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Original scientific paper

GENETIC DIVERSITY OF WILD SUNFLOWER (*Helianthus* sp.) ACCESSIONS WITH DIFFERENT TOLERANCE TO MID-STALK WHITE ROT

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Random amplified polymorphic DNA (RAPD) markers were used to detect polymorphism among accessions of wild sunflower species *Helianthus maximiliani*, *Helianthus tuberosus, Helianthus mollis* and *Helianthus rigidus* with different tolerance to mid-stalk white rot and selection of potential markers for different levels of tolerance to this disease. Estimates of genetic variation showed that genetic diversity was equally distributed between *Helianthus* species and within them. Cluster analysis corresponded to the phylogenetic relations within the genus *Helianthus*. The results obtained by principal coordinates analysis (PCoA), where the first two principal coordinates accounted for 83.7% of total variation, perfectly coincided with the results of cluster analysis. Contingency coefficient significance test showed that most of the used primers generated bands associated with some level of tolerance or susceptibility to mid-stalk white rot. Furthermore, contingency analysis showed that primer C12 generated bands associated with resistance (100%) to mid-stalk white rot both in *H. mollis* and in all accessions, while primer X18 generated bands significantly associated with high tolerance (75%) in *H. rigidus*, *H. mollis* as well as in all tested accessions. The C15-600 bp locus was found to be significantly associated with high tolerance (75%) in all accessions, and medium tolerance (50%) in *H. mollis*.

Key words: breeding, contingency test, *Helianthus*, multivariate analysis, RAPD markers, *Sclerotinia sclerotiorum*

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INTRODUCTION

White rot caused by the fungus (*Sclerotinia sclerotiorum* (Lib.) de Bary is the major disease of sunflower in countries with the humid climate, while in countries with moderate climate it causes the yield loss in rainy years (KAYA *et al.*, 2012). It is a facultative polyphagous parasite which attacks a variety of wild plants and agricultural crops.

Wild sunflower species have been a valuable source of resistance genes for many of the common pathogens of cultivated sunflower (*Helianthus annuus* L.), including *S. sclerotiorum* (JAN and SEILER, 2007). Tolerance to mid-stalk white rot was observed in annual *H. praecox* and perennials *Helianthus pauciflorus*, *Helianthus giganteus*, *Helianthus maximiliani*, *Helianthus resinosus*, *Helianthus mollis*, *Helianthus rigidus* and *Helianthus tuberosus* (VASIĆ *et al.*, 2002; JAN and SEILER, 2007).

Progress in sunflower breeding for white rot tolerance is limited due to the lack of effective sources of tolerance. Breeding relies on identification and incorporation of partial tolerance from diverse genotypes, mostly wild species. Evaluation of white rot tolerance in the field is difficult since environmental factors play a significant role in disease development. However, molecular markers tightly linked to white rot tolerance genes can be employed to assist the selection for tolerance genes during variety development (YUE *et al.*, 2008).

Random amplified polymorphic DNA (RAPD) markers are dominant markers and extensively used in genetic mapping and identification of markers linked with different traits. Due to technical simplicity and speed, the RAPD method has been used for diversity analyses in several crops (BAI *et al.*, 2003; CHUNG *et al.*, 2008). In sunflower, RAPD markers have been used for evaluation of genetic diversity in wild species and cultivated genotypes (VASIĆ *et al.*, 2003; IQBAL *et al.*, 2010; RIBEIRO *et al.*, 2010), identification of progenies from interspecific crosses (TAŠKI-AJDUKOVIĆ *et al.*, 2006), as well as for genome mapping and for tagging tolerance genes to rust (LAWSON *et al.*, 1998) and broomrape (LU *et al.*, 2000). Despite a few drawbacks, such as their dominant nature and poor reproducibility, RAPD markers are still often used, since they allow an inexpensive and rapid analysis of the polymorphisms in many individuals with good coverage of the entire genome.

The aim of our research was detection of DNA polymorphisms among accessions of wild sunflower species differing in their tolerance to mid-stalk white rot and selection of potential markers for different levels of tolerance to this disease.

