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AFLP MARKERS FOR THE STUDY OF INTROGRESSION BETWEEN WILD SUNFLOWER SPECIES (*H. argophyllus* AND *H. debilis* ssp. *cucumerifolius*) AND IN RELATION WITH *H. annuus*

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SUMMARY

Sunflower is a plant of the American continent, but now many wild species are spread in different part of the world. Along the coast of the Inhambane Bay, Mozambique, two sunflower species, *H. argophyllus* and *H. debilis*, both of Texas origin, grow far apart. However, in two sites on sandy soil they grow together and many plants have been recognized as hybrids, sharing morphological traits typical of the two species. Some putative hybrid plants produced seed from which a new generation was raised at University of Udine.

We present data coming from morphological and AFLP analyses of plants grown in the spring 2000. With morphological traits typical of the two species we have build up a classical hybrid index by adding the scores of each species. Clear evidence of introgressed plants results also by looking at the unequality of the AFLP bands present in *H. debilis* and *H. argophyllus* and shared in the introgressed material. A partial pollen grain staining confirms the presence of reduced fertility of some morphologically introgressed plants. From a breeding point of view, these wild species and the introgressed material seems to have great potential value for their specificity. The AFLP band pattern from *H. annuus* material appears to be quite unique and a cluster analysis shows a clear diversity of the three species. Deeper investigation is required in order to assess the extent of these data and to establish whether plants with intermediate traits can represent a new taxonomic unit.

Keys words: wild sunflower, introgression, AFLPs, hybrid index

INTRODUCTION

Sunflower is a plant of the American continent where *Helianthus annuus,* together with *H. tuberosus*, has been used since antiquity (Seiler and Rieseberg, 1997). Because of the increasing importance of *H. annuus* all around the world,

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wild sunflowers are studied as genetic resources for breeding programs. Rogers *et al.* (1982) described more than 50 taxonomic units found in the United States and many wild species are spread out in different parts of the world. Many species produce hybrids when they grow close to each other and Heiser has repeatedly reported (1947, 1949, 1951) about the origin of several American races or species resulting from introgressive hybridization. Recently, using molecular analysis, Rieseberg *et al.* (1988, 1990, 1995) investigated the origin of many of these species. Anderson (1971) pointed out that the contact of sunflower species caused by antropic activities has enlarged their genetic variability, thus permitting a further expansion in new areas of new genetic pools.

During last centuries, two annual wild species, *H. argophyllus* and *H. debilis* ssp. *cucumerifolius*, have reached Inhambane Bay, Mozambique (Vischi *et al.*, 2001), and now they grow in some places along the seashore. *H. argophyllus* is a tall and possibly perennial plant (Olivieri *et al.*, 1999) with dense silver pubescence over leaves, stem and inflorescence. It assumes xeric characteristics in comparison with normal sunflower. *H. debilis* ssp. *cucumerifolius* is a short plant with small, typically shaped green leaves, presenting a vegetative cycle lasting only a few months. In a few sites they grow together and many plants share morphological traits typical of the two species. Some putative hybrids produced seed from which a new generation was grown in Udine, Italy. This material, being watered by seawater, could be of breeding interest at least for salt and drought resistance.

We report data coming from morphological and AFLP analyses of plants belonging to the two above-mentioned species and to populations with intermediate traits concerning the introgressive process occurring between *H. argophyllus* and *H. debilis* ssp. *cucumerifolius* and a comparison, by molecular markers, between wild material and the cultivated sunflower so as to assess its potential value in breeding programs.

MATERIALS AND METHODS

Plant material

Around Inhambane area, Mozambique, in December 1998 and 1999, seed samples of *H. argophyllus*, *H. debilis* ssp. *cucumerifolius* and plants with intermediate traits were collected from plants in ten locations (Olivieri *et al.*, 1999; Vischi *et al.*, 2001) keeping separated the plants and heads of each plant. At the University of Udine during the following summer, first observations were carried out on plants developed from seed samples of each sunflower head. In summer 2000, seed of material coming from the most distant sites of Inhambane, i.e., the two standard species, were sown together with the putative introgressed material as appeared from the original sampled mother plants and the progeny observed in the first summer. To overcome seed dormancy the achenes were scarified and dehulled accord-

ing to Benvenuti *et al*. (1991). Plants were grown in pots and under identical environmental conditions at the Experiment Farm of the University of Udine.

