4
E. woodii Sander	RSA (extinct in nature)	1	0	1
E. sp. *"Msinga"*	RSA	4	4	2
E. sp. *"Piet Retiefit"*	RSA	4	3	4

Tris-HCl (pH 8.0) 10 mM EDTA, 7.5% polyvinylpyrrolidone (PVP m.w. 40000), 2 mM phenylmethylsulphonylfluoride (PMSF), 14 mM 2-mercaptoethanol and 2.2 mM dithiothreitol. The leaflet weight to buffer volume ratio was 1 to 5. The homogenate was filtered through Miracloth paper, partially purified through successive ammonium sulphate precipitations (20-60%) and extensively dialysed against 100 mM Tris-HCl (pH 8.0), 10 mM EDTA. The extract was then either electrophoresed or stored at -80 °C for long time preservation. No detectable enzymatic activity has been observed after 6 months of such storage.

Three buffer systems were used in a horizontal starch gel electrophoresis apparatus (Connaught Laboratories, Canada); electrophoreses (11% starch gel) were carried out for 18 h at 4 °C (4 V/cm, with 200 x 100 x 5 mm slabs); standard staining procedures for these isozymes were used (Tab. 2).

Tab. 2 - Buffers, running conditions and staining methods used.

Enzyme	Gel buffer		Electrode buffer		Staining	
PGM	Tris	15 mM	Tris	0.2 M	NADP	0.5 mM
	EDTA	2 mM	EDTA	2 mM	G1P	2.6 mM
	Boric acid	50 mM	Boric acid	0.23 M	G1,6 P	0.05 mM
	pH	7.5	pH	8.0	PMS	0.3 mM
					MTT	1.4 mM
			60 V; 9 mA; 18 h		G6PDH	1.4 U
					$MgCl_2$	5 mM
					pH	8.0
6PGD	Tris	8 mM	Tris	0.15 M	NADP	0.5 mM
	Citric acid	2 mM	Citric acid	50 mM	6PG	2.6 mM
	pH	7.2	pH	7.2	PMS	0.3 mM
					MTT	1.4 mM
			40 V; 15 mA; 18 h		$MgCl_2$	5 mM
					pH	8.0
IDH	Tris	9 mM	Tris	135 mM	NADP	0.625 mM
	Citric acid	2 mM	Citric acid	32 mM	Isoc. Ac.	40 mM
	pH	8.0	pH	8.0	PMS	0.2 mM
					MTT	1.75 mM
			50 V; 12 mA; 18 h		$MgCl_2$	5 mM
					pH	8.0

All enzymes detected migrated anodally. Four loci were inferred from the observed banding patterns. In the case of the two PGM loci, the locus with the slowest mobility was numbered *1* and the fastest *2*. Alleles are named in a similar fashion, being *a* the slowest and *d* the fastest one (Tab. 3).

Tab. 3 – Allele mobility (in cm from origin).

Enzyme	Allele			
	a	*b*	*c*	*d*
PGM*1*	2.0	2.5	3.0	3.5
PGM*2*	4.0	4.5	5.0	5.5
6PGD	1.5	2.0	2.5	3.0
IDH	1.5	2.0	2.5	

RESULTS

The genetic interpretation of the banding patterns was based on several lines of evidences (Tab 4). When a single activity band occurred in the majority of the sampled plants, it was assumed to represent homozygous status for the involved locus or loci. In this regard, previous literature relative to subunit composition and typical number of isozyme loci for a particular enzyme was taken into account.

All the species examined showed two isoenzymatic forms for PGM (PGM*1* and PGM*2*) and a single form for 6-PGD and IDH. As far as the probable number of different alleles is concerned, the electropherograms indicated the possible presence of 4 alleles at the 6-PGD locus, 3 alleles at the IDH locus and 8 at both PGM loci (4 each). Five different phenotypes for IDH and 6-PGD, and 14 for PGM were detected.

6-PGD shows band *b* (distance from origin 2 cm) in all the species but four (Tab. 4). Among these four (all represented by a single individual), *E. latifrons*, *E. laurentianus* and *E. sclavoi* exhibit only band *d* (distance from origin 3 cm), while *E. gratus* exhibits

only band *a* (distance from origin 1,5 cm). The latter two bands are present also in other species (Tab. 4).

Tab. 4 – Allele forms in the examined species.