MATERIALS AND METHODS

Plant material

Four accessions of each *Helianthus maximiliani* Schrad. and *Helianthus tuberosus* L., and five accessions of each *Helianthus mollis* Lam. and *Helianthus rigidus* (Cass.) Desf., originating from the wild sunflower species collection of Institute of Field and Vegetable Crops, Novi Sad, Serbia, were used in the study (Table 1). The accessions were pre-screened for their tolerance to mid-stalk white rot by incorporating of sclerotias into the stems at the beginning of bud formation (VASIĆ *et al.*, 2002).

DNA isolation

Genomic DNA was isolated from 1 g of frozen leaf tissue taken from individual plants, according to the protocol of SOMMA (2004). Purified DNA was quantified on 1% agarose gel

with λDNA as the reference. After concentration adjustment, DNAs from 10 individual plants were mixed in equal volume to form bulks for each accession.

Table 1. Wild sunflower accessions used in the study and their tolerance to mid-stalk white rot

Accession name[a]	Genebank	Synonym	Country	Tolerance (%)
MAX 34	SRBIFVCNS	-	USA	75
MAX 2007	SRBIFVCNS	Pl 531041	USA	75
MAX 2010	SRBIFVCNS	Pl 531042	USA	75
MAX M	SRBIFVCNS	-	Unknown	0
MOL 285	SRBIFVCNS	-	Russia	50
MOL 1298	SRBIFVCNS	Pl 468759	USA	100
MOL 1530	SRBIFVCNS	Pl 468760	USA	25
MOL 1629	SRBIFVCNS	Pl 468762	USA	25
MOL X	SRBIFVCNS	-	Unknown	100
RIG 1692	SRBIFVCNS	-	Unknown	50
RIG 1696	SRBIFVCNS	-	Unknown	25
RIG 1843	SRBIFVCNS	Pl 494612	USA	25
RIG 1844	SRBIFVCNS	Pl 494613	USA	0
RIG 2012	SRBIFVCNS	Pl 531060	USA	75
TUB 6	SRBIFVCNS	-	Montenegro	0
TUB 7	SRBIFVCNS	-	Montenegro	25
TUB 675	SRBIFVCNS	-	Unknown	50
TUB 1699	SRBIFVCNS	-	Unknown	0

[a]MAX - *Helianthus maximiliani*, MOL - *Helianthus mollis*, RIG - *Helianthus rigidus*, TUB - *Helianthus tuberosus*

RAPD analysis

In order to test amplification profiles for polymorphism, readability and reproducibility, twenty three 10-base primers and six primer combinations were initially tested, from which fourteen individual primers and two primer mixtures were used for further RAPD analysis (Table 2). Polymerase chain reaction (PCR) was carried out in a 25-µl reaction volume containing 2.5 µl of buffer; 0.2 mM of dNTP; 0.5 µM of primer (in reactions with two primers there was 0.25 µM of each primer); 2 units of *Taq* polymerase (Fermentas) and 30 ng of DNA. The PCR reaction was run on Mastercycler Ep Gradient S termocycler (Eppendorf). Amplification conditions included denaturation at 94ºC for 4min, followed by 40 cycles with 94ºC for 2 min, 36ºC for 1 min and 72ºC for 2 min, with final elongation on 72ºC for 10 min. PCR products were separated on 1.7% agarose gels containing 0.005% ethidium bromide and visualized under UV light.

Data analysis

For statistical analysis the binary data matrix was used under the assumption that RAPD fragments behave as diploid dominant markers with alleles being either present (amplified) or absent (not amplified) and that each amplified fragment corresponds to a different RAPD locus. In order to measure informativeness of the markers, the polymorphism information content (PIC)

for each primer was calculated. POPGENE software package version 1.32 (YEH *et al.*, 1997) was used for calculation of the following parameters of genetic variation: total number of loci, number of polymorphic loci and their percentage, effective number of alleles per loci (KIMURA and CROW, 1964), expected heterozygosity (NEI, 1973) based on allelic frequencies and Shannon's index of phenotypic diversity (LEWENTIN, 1972) based on marker frequencies. The genetic diversity was estimated by calculating total gene diversity (Ht), gene diversity within species (Hs), genetic differentiation (Gst) and gene flow (Nm) (NEI, 1987; MCDERMOTT and MCDONALD, 1993). Cluster analysis was done by an unweighted pair-group arithmetic mean method (UPGMA), based on the matrix of Dice's distances (D' = b/(2a+b)). This matrix was also used for principal coordinate analysis (PCoA). The goodness of fit of the tree matrix was determined by cophenetic correlation and was interpreted according to ROHLF (1997). The contingency coefficient was used as a measure of association between RAPD markers and tolerance to the *S. sclerotiorum*. The statistical significance of the association was evaluated by independence test. Statistical analysis was carried out using STATISTICA 10 (StatSoft, 2011).

