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COMPARISON OF POPULATIONS OF *Helianthus argophyllus* AND *H. debilis* ssp. *cucumerifolius* AND THEIR HYBRIDS FROM THE AFRICAN COAST OF THE INDIAN OCEAN AND THE USA USING MOLECULAR MARKERS

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SUMMARY

A comparison of *H. argophyllus* and *H. debilis* ssp. *cucumerifolius* populations from the coast of Mozambique and Eastern South Africa and Texas, USA was carried out at Udine University (Italy). American populations were supplied by the USDA Northern Crop Science Lab, while the populations from Africa were collected over several years by Udine researchers. Populations comparison were based on morphological traits and nuclear and chloroplastic SSR markers of plants grown in pots in a growth chamber.

All populations are of potential interest to breeding programs as well as for evolutionary studies. Hybrid populations involved both species and for some African swarms possibly *H. annuus*. For *H. argophyllus*, there is evidence of a bottleneck effect. Material coming from Texas showed a lower number of alleles in nuclear SSR compared with the African material.

Key words: **sunflower, *Helianthus argophyllus*, *Helianthus debilis*, wild species population, SSR markers, DNA**

INTRODUCTION

Sunflower is a native American species described for the first time by Matthioli (1568) after growing *Helianthus annuus* in the Botanical Garden, former Horto dei Simplici, in Padua. After improving it as an oilseed crop in Europe, interest increased all over the world with Americans beginning to study the wild and cultivated sunflowers (Heiser, 1976; Rogers *et al.*, 1982;, Rieseberg *et al.*, 1998, 1990;

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Seiler, 1997; Seiler and Rieseberg, 1997; Whitton *et al*., 1997; Linder *et al*., 1998; Carney *et al*., 2000; Yu *et al*., 2002; Tang and Knapp, 2003; Tang *et al*., 2002, 2003). Rogers *et al*. (1982) list more than 50 *Helianthus* species present in USA, and other species and related genus such as *Tithonia* and *Viguiera* that grow in Central and South America. *Helianthus argophyllus* and *H. debilis* ssp. *cucumerifolius* have been found in Africa and are a potential genetic resource since they grow in sites saturated by sea water. Cagiotti *et al*. (1999) reported more xeric characteristics in *Helianthus argophyllus* compared with *Helianthus annuus*.

Helianthus argophyllus was found in places along the seaside in Mozambique (Olivieri *et al*., 1999) and studied using AFLP markers (Quagliaro *et al*., 2001). Recently, other *H. argophyllus* populations have been found in areas around harbors in the Republic of South Africa.

A second wild sunflower species, *H. debilis* ssp. *cucumerifolius*, was found in the Inhambane bay area (Olivieri *et al*., 1999), as well as at two sites where it appears to be a hybrid population with an introgression of *H. debilis* ssp. *cucumerifolius* with *H. argophyllus*. Vischi *et al*. (2002) reported the first evidence of this based on morphological traits and AFLP markers.

In the present study we present the first report of the differences between populations of *H. argophyllus* and *H. debilis* ssp. *cucumerifolius* from South-East Africa and Texas grown in common environment. To investigate the origin of African populations, we compared them with the American populations both at the morphological and molecular level using 20 nuclear *H. annuus* SSRs (Pianiego *et al*., 2002) and 10 universal chloroplast SSRs (Weising and Gardner, 1999).

MATERIAL AND METHODS

Wild species populations

Populations of American sunflower, *H. argophyllus* and *H debilis*, were collected along the Texas coast (Rockport, North Padre Island) and inland locations (Victoria and Falfurias). Coastal locations for *H. argophyllus* were Rockport, North Padre Island and Liberty, and Rockport for *H. debilis*. The inland locations for *H. argophyllus* were Victoria and Falfurias, and Sublime for *H. debilis*. Rockport populations PI 435625 and PI 435655 representing *H. argophyllus* and *H. debilis* spp. *cucumerifolius*, respectively, were originally collected only three km apart.