Twelve *H. annuus* plants (from different experimental lines) were included as a control to test the discriminative efficiency of the AFLP technique as well as to ascertain the molecular differences in relation to the wild material.

Morphological hybrid indices

Observations were carried out on single plants at one-month intervals since germination. *H. argophyllus* and *H. debilis* ssp. *cucumerifolius* are easily recognizable for many distinctive morphological characters. In this study in order to construct an hybrid score index (Anderson, 1949; Heiser, 1949; Grant, 1971; Olivieri and Jain, 1977) three diagnostic qualitative traits were considered. Based on the terminology of Seiler (1997) hairiness and color of leaves were scored using a scale 0-1, hispid (short stiff hairs) or hirsute (long silky hairs) and bright green or silver green, respectively, whereas leaf margin was partitioned on a scale 0-3 (entire, undulate, crenate, dentate), where the extremes of the scale represented *H. argophyllus* and *H. debilis* ssp. *cucumerifolius*, respectively. The hybrid indices were the sum of the scores for each trait being reach value of 0-6. For morphological index we used quantitative data concerning leaf measures:

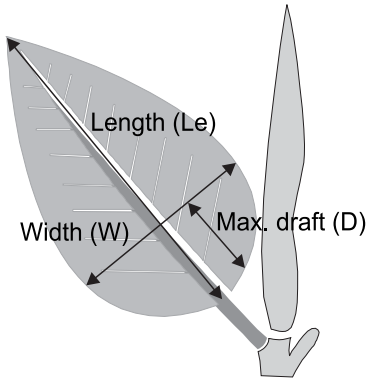

Figure 1: Leaf shape measures

length (Le) width (W) position of maximum draft (D)

and three parameters for leaf shape, A = (Le-D)/D; B = Le/W; A x B (Figure 1).

The other two distinctive traits for the species as plant height and beginning of flowering were also used. For quantitative traits all material was considered as a whole and the index of hybridization was calculated jointing the values of each plant trait after standardization.

AFLP methodology

The genomic DNA was isolated from fresh leaf tissue using the method of Doyle and Doyle (1990). AFLP analysis was carried out according to Vos *et al*. (1995) with slight modifications (Quagliaro *et al*., 2001). A total of 4 primer pair combinations were used (E41/M51,M52; E65/M58,M60). Adapters and primers are reported in Table 1. To check the reproducibility of the methodology the amplifications were repeated twice for each pair primer combinations. Only clear and unambiguous bands were scored.

Table 1: Sequence of AFLP adapters and primers

Name		Sequence
*Eco*RI adapter		5'-CTCGTAGACTGCGTACC-3' 3'-CTGACGCATGGTTAA-5'
*Mse*I adapter		5'-GACGATGAGTCCTGAG-3' 3'-TACTCAGGACTCAT-5'
*Eco*RI +1 primer	E01:	5'-GACTGCGTACCAATTC**A**-3'
	E03:	5'-GACTGCGTACCAATTC**G**-3'
*Eco*RI +3 primers	E41:	5'-GACTGCGTACCAATTC**AGC**-3'
	E65:	5'-GACTGCGTACCAATTC**GAG**-3'
*Mse*I + 1 primers	M02:	5'-GATGAGTCCTGAGTAA**C**-3'
*Mse*I + 3 primers	M58:	5'-GATGAGTCCTGAGTAA**CGT**-3'
	M60:	5'-GATGAGTCCTGAGTAA**CTC**-3'

AFLP data analysis

The resultant band patterns obtained were manually scored for the presence or absence of bands. The genetic similarities were calculated from AFLP data using the Dice similarity index (Dice, 1945). The matrix values estimated the number of AFLP fragments shared (or not shared) between two individuals. Similarity matrix was used to construct a neighbor-joining phenogram using NEIGH-BOUR and DRAW-TREE options in the PHYLIP package (version 3,57c; Joe Felsenstein, University of Washinghton, USA).

Pollen viability

Pollen was collected from flowers on each plant and stained with both acetocarmine and lactophenolcotton blue (Heiser, 1949) before microscopic observation. Unstained pollen grains and those with stained but shriveled cytoplasm were scored as not viable. At least 300 grains were randomly counted per plant to estimate the percentage of viable pollen grains.