Species	PGM*1*	PGM*2*	6PGD	IDH
E. altensteinii	2.5 - 3.0	4.5 - 5.0	1.5 - 2.0	2.0
E. arenarius	2.5	4.5	2.0	2.0 - 2.5
E. bubalinus	3.0	5.0	2.0	2.0
E. caffer	2.5 - 3.0	4.5 - 5.0	2.0	1.5 - 2.5
E. cycadifolius	2.5 - 3.0	4.5 - 5.0	2.0	1.5 - 2.0
E. eugene-maraisii	2.5 - 3.0	4.5 - 5.0	2.0	1.5 - 2.0 - 2.5
E. ferox	2.5 - 3.0	4.0 - 4.5 - 5.0	2.0	2.0 - 2.5
E. friderici-guilielmi	2.5	4.0 - 4.5	2.0	2.0
E. gratus	2.0 - 2.5	4.0 - 4.5	1.5	2.0 - 2.5
E. heenanii	2.5	4.0	2.0	2.0
E. hildebrandtii	2.5 - 3.0	4.5 - 5.0	2.0	2.0 - 2.5
E. horridus	2.5 - 3.5	4.5 - 5.0	2.0	1.5 - 2.0 - 2.5
E. inopinus	2.5 - 3.0	4.0 - 4.5 - 5.0	1.5 - 2.0	2.0 - 2.5
E. kisambo	3.0 - 3.5	5.5	2.0	1.5
E. lanatus	2.5 - 3.0	4.0 - 4.5 - 5.0	1.5 - 2.0	1.5 - 2.0
E. latifrons	2.5	5.0	3.0	2.0
E. laurentianus	2.5	4.5	3.0	1.5 - 2.0
E. lebomboensis	2.5 - 3.0	4.5 - 5.0	2.0	2.0
E. lehmannii	2.5	4.5	2.0	1.5 - 2.0 - 2.5
E. longifolius	2.0 - 2.5 - 3.0	4.0 - 4.5	1.5 - 2.0	1.5 - 2.0 - 2.5
E. manikensis	2.5 - 3.5	4.5 - 5.5	1.5 - 2.0	1.5 - 2.5
E. marunguensis	2.5 - 3.0	4.5 - 5.0	2.0	1.5 - 2.0 - 2.5
E. natalensis	2.5 - 3.0 - 3.5	4.0 - 4.5 - 5.5	1.5 - 2.0	1.5 - 2.0 - 2.5
E. ngoyanus	2.5	4.5	2.0	1.5
E. paucidentatus	2.0 - 2.5 - 3.0	4.5 - 5.0	1.5 - 2.0	2.0 - 2.5
E. poggei	2.0 - 2.5	4.0 - 4.5	1.5 - 2.0	2.0 - 2.5
E. sclavoi	3.0	5.0	3.0	2.0
E. transvenosus	2.5	4.0 - 4.5	2.0	2.0
E. trispinosus	2.5 - 3.5	4.5 - 5.5	1.5 - 2.0	1.5 - 2.0 - 2.5
E. umbeluziensis	2.5 - 3.0	5.0	2.0 - 3.0	1.5 - 2.0 - 2.5
E. villosus	2.0 - 3.0	4.5 - 5.0	2.0 - 3.0	2.0 - 2.5
E. woodii	3.5	5.5	Not available	1.5
E. sp. *"Msinga"*	2.5	4.5	1.5 - 2.0	1.5 - 2.0
E. sp. *"Piet Retiefii"*	2.5	4.5	2.0 - 2.5	1.5 - 2.0 - 2.5

As far as IDH is concerned, in almost all the species band *b* (distance from origin 2 cm) is always present (Tab. 4). *E. kisambo*, *E. woodii* and *E. ngoyanus* exhibit only band *a* (distance from origin 1.5 cm).

Typically, plants contain two isozymes for PGM, one cytosolic and one plastidial (SOLTIS & SOLTIS, 1989). Also in our case, two PGM forms are present, but we cannot attribute them with certainty to cytosolic or plastidial compartments. In all specimens, the PGM*1* bands *b* (distance from origin 2.5 cm) and *c* (distance from origin 3 cm) are present alone or together, with the exception of the single individual of *E. woodii* which exhibits only band *d* (distance from origin 3.5 cm). In most specimens, PGM*2* phenotypes are represented by a single band, either band *b* (distance from origin 4.5 cm) or band *c* (distance from origin 5 cm). The exception are *E. woodii* and *E. kisambo,* which exhibit only band *d* (distance from origin 5.5 cm), and *E. heenanii*, which exhibits only band *a* (distance from origin 4 cm) (Tab. 4).

The presence of at least two allelic form in some of examined samples indicates that all the investigated enzymes (with the possible exception of PGM*2* locus) are nuclear-encoded in *Encephalartos*.

DISCUSSION

The isozyme patterns shown here are similar to those observed in other seed plants (GOTTLIEB, 1981).

The three enzymatic systems investigated show an average gene diversity of 8%, polymorphic loci were 80%, and the average number of alleles per locus was 2.1 (for this computation we used only the species for which more than one specimen was available) (Tab. 1).