African populations of *H. argophyllus* and *H. debils* were collected along the Inhambane bay at different sites (Figure 1). Site 1 and 2 were located along the road connecting Inhambane and Tofo beach, close to the villages of Chamane and Marambone. In site 1, some plants of *H. argophyllus* were growing around deep holes and soil depressions, showing the perennial habit and basal stalks reaching 7-10 cm in diameter. *Helianthus debilis* ssp. *cucumerifolius* was found at Sites 7 and 9, in the city of Inhambane, about 100 meters from the *H. argophyllus* population

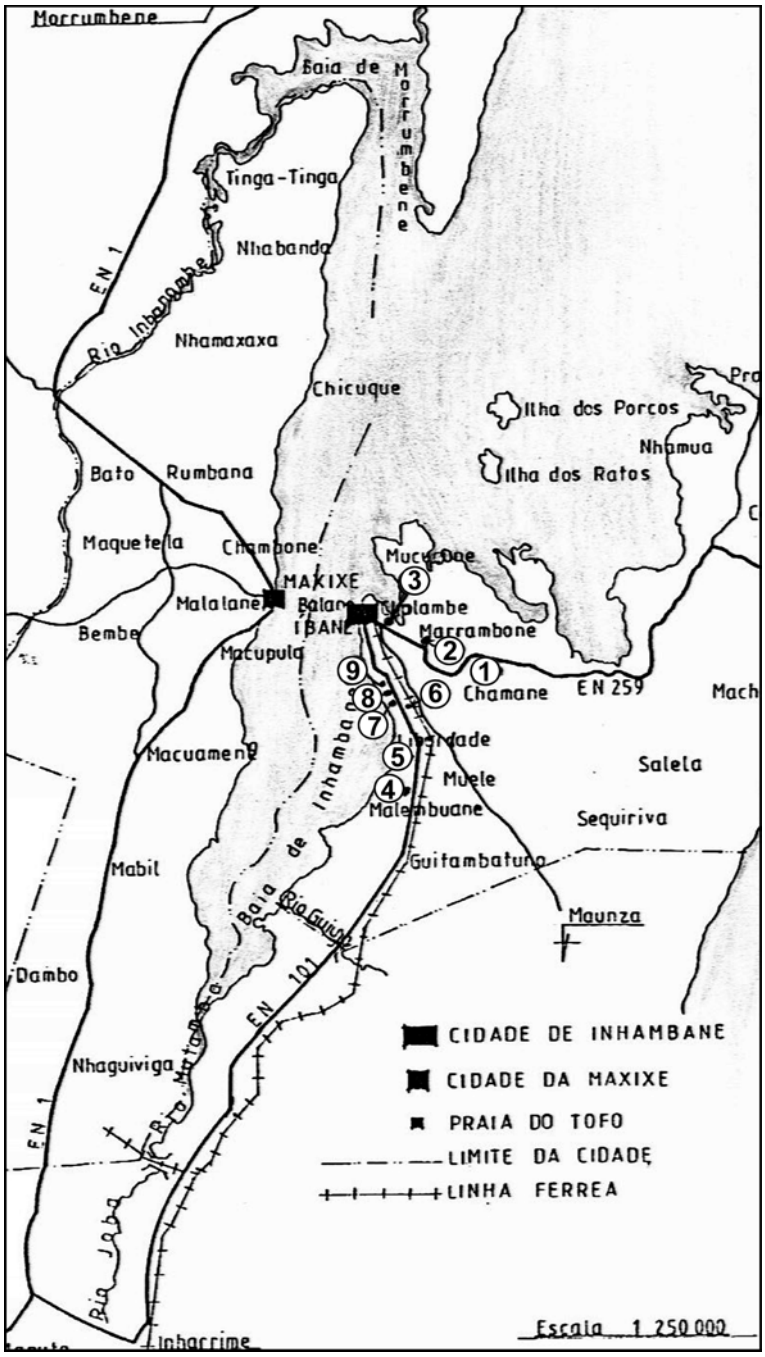

Figure 1: Distribution of H. argophyllus and H. debilis populations in Inhambane bay, Mozambique (see text for details)

growing in a sandy coastal slope in front of a cemetery and the Direccion Provincial de Agricultura. They are endangered populations.

Site 10 was on the docks and surrounding area of the town of Maxixe where *H. debilis* ssp. *cucumerifolius* grows on areas supplied by sea water, while patches of *H. argophyllus* grow about 100 meter outside the beach.

Additional populations were collected in the Republic of South Africa, after visiting the Herbarium Natalense at Durban, where specimens of both species are stored. *Helianthus argophyllus* had been collected at Isipingo in 1966 and recollected from a population still growing near a cliff. In downtown Durban, a swarm of *H. argophyllus* grows as a weed in a sandy soil close to the beach and was recollected.

Plant material and DNA extraction

Seeds were sown in boxes with a sandy soil on 14 June 2002 at Udine, Italy. Germination occurred slowly over two months and plants with 3 or 4 true leaves were transplanted in pots of different size in relation to emergence time. Plants of the same age were kept in the same conditions and observations were done on the morphological and flowering traits (Seiler, 1997).