RESULTS AND DISCUSSION

Distribution of hybrid index scores

Table 2 reports the morphological observations of quantitative traits of plants relevant to the two periods considered. These results are consistent with visual observation of plants especially during the early stage, when plants of *H. argophyllus* and plants with intermediate traits were very similar, whereas only after a month the differences became recognizable. *H. debilis* plants were always significantly different from intermediate ones regarding plant height and beginning of flowering. Clear differences appeared also for qualitative traits. Table 3 shows, for *H. argophyllus*, *H. debilis* ssp. *cucumerifolius* and their hybrids, the number of

plants classified according to hairiness and leaf color, whereas the classification in Table 4 is based on leaf margin. It appears that *H. argophyllus* has mostly hirsute and silver green leaf with entire margin, quite different traits in respect to *H. debilis* ssp. *cucumerifolius*. Hybrid score indices for qualitative and quantitative traits are reported in Figures 2 and 3 for the two dates considered.

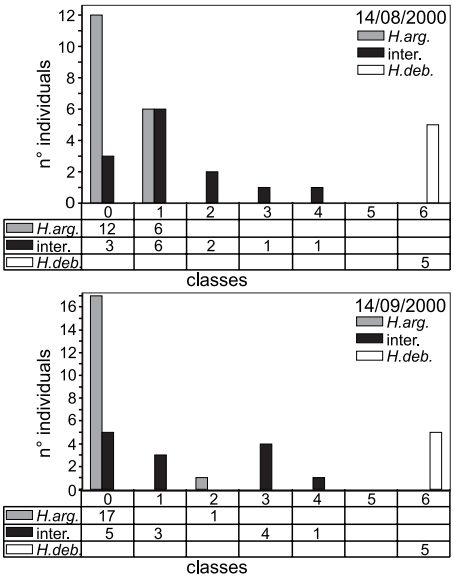

Figure 2: Hybrid indices for qualitative traits (14/08-14/09/2000)

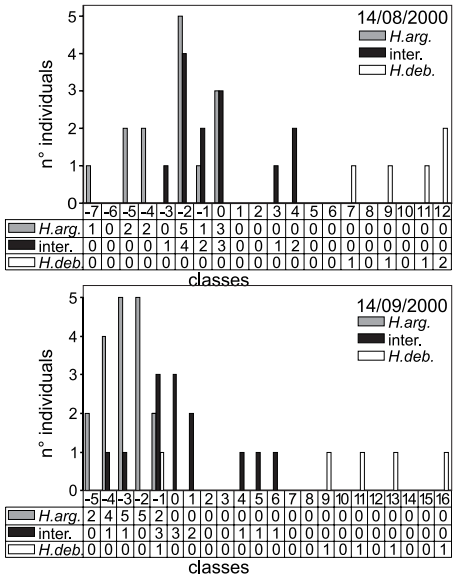

Figure 3: Hybrid indices for quantitative traits (14/08-14/09/2000)

Table 2: Variability of wild material for morphological traits

Statistical significance	Trait	*H.argophyllus*	Hybrids	*H. debilis*
	Leaf width (14/08/00)	28.3 ± 6.6	33.9 ± 7.6	33.8 ± 7.3
	Leaf width (14/09/00)	29.9 ± 4.6	32.2 ± 6.2	31.2 ±11.1
*	Leaf length (14/08/00)	70.6 ±12.1	64.7 ± 18.4	51.8 ± 4.9
*	Leaf length (14/09/00)	78.8 ± 9.6	68.5 ± 11.0	49.2 ± 4.6
*	A (14/08/00)	1.44 ± 0.29	1.79 ± 0.51	5.20 ± 1.98
*	A (14/09/00)	1.56 ± 0.27	2.13 ± 0.48	5.26 ± 2.51
*	B (14/08/00)	2.56 ± 0.40	2.07 ± 0.34	1.59 ±0.34
*	B (14/09/00)	2.66 ± 0.24	2.18 ± 0.38	1.83 ± 0.89
*	A x B (14/08/00)	3.64 ± 0.69	3.57 ± 0.62	8.69 ± 5.38
*	A*B (14/09/00)	4.10 ± 0.68	4.52 ± 0.60	8.22 ± 2.21
*	Plant height (cm) (14/08/00)	27.1 ± 4.9	27.7 ± 3.3	41.0 ± 7.2
	Plant height (cm) (14/09/00)	41.0 ± 6.3	52.0 ± 9.9	57.3 ± 9.6
*	Flowering time from 31/7/00	60.3 ± 2.5	54.7 ± 12.2	14.2 ± 0.5