Table 2. Description of oligonucleotide primers used for RAPD analysis

Primer	Sequence (5'-3')	Max number of bands	Band size range (bp)	PIC[a]
C04	CCGCATCTAC	19	300-1500	0.256
C12	TGCGTGCTTG	9	225-1600	0.327
C15	GACGGATCAG	11	225-2000	0.169
x02	TTCCGCCACC	15	425-1500	0.322
x03	TGGCGCAGTG	11	500-1700	0.212
x05	CCTTTCCCTC	1	400	0.178
x07	GAGCGAGGCT	7	250-1800	0.150
x10	CCCTAGACTG	11	400-1800	0.331
x11	GGAGCCTCAG	14	500-2500	0.343
x12	TCGCCAGCCA	11	450-1500	0.288
x14	ACAGGTGCTG	5	500-1000	0.162
x16	CTCTGTTCGG	11	700-2800	0.313
x17	GACACGGACC	8	400-1100	0.397
x18	GACTAGGTGG	9	800-1700	0.203
Primer mixtures		No. of new bands	Band size (bp)	
x02/x05		1	325	0.318
x07/x14		2	900, 1500	0.168

[a] PIC: Polymorphism information content

RESULTS

Genetic diversity

Out of 29 primers and primer combinations tested, 16 generated stable and reproducible bands, ranging from 225 to 2800 bp (Table 2). A total of 145 bands were generated, with average

number of bands per primer of 9.06 and polymorphism information content (PIC) value ranging from 0.150 to 0.394.

The estimation of genetic variation among and within tested *Helianthus* species based on RAPD markers is presented in Table 3. The number and percentage of polymorphic loci, as well as effective number of alleles, were the highest in *H. mollis* accessions and the lowest in the accessions of *H. maximiliani*. The genetic diversity estimated by expected heterozygosity ranged from 0.101 in *H. maximiliani* to 0.187 in *H. mollis*, with an average of 1.237 within species and 1.458 among *Helianthus* species. In terms of Shannon's diversity index, *H. mollis* showed the highest value (I = 0.279). The mean value of genetic diversity within the species was 0.206 and total genetic diversity across species was 0.418.

The data obtained from RAPD loci analysis were also used for the assessment of distribution of genetic diversity among and within wild *Helianthus* species (Table 3). The total gene diversity (*Ht*) and gene diversity within population (*Hs*) were 0.275 and 0.143 respectively. The coefficient of gene differentiation (*Gst*) among populations was 0.479 indicating that 47.9% genetic diversity occurred between *Helianthus* species and 52.1% occurred within them. The genetic structure predicted by Shannon's diversity index analysis showed that 50.7% of the total variation occurred between *Helianthus* species. The gene flow (*Nm*) among the *Helianthus* species was estimated to be 0.543 individuals per generation (<1) indicating that there is relatively low gene flow among them.

Table 3. Estimated genetic variation in wild Helianthus sp. using RAPD markers

Species	Polymorphic loci (no.)	Polymorphic loci (%)	Effective no. of alleles (Ne)	Expected heterozygosity (He)	Shanon's index (I)
H. maximilliani	36	24.83	1.174 ±0.316	0.101 ± 0.178	0.148 ± 0.259
H. mollis	72	49.66	1.315 ±0.353	0.187 ± 0.197	0.279 ± 0.288
H. rigidus	58	40.00	1.260 ±0.349	0.153 ± 0.195	0.227 ± 0.285
H. tuberosus	42	28.97	1.201 ± 0.330	0.117 ± 0.186	0.172 ± 0.272
Mean	52	35.86	1.237 ± 0.337	0.140 ± 0.189	0.206 ± 0.276
Overall	123	84.83	1.458 ± 0.341	0.275 ± 0.172	0.418 ± 0.234

Inter-species and inter-accession genetic relationships

The phenogram generated by UPGMA analysis, based on the matrix of Dice's distances is shown in Figure 1a. The cophenetic correlation value (*r)*, used as a measure of goodness-of-fit for cluster analysis, was 0.8816, indicating a good representation of the original data. Three major clusters were identified from the phenogram at 0.30 distance. *H. maximiliani* and *H. mollis* are well distinguished, and form separate clusters, while *H. rigidus* and *H. tuberosus* are relatively similar and are grouped within the same cluster. The accessions of the same species are grouped in two sub-clusters within this cluster, except the accession RIG 1844 that is in the sub-cluster with *H. tuberosus* accessions.