The analyses were carried out on 68 plants of *H. argophyllus* from Texas (Rockport, North Padre Island, Victoria, Falfurrias) and 137 plants from Africa (Mozambique: Inhambane, Xai-Xai, Maputo and South Africa: Durban, Isipingo). Seventeen plants of *H. debilis* collected in Texas (Sublime, Liberty, Rockport) and 35 plants from Mozambique (Maxixe, Inhambane) were also analyzed. DNA was extracted from leaf tissue by cetyltrimethylammomium bromide (CTAB) protocol (Doyle and Doyle, 1972) and quantified by gel-electrophoresis staining comparison.

Nuclear SSR genotyping

In this study we used a set of 20 microsatellite primers published by Paniego *et al.* (2002), developed for cultivated sunflower. To determine their usefulness for identifying the wild species, we used a sub-set of about 8 plants for each species and geographical origin. The set of 20 SSR markers was amplified with primers end-labeled with [^{33}P]γ-ATP and T4 polynucleotide kinase. PCR reactions were performed in a total volume of 10 μl using a Gene Amp 700 DNA thermocycler (PE Applied Biosystem, USA). The reaction mixture contained 1.5 ng of genomic DNA, 1.5 mM $MgCl_2$, 0.2 mM each of dNTPs; 0.25 μM each of primers, 1 x PCR buffer, 0.4U Taq DNA polymerase (Amersham Science, UK). Touchdown PCR was performed for enrichment of the template. The initial denaturation step was performed at 94°C for 5 min, followed by one cycle of 94°C for 30 s, 59°C for 30 s, 72°C for 30 s. The annealing temperature was decreased 1°C per cycle in the subsequent cycles until reaching 52°C. Products were subsequently amplified for 25 cycles at 94°C for 30 s, 52°C for 30 s, 72°C for 30 s with a final extension of 7 min. The products of

PCR amplification were separated on 6% acrylamide gel under denaturing conditions and visualized by autoradiography after 1-2 days exposure.

Chloroplast SSR genotyping

The 10 universal chloroplast SSRs were amplified according to the protocol of Weising and Gardner, (1999) using radioactively labeled primers. Bands were scored by visual inspection.

RESULTS

Morphological observations

Seeds of *H. debilis* ssp. *cucumerifolius* from Texas were particularly recalcitrant to germination, possibly because of different soil requirement or high dormancy. Seeds of *H. argophyllus* from Rockport, PI 435625, and to a lesser extent seed from Victoria (PI 494582) and Isipingo, had a germination rate of less than 50%. Hybrid seedlings were distinguishable from parental species by cotyledon leaflet shape and color (Vischi *et al*., 2002). About 2-3% of the seedlings of the hybrid population showed lack of symmetry in cotyledon leaves and abnormality in their development. Some were stunted in growth and some died before producing the first true leaf. Plant height and leaf size were characterized by high plasticity but no differences were found among American and African populations for both species (data not presented).

Table 1 shows the number of plants sampled from each population of *H. argophyllus*, *H. debilis* ssp. *cucumerifolius* and their hybrids, the index of hairiness of stem and flowering time. Although there was variability within all *H. argophyllus* populations, plants from Texas were hairier than those from African.

Helinaathus debilis ssp. *cucumerifolius* plants started flowering 50-60 days after sowing, whereas *H. argophyllus* required a short day photoperiod flowering after more than 100 days. There were differences among populations regarding earliness of flowering as shown in Table 1.

Each population had its own variability, but it seems that earliness in flowering was basically correlated with latitude.

Nuclear SSR

Thirteen nuclear SSRs primers gave amplification signals among the set of 20 primer combinations tested. The polymorphic loci with the number of alleles are reported in Table 2.

The score of nuclear SSR markers was sometimes very difficult to interpret because of the very high number of alleles compounded with band artifacts and stutter bands. A possible explanation for this is that the SSR markers used had been developed for *H. annuus* and the parameters of PCR amplification need to be

optimized for each wild species. As a general observation, more polymorphic bands were observed in *H. argophyllus* populations coming from Texas compared with the African populations, reflecting a major level of genetic variability of these populations as expected from material coming from the center of origin.