Table 3: Classification of plants according to hairiness (leaf and stem) and leaf color

Trait	Date	*H.argophyllus*		Pop. intermedia		*H. debilis*	
Hairiness		Hr	Hst	Hr	Hs	Hr	Hs
Leaf	14/08	18	-	12	1	-	5
	14/09	17	1	9	4	-	5
Stem	14/08	18	1	11	2	-	5
	14/09	17	1	9	4	-	5
Color of leaves		Sg	Bg	Sg	Bg	Sg	Bg
	14/08	18	-	11	2	-	5
	14/09	18	-	10	3	-	5

Legend - Hr: hirsute; Hs: hispid; Sg: silver green; Bg: bright green (see Seiler 1997)

Hybrid index scores for qualitative traits were on a scale 0 to 6 and quantitative traits were on a scale -7 to (+)16. *H. argophyllus* and *H. debilis* individuals were positioned in the distribution extremes (0=*H. argophyllus*; 6=*H. debilis*) for qualitative traits as well as for quantitative traits (positive values for *H. debilis*, negative values for *H. argophyllus*), whereas hybrid individuals were positioned in between these opposite populations but skewed towards *H. argophyllus* individuals.

Table 4: Classification of plants according to leaf margin

Material	14/08/00				14/09/00			
	E	U	C	D	E	U	C	D
H. argophyllus	12	6			18			
Pop. intermedia	3	8	2		7	3	2	1
H. debilis				5				5

Legend. E: entire; U: undulate; C: crenate; D: dentate (see Seiler 1997)

AFLP analysis

Thirty-eight *H. argophyllus* plants coming from two sites, 13 *H. debilis* plants, 28 plants with hybrid traits and 12 *H. annuus* individuals were considered in the analysis. Four combinations of AFLP primers were used in the experiment. AFLP fragment size ranged from approximately 35 to 400 bp. Polymorphic fragments were distributed across the entire size range. Repeated amplification with each primer combination gave rise to the same electrophoretic pattern. The total number of scoreable bands and polymorphic fragments for each primer combination are reported in Table 5. The combination E41/M51 produced the best result with 117 fragments and detected the higher level of polymorphism among the species, in accordance with previous studies in sunflower (Quagliaro *et al.*, 2001). Table 5 also reports the numbers of polymorphic species-specific markers identified, which are 30 and 21 for *H. debilis* and *H. argophyllus*, respectively. Some of these species-diagnostic markers were always detected in the hybrid population: 15 *H. argophyllus*-specific and only one *H. debilis*-specific.

Table 5: Total, polymorphic and species-specific bands for each combination of AFLP primers

Primer pair	Band				
	Total	Polymorphic among *H. arg., H. deb* and *H. ann.*	Polymorphic between *H. arg*. and *H. deb.*	*H. debilis* species -specific	*H. argophyllus* species -specific
E41/M51	117	115	105	13	2
E41/M52	84	82	62	10	7
E65/M58	81	77	70	3	7
E65/M60	90	87	72	4	5
Total	372	361	309	30	21

To complement the analysis, based on phenotypic frequencies, the Dice index was used to generate a similarity matrix. The neighbor-joining dendrogram representing relationships between individuals and populations is shown in Figure 4.