Principal coordinates analysis (PCoA) was done in order to get a closer look into the relationships among examined accessions and species (Figure 1b). The first two principal coordinates accounted for 83.7% of total variation (51.7 and 32.0%, respectively). These two

coordinates indicated three distinct groups, one formed of *H. maximiliani* accessions, the other of *H. mollis* accessions, and the third that included both *H. rigidus* and *H. tuberosus* accessions, that appeared to be very similar with considerable overlap of their respective clouds. This perfectly coincides with the results of cluster analysis (Figure 1a). *H. rigidus* and *H. tuberosus* accessions are additionally discriminated by the third principal coordinate (Figure 1b).

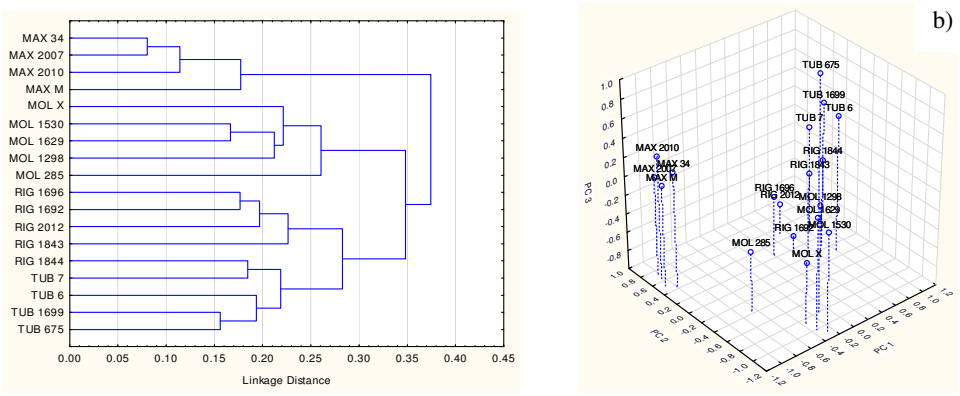

Figure 1. Relationships among different accessions of tested Helianthus species based on RAPD markers according to: a) Dendrogram obtained using UPGMA algorithm and Dice's distances, b) PCoA

Loci and tolerance association

Contingency coefficient test showed that most of the used primers generated bands associated with particular tolerance level to mid-stalk white rot, with exception of primers X05, X17 and both primer mixtures (Table 4). Primers C04, X10, X12 and X16 generated bands associated either with tolerance or susceptibility, while locus X14-1000 bp was associated with susceptibility (0%). All other primers generated bands significantly associated with either total resistance (100%) or high tolerance (75%).

Contingency coefficients were also calculated for each species. Review of the loci significantly associated with tolerance or susceptibility to mid-stalk white rot within a certain species is given in Table 5. The primers that generated bands associated with tolerance in all accessions, also generated bands associated with tolerance or susceptibility within the species. Exceptions were primer x17 and primer mixture x07/x14, that generated bands significantly associated with susceptibility (0%) in *H. maximiliani* and *H. rigidus*, and high tolerance (75%) to mid-stalk rot in *H. maximiliani*, respectively. Loci C12-500 bp and C12-1400 bp were associated with resistance (100%) to mid-stalk white rot both in *H. mollis* and in all accessions. Locus X18-1650 was significantly associated with high tolerance (75%) in *H. rigidus*, while loci X18-1300 bp and X18-1700 bp were associated with high tolerance in all tested accessions. Locus C15-600

bp was found to be significantly associated with both, high tolerance (75%) in all accessions, and medium tolerance (50%) in *H. mollis*.