Table 1: Origin and morphological data of the populations studied

Pop. no.	Origin	Plant number	Index of hairiness (*)	No flowering plants I II III IV Oct. week			
	H. argophyllus from Texas:			I	II	III	IV
1	Victoria, PI 494582	10	3-5	-	3	2	-
2	Rockport, PI 435625	3	3-4	-	3	-	-
3	North Padre Island PI 494572	27	3-5	1	4	6	1
4	Falfurrias, PI 494578	28	3-4	-	1	6	4
	H. argophyllus from Africa:						
5	Inhambane, site 1 and 2, Mozamb.	31	2-4	1	2	3	2
6	Xai-Xai town, Mozambique	27	2-3	3	1	2	2
7	Maputo, Costa do Sol, Mozambique	32	2-3	-	1	8	5
8	Durban, City, Rep.Africa South	42	2-3	8	6	-	-
9	Isipingo, Rep. Africa South	5	2-3				
	H. debilis var. *cucumerifolius* from Texas:						
10	Liberty, PI 468667	9	1-2				
11	Sublime, PI 435654	7	0-2				
12	Rockport, PI 435655	1	1				
	H. debilis var. *cucumerifolius* from Africa:						
13	Maxixe, site 10, Mozambique	22	0-1				
14	Inhambane, site 9, Mozambique	13	1-2				
	Hybrids in Africa						
15	Inhambane, site 7	26	0-3	4	2	2	-

*0=no hairiness; 5=max. hairiness

Table 2: Number of alleles in 5 nuclear SSR loci

Locus	Allele no.			
	H. argophyllus		*H. debilis*	
	Africa	Texas	Africa	Texas
Ha360-ar	1	?	?	1
Ha494-ar	3	6	2	7
Ha806-ar	4	8	5	4
Ha1287-ar	2	2	4	4
Ha1796-ar	1	2	4	1

Chloroplast SSR

In preliminary tests carried out on a limited number of populations of the two species, 9 chloroplast SSR markers produced amplification signals, except *ccmp8*. The amplification profile of chloroplast SSR markers did not have band artifacts.

Considering the amplification profiles and the level of polymorphism, we selected the *ccmp2* locus for analyzing the complete set of plants. The *H. debilis* ssp *cucumerifolius* populations from Texas were monomorphic with only one allele found. Two alleles (named ***A*** and ***B***) were found in *H. argophyllus* populations (Figure 2).

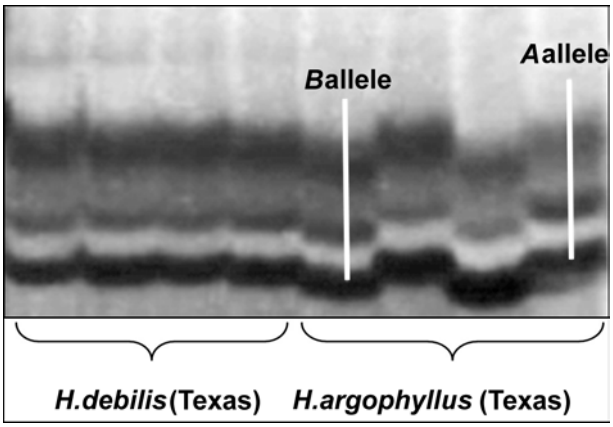

Figure 2: Chloroplast SSR analysis of H.argophyllus and H. debilis at ccmp2 locus; two alleles (A and B) were detected in H. argophyllus plants

The distribution of *ccmp2* alleles in Texas and African populations of *H. argophyllus* is reported in Table 3.

The frequency of the *B* allele in African populations varied from 50 to 100%. The Texas populations from Falfurias and Rockport were in the same range. Conversely, the frequency of this allele was very low or absent in Padre Island and Victoria populations. This suggests that a small American population may have served as the origin of the African populations, but further analyses with the other *ccmp* markers need to be performed to confirm this hypothesis.

Table 3: Chloroplast SSR *ccmp2* locus; alleles distribution in *H. argophyllus* populations

Country	Location	*A* alleles no.	*B* alleles no.	% *B* allele
Texas	Rockport	1	2	66
	Padre Island	27	0	0
	Victoria	8	1	11
	Falfurias	7	20	74
SE Africa	Inhambane	7	21	75
	Xai Xai	4	4	50
	Durban	8	24	75
	Maputo	1	12	92
	Isipingo	0	5	100

P-value for identity of allele frequency among Texan populations=0.0000 (highly significant)
P-value for identity of allele frequency among African populations=0.2070 (not significant)