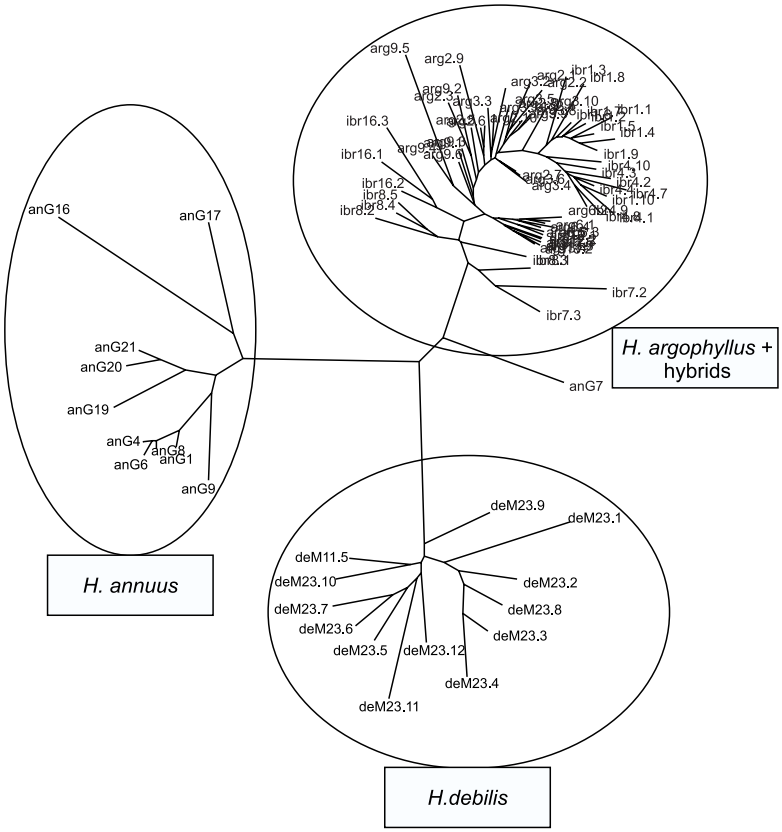

Figure 4: Neighbor-joining phenetic tree of relationships between H. argophyllus, H. debilis, hybrids and H. annuus individuals based on Dice's genetic distance index

The unrooted dendrogram based on shared fragments divided the genotypes into distinct groups resembling the different species. Introgressed material was included in the *H. argophyllus* cluster but in general they formed distinguishable subgroups mainly composed of half-sibs individuals. It is to note that wild material is placed far away from *H. annuus*, pointing out its difference and potential value for breeding programs.

Pollen viability

Pollen viability analysis provided further evidences of the hybrid origin of most of the intermediate trait plants. No significant differences were observed between stainability percentages using the two dyes. It is to observe that a few plants classified as *H. argophyllus* showed a small percentage of non-stainable pollen. Mean viability in *H. argophyllus* and *H. debilis* samples was high (>90%) having 80-100% pollen viability as a range. On the contrary in intermediate trait plants, the mean pollen viability was about 75% with a wider variation ranging from complete (100%) to less than 27% viability, suggesting chromosome abnormalities at the meiotic level.

CONCLUSIONS

Morphological observations, molecular marker and pollen viability analysis strongly support an introgressive process taking place between *H. argophyllus* and *H. debilis* ssp. *cucumerifolius* populations found along the shores of the Inhambane Bay. Plants with intermediate traits were highly variable at the morphological level for plant height, beginning of flowering, hairiness of stem and leaves, flower size, leaf shape and pigmentation, leaf margin, plant structure. These traits bear evidence of repeated back-crossing (introgression) resulting in a broad recombination of morphological traits after parental hybridization.

The study of introgression by mean of hybrid indices and AFLP analysis indicated that *H. argophyllus* and *H. debilis* species were clearly distinguishable at both the morphological and molecular level. AFLP technology, as previously reported for many species, is robust and proficient in estimating genetic diversity between individuals, populations and species (Zhu *et al.*, 1998;, Hill *et al.*, 1996; Pejic *et al.*, 1998;, Travis *et al.*, 1996; Winfield *et al.*, 1998; Quagliaro *et al.*, 2001). Four primer combinations were enough to detect 361 polymorphic bands among the populations. This number of bands was sufficient to unambiguously assign individuals to species since 30 and 21 fragments were species-specific for *H. argophyllus* and *H. debilis*, respectively, whereas more than 50 bands were diagnostic at the 90% level (data not shown). The introgressed material was found to be more similar to *H. argophyllus* at the molecular level than at the morphological level.

Two hypotheses can be formulated:

1. *H. argophyllus* alleles are dominant with respect to the *H. debilis* alleles and AFLPs as dominant markers do not reveal the heterozigote genotypes;
2. AFLP markers are not linked to genes controlling the observed traits.

The wild material appeared quite different at the molecular level in comparison with *H. annuus* individuals suggesting great potentiality for its utilization in breeding programs.

From a taxonomic point of view further analyses are required to determine whether the population with intermediate traits could be assigned to a new species considering the morphology and the level of plant fertility. This material, of great scientific importance for evolutionary and genetic studies, grows in endangered areas for antropic pressure along the coast in Inhambane Town. It should be carefully monitored and preserved by local and international organizations.