It is interesting to compare these values with allozyme data set compiled by HAMRICK & GODT (1990) for 473 plants. These authors found that the average gene diversity was 15%, the percentage of polymorphic loci was 50.5% and the average number of alleles per locus was 1.96. It must be noted that in our sample the same alleles are generally present in different species and this causes the high level of polymorphism coupled with the low value of gene diversity.

In most plant species, polymorphic loci are characterised by two or more relatively common alleles and tend to be polymorphic throughout individuals. This is true also for *Encephalarthos*. In previous studies on the genetic structure of *Macrozamia communis* (ELLSTRAND et al., 1990) and *Zamia pumila* (WALTERS & DECKER-WALTERS, 1991), the authors proposed the existence of an unusual genetic structure with relatively low polimorphic loci and rare alternate alleles.

In *Encephalartos* species in which more than a couple of specimens have been analysed, the presence of polymorphic loci, particularly for IDH and PGM*1*, is clearly revealed (Tab. 4). The just mentioned two enzymes are monomorphic in *Macrozamia* and poorly variable in *Zamia pumila*. In the latter species, differently from other gymnospermous taxa (HAMRICK & GODT, 1990), much of this polymorphism is due to rare alleles, each of which occurs in one or few populations.

Given the biology of cycads (long-lived, dioecious, insect pollinated, animal dispersed), a relatively high level of genetic variation would be expected (as in *Encephalartos*), rather than the homogeneity shown by *Zamia* and *Macrozamia* species. The unusual genetic structure of the latter two genera could be related to their possibly more recent origin (CAPUTO et al., 1993).

In conclusion, the presence of polymorphic loci at inter- and intraspecific level in *Encephalartos* reveals the potential usefulness of this approach for the study of relationship among closely related *Encephalartos* species, whose morphological features and taxonomic range are often controversial. Moreover, this investigation indicates that testing genetic variation may be accomplished even if the plants are from botanical garden collections, provided that the size of the sample is large enough to provide a complete representation of both the range of diversity in the genus and intraspecific variability.

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REFERENCES

CAPUTO P., MARQUIS C., STEVENSON D. W. & WURTZEL E. T. 1993. Molecular biology in cycad systematics. In: Stevenson D. W. & Norstog K. (Eds.). The biology, structure, and systematics of the Cycadales. p. 213-219. Palm & Cycad Societies of Australia, Milton, Queensland, Australia.

CRANE P. R. 1988. Major clades and the relationship in the "higher" gymnosperm. In: Beck C. S. (Ed.). Origin and evolution of the gymnosperm. p. 218-272. Columbia University Press, New York.

ELLSTRAND N. C., ORNDUFF R. & CLEGG J. M. 1990. Genetic structure of the Australian cycad *Macrozamia communis* (Zamiaceae). American Journal of Botany, 77: 677-681.

GOODE D. 1993. Cycads of Africa. Comrie-Greig J. (Ed.). Leefung-Asco Printers, Hong Kong.

GOTTLIEB L. D. 1981. Electrophoretic evidence and plant populations. Progress Phytochemistry, 7: 1-46.

HAMRICK J. L. & GODT M. J. 1990. Allozyme diversity in plant species. In: Brown A. H. D., Clegg M. T., Kahler A. L. & Weir B. S. (Eds.). Populations genetics and germplasm resources in crop improvment. p. 43-63. Sinauer Ass., Sunderland, MA, U.S.A.

LEIBENGUTH F. 1984. Genetic polymorphism in *Cycas rumphii.* Annales Bogorienses, 8: 57-66.

LEIBENGUTH F. 1985. Enzyme polymorphism in the phylogenetic relicts *Cycas rumphii* and *Dracaena draco.* Biochemie und Physiologie der Planfzen, 180: 465-471.

MORETTI A., DE LUCA P. & STEVENSON D. W. 1989. *Encephalartos sclavoi* De Luca, Stevenson and Moretti (Zamiaceae), a new species from Tanzania. Annals of Missouri Botanical Garden, 76: 934-938.

SOLTIS D. E. & SOLTIS P. S. 1989. Isozymes in plant biology. Dioscorides Press, Portland, OR, U.S.A.

STEVENSON D. W. 1990. *Chigua*, a new genus in the Zamiaceae with comments on its biogeographic significance. Memoires of the New York Botanical Garden, 57: 169-172.

STEVENSON D. W. & OSBORNE R. 1993. The world list of cycads. Encephalartos, 33: 19-25

WALTERS W. T. & DECKER-WALTERS D. S. 1991. Patterns of allozyme diversity in the West Indies cycad *Zamia pumila* (Zamiaceae). American Journal of Botany, 78: 436-445.

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