DISCUSSION

There are few reports of *H. argophyllus* and *H. debilis* ssp. *Cucumerifolius* occurring *in* Africa. No information is available for Mozambique, whereas Ross (1972), Arnold and de Wet (1993), and Liestner (2000) mentioned the presence of these species in the Kwazulu Natal region of South Africa. Pooley (1998) in her guide to wild flowers does not report any sunflower species but mentions *Tithonia diversifolia* (Mexican sunflower) and *T. rotundifolia* (red sunflower) as invasive alien weeds introduced from America. Specimens collected mainly in Durban area during the last century are stored at the Herbarium Natalense. Concerning the origin of these sunflower species in Africa, some authors justify their origin as escaped from gardens. However, the simultaneous presence of both species and their hybrids in two sites in Inhambane bay (Maxixe site 10, and Inhambane site 9), suggests that they had been introduced in Africa together, possibly from the same area of origin. We suspect that they arrived together from Texas, in relation to the slave trade practiced in past centuries (Capela and Medeiros, 1987). It is possible that plants of these two species were stowed in vessels as forage and litter for animals or used as food for sailors. Once unloaded at the African coast, some seeds had become established in Texas-like soil and climate conditions. The latitudes of both areas range between 20 to 32 degrees, although in the opposite hemispheres.

Rogers *et al.* (1982) reported that *H. argophyllus* and *H. debilis* ssp. *cucumerifolius* were sympatric in Texas, growing close to each other as observed for Rockport populations. Temporal barriers of crossability between the two species do not seem to exist since they have a 2-3 month-overlap in flowering period according to the observations at the experiment farm of Udine University. Because of photoperiod sensitivity, cross pollination should occur easily in plants growing at a low latitude, just like the American and African populations.

Whatever the way of colonization of wild sunflower in Africa may have been, they appear to be sub-populations of the native American species. African *H. argophyllus* shows a smaller number of alleles in nuclear SSR loci compared with the Texas populations. Also, a bottleneck effect can be seen by hairiness of the stems. However, due to the paucity of data, we cannot apply an appropriate statistical test.

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ANÁLISIS COMPARATIVO DE LAS POBLACIONES DE *Helianthus argophyllus* Y *H. debilis* ssp. *cucumerifolius* DE LA COSTA AFRICANA DEL OCÉANO INDIO Y DE LOS E.E.U.U. Y SUS HÍBRIDOS, UTILIZANDO LOS MARCADORES MOLECULARES

RESUMEN

En la Universidad de Udine (Italia) se efectuó un análisis comparativo de las poblaciones de las especies *H. argophyllus* y *H. debilis* ssp. *cucumerifolius*, de los E.E.U.U., de la costa de Mozambique, y de la costa oriental de África del Sur. Las poblaciones estadounidenses fueron obtenidas de USDA Northern Crop Science Lab, mientras que las africanas fueron recolectadas por parte de los investigadores de Udine, durante varios anos. Las poblaciones fueron comparadas a base de las características morfológicas y de los marcadores nucleares y cloroplásticos SSR, de las plantas cultivadas en jarrones en la cámara. Todas las poblaciones investigadas son de interés potencial para los programas de mejoramiento, tanto como para las investigaciones evolutivas. En las poblaciones híbridas, estaban incluidas ambas especias investigadas, y en algunas poblaciones africanas, se determinó la posible presencia de la especie *H. annuus*. En la especie *H. argophyllus,* se encontraron ciertas pruebas de la presencia del efecto del cuello de botella. El material de Tejas tuvo el menor número de alelos en el SSR nuclear, en comparación con el material africano.

ANALYSE COMPARATIVE DES POPULATIONS D'ESPÈCES *Helianthus argophyllus* ET *H. debilis* ssp. *cucumerifolius* ET LEURS HYBRIDES DE LA CÔTE AFRICAINE DE L'OCÉAN INDIEN ET DES ETATS UNIS UTILISANT LES MARQUEURS MOLÉCULAIRES

RÉSUMÉ

Une analyse comparative des populations d'espèces *H. argophyllus* et *H. debilis*, sous-espèce cucumerifolius des Etats Unis et de la côte du Mozambique et la côte Est de l'Afrique du Sud a été effectuée à l'Université à Udine en Italie. Les populations d'espèces des Etats Unis ont été reçues de USDA Northern Crop Science Lab, tandis que celles de l'Afrique ont été recueillies par les chercheurs de l'Université à Udine pendant quelques années. Les populations d'espèces ont été comparées à la base des traits morphologiques et marqueurs SSR de noyau et chloroplaste des plantes poussées dans les pots en chambre pollinique. Toutes les populations examinées sont d'un intérêt potentiel pour les programmes de développement de plantes cultivées et de recherches d'évolution. Dans les populations d'hybrides les deux espèces examinées ont été inclues, tandis que dans certaines populations africaines la présence possible de l'espèce *H. annuus* est confirmée. Pour l'espèce *H. argophyllus* certaines preuves de la présence de l'effet d'embouteillage ont été trouvées. Le matériel examiné de Texas avait peu d'allèles dans les marqueurs SSR de noyau par rapport au matériel de l'Afrique.