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MARCADORES AFLP PARA LA INVESTIGACION DEL INTERCAMBIO DE GENES ENTRE LAS ESPECIES SILVESTRES (*H. argophyllus* y *H. debilis* ssp. *cucumerifolius*) Y INSTRODUCCION DE GELNES EN *H. annuus*

RESUMEN

El girasol es una planta que proviene del continente americano, pero ahora muchas especies silvestres son extendidas en todo el mundo. A lo largo de la costa del golfo Inhambane, Mozammbique, dos especies del girasol, *H. argophyllus* y *H. debilis*, ambas proveniendo de Texas, crecen en los lugares reciprocamente distantes. Entretanto, en dos lugares con el suelo arenoso, estas especies crecen juntas y se encontraron muchas plantas hibridas, con las caracteristicas morfologicas tipicas de ambas especies. Algunas de estas plantas para las cuales se supone de ser hibridas han producido las semillas coleccionadas y sembradas en la Universidad de Udine.

Los datos observados son el resultado de analisis morfologicos I de AFLP relativos a las plantas cultivadas en la primavera de 2000. Para las propiedades morfologicas siendo tipicas para ambas especies hemos formado el indice clasico de hibridos en el cual eran registradas las evaluaciones para ambas especies. La prueba cierta de la existencia de plantas hibridas son las

cintas de AFLP diversamente largas, que son presentes en *H. debilis* y *H. argophyllus*, y se encuentran tambien en el material hibrido. La coloracion parcial del polen confirma que algunas de las plantas hibridas tienen la fertilidad reducida. Del punto de vista de seleccion, estas especies silvestres y el material hibrido tienen un grande valor potencial a causa de su caracter especifico. La disposicion de las cintas AFLP es completamente diferente del material de *H. annuus*, y el analisis de agrupamiento muestra la existencia de diferencias claras entre tres especies investigadas. Es necesario un analisis mas profundo para evaluar hasta cual grado llegan esas difrencias y para constatar si las plantas con las caracteristicas combinadas pueden ser consideradas como una nueva unidad taxonomica.

MARQUEURS AFLP DANS L'ÉTUDE DES ÉCHANGES DE GÈNES ENTRE LES ESPÈCES SAUVAGES (*H. argophyllus* et *H. debilis* ssp. *cucumerifolius)* ET INTRODUCTION DE GÈNES DANS L'*H. annuus*

RÉSUMÉ

Le tournesol est originaire du continent américain, mais plusieurs espèces sauvages sont maintenant répandues dans différentes parties du monde. Deux espèces de tournesol, l'*H. argophyllus* et l'*H. debilis,* toutes les deux d'origine texane poussent, éloignées l'une de l'autre, le long de la côte de la baie d'Inhambane au Mozambique. Cependant, dans deux localités au sol sablonneux, elles poussent ensemble et plusieurs plantes ont été reconnues comme hybrides, partageant les traits typiques des deux espèces. Quelques-unes de ces plantes dont on pense qu'elles sont hybrides ont produits des graines qui ont été recueillies pour être semées à l'Université d'Udine.

Nous présentons des données qui sont le résultat des analyses morphologiques et AFLP de ces plantes cultivées au printemps 2000. Nous avons établi un index classique d'hybrides pour les caractéristiques morphologiques qui sont typiques des deux espèces et y avons ajouté l'évaluation de chaque espèce. Les bandes AFLP de longueurs différentes présentes dans l'*H. debilis* et l'*H. argophyllus* et que l'on retrouve dans le matériel hybride sont une preuve certaine de l'existence de plantes hybrides. La coloration partielle du pollen confirme que certaines des plantes hybrides ont une fertilité réduite. Du point de vue de la sélection, ces espèces sauvages et le matériel hybride ont une grande valeur potentielle à cause de leur spécificité. La distribution des bandes AFLP est tout à fait différente du matériel de l'*H. annuus* et l'analyse de groupement montre l'existence de différences évidentes entre les trois espèces examinées. Une analyse plus profonde est nécessaire pour que puisse être évaluée l'ampleur de ces différences et confirmé si les plantes à caractéristiques combinées peuvent être considérées comme une nouvelle unité taxinomique.