Table 4. Association of loci with the level of mid-stalk white rot tolerance in all tested accessions expressed by contingency coefficients

	Tolerance (%)						
Loci	100	75	50	25	0	Total	Species
C04-300bp[a]	0.271	0.557 *	0.051	0.234	0.151	0.57	0.647 *
C04-400bp	0.186	0.562 *	0.232	0.315	0.036	0.584	0.707 *
C04-550bp	0.552 *	0.275	0.232	0.256	0.275	0.617 *	0.653 *
C04-820bp	0.156	0.119	0.514 *	0.267	0.232	0.55	0.408
C04-1500bp	0.23	0.275	0.232	0.315	0.562 *	0.612 *	0.376
C12-700bp	0.447 *	0.094	-	0.087	0.354	0.507	0.669 *
C15-600bp	0.243	0.603 *	-	0.402	0.094	0.626 *	0.612 *
x02-1200bp	0.495 *	0.315	0.055	0.167	0.315	0.571	0.707 *
x03-850bp	0.124	0.427 *	0.302	0.173	0.094	0.48	0.642 *
x03-1300bp	0.214	0.491 *	0.055	0.359	0.033	0.535	0.662 *
x07-250bp	0.214	0.491 *	0.055	0.359	0.033	0.535	0.661 *
x07-700bp	0.552 *	0.275	0.232	0.256	0.275	0.617 *	0.653 *
x10-680bp	0.243	0.186	0.302	0.173	0.427 *	0.516	0.456
x10-700bp	0.173	0.491 *	0.267	0.359	0.033	0.555	0.662 *
x10-1000bp	0.447 *	0.094	-	0.087	0.354	0.507	0.669 *
x11-800bp	0.039	0.431 *	0.196	0.191	0.06	0.442	0.475 *
x12-450bp	0.186	0.562 *	0.232	0.315	0.036	0.584	0.707 *
x12-500bp	0.156	0.642 *	0.196	0.267	0.232	0.642 *	0.642 *
x12-700bp	0.186	0.275	0.431 *	0.033	0.036	0.473	0.376
x12-720bp	0.186	0.562 *	0.232	0.315	0.036	0.584	0.707 *
x14-1000bp	0.214	0.315	0.055	0.107	0.491 *	0.528	0.662 *
x16-900bp	0.214	0.491 *	0.055	0.359	0.033	0.535	0.665 *
x16-2200bp	0.243	0.094	-	0.173	0.427 *	0.453	0.582 *
x18-1300bp	0.271	0.557 *	0.247	0.234	0.392	0.63 *	0.513
x18-1700bp	0.214	0.491 *	0.055	0.359	0.033	0.535	0.662 *
Species	0.495	0.578 *	0.238	0.337	0.391	0.7	-

[a] Only loci that showed significant association with any level of tolerance or susceptibility are presented in the table

* Statistically significant at α=0.05

Table 5. Loci significantly associated with particular level of mid-stalk white rot tolerance based on contingency coefficient test, in all tested accessions (total) and within the species

	Tolerance (%)				
Primer	100	75	50	25	0
C04	Total	MAX (600) Total (300,400)	MOL (700,820) Total (820)	TUB (600)	Total (1500)
C12	MOL Total			RIG (1400)	
C15		RIG (600) Total (600)	MOL (600)	TUB (800)	
x02	Total	MAX (900)		TUB (850)	MAX
x03		Total (850,1300)	MOL (1300)		RIG (650) MAX (1200)
x07	Total	Total (250)	MOL (250)		MAX (300)
x10	Total	Total (700)	MOL (450,1700)	TUB (1800)	RIG (450) TUB (680) Total (680)
x11		Total (800)		MOL (500,1200)	RIG (680)
x12		MAX (500) Total (450,500,720)	MOL (700,800) Total (700)		RIG (700) MAX (1000)
x14	Total				RIG (700,1000) MAX (500)
x16		RIG (700,850) Total (900)	MOL (1100) TUB (1900)	TUB (900)	TUB (750) Total (2200)
x17					RIG (900,1100) MAX (1000)
x18		RIG (1650) MAX (1300) Total (1300,1700)	RIG (1700)		MAX (1200)
x07/x14		MAX (900)			

MAX - *Helianthus maximiliani*, MOL - *Helianthus mollis*, RIG - *Helianthus rigidus*, TUB - *Helianthus tuberosus*

DISCUSSION

Estimation of gene variation showed that selected accessions of *H. mollis* and *H. rigidus* had the highest genetic variability. The lowest variation was observed in *H. maximiliani* accessions, which disagrees with the results of VASIĆ *et al.* (2003) who observed high variability within this species using RAPD analysis. *H. mollis* accessions had the highest genetic diversity, as calculated both by Nei's gene diversity and Shannon's diversity index. Similar variability within this species was observed by HU *et al.* (2003) with the use of TRAP markers. The overall gene flow was 0.543, indicating that gene permutation and interaction among accessions was relatively low, which is expected, as the accessions were grown in the controlled environment within the collection. Low gene flow among wild sunflower accessions was even observed in the natural conditions (RIBEIRO *et al.*, 2010).

Cluster analysis corresponded to the phylogenetic relations within the genus *Helianthus*. This is in accordance with work of several authors who used RAPD markers to determine phylogenetic relations among wild sunflower species (VASIĆ *et al.*, 2003; RIBEIRO *et al.*, 2010). As in work of VASIĆ *et al.* (2003) *H. maximiliani* and *H. mollis* were well distinguished. *H. rigidus* and *H. tuberosus* accessions formed one cluster with one *H. rigidus* accession being in the same cluster with *H. tuberosus* accessions. This may be due to wrong classification or hybridization of these two species which is common in natural conditions (ROGERS *et al.*, 1982).

Both cluster analysis and PCoA analysis failed to group examined accessions according to their tolerance to mid-stalk white rot, so in order to find association of certain bands with tolerance contingency coefficients were used. As in work of HASSAN *et al.* (2011) and IMEROVSKI *et al.* (2013), who used contingency coefficients for identifying genetic markers for *Orobanche* resistance in sunflower, the coefficients enabled us to determine that two amplification products of both primer C12 and primer X18, as well as locus C15-600 bp were significantly associated with either high or medium tolerance to mid-stalk white rot in all tested accessions. Furthermore, contingency coefficients with the independence test provided us information on association of tolerance and primers in each tested species, which is in accordance with the results of MULLER *et al.* (2009), who used this test for association of traits among and within sunflower populations. Bands produced by primer C15 were also found to be associated to increased oxalic acid tolerance in product of somatic fusion between cultivated sunflower and *H. maximiliani* (VASIĆ, 2003), as well as mid-stalk white rot tolerance in *H. mollis* (MILADINOVIĆ *et al.*, 2011).

Up to our knowledge, no similar work, i.e. search for molecular markers for *S. sclerotiorum* resistance in wild sunflower species have been done. RAPD markers provided adequate level of information for determination of genetic variability among and within wild sunflower species regarding mid-stalk white rot tolerance, showing that this simple and quick molecular technique could be used for this purpose. Contingency analysis indicated that some loci were significantly associated with resistance and high tolerance in all accessions as well as in specific wild *Helianthus* species, and that one locus (C15-600 bp) was significantly associated with high tolerance in *H. rigidus* and all accessions, and medium tolerance in *H. mollis*. They would be tested further as potential SCAR markers for early identification of wild sunflower plants and accessions tolerant to mid-stalk white rot, with the goal to use them for facilitation of crosses and backcrosses during introduction of tolerance to this disease from wild to cultivated sunflower.

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GENETIČKA VARIJABILNOST POPULACIJA DIVLJEG SUNCOKRETA (*Helianthus* sp.) RAZLIČITO TOLERANTNIH NA BELU TRULEŽ STABLA

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Izvod

U radu su korišćeni RAPD markeri za detekciju polimorfizma između populacija *H. maximiliani*, *H. tuberosus, H. mollis* i *H. rigidus* različito tolerantnih na belu trulež stabla i odabir potencijalnih markera za različite nivoe tolerantnosti prema ovom oboljenju. Od 29 testiranih prajmera i kombinacija prajmera, 16 je dalo stabilne i ponovljive trake. Procena genetičke varijacije je pokazala da je genetička divergentnost podjednako raspodeljena između testiranih vrsta i u okviru njih. Dendrogram dobijen klaster analizom je odgovarao filogenetskim vezama u okviru roda *Helianthus*. Rezultati dobijeni PCoA analizom, gde su prve dve glavne koordinate objašnjavale 83,7% ukupne varijacije, su se podudarali sa rezultatima klaster analize. Test značajnosti koeficijenata kontigencije je pokazao da je većina korišćenih prajmera dala trake povezane sa nekim od nivoa tolerantnosti ili osetljivosti na belu trulež stabla. Analiza kontigencije je pokazala da je prajmer C12 dao trake povezane sa otpornošću (100%) i kod *H. mollis* i kod svih testiranih populacija, dok je prajmer X18 dao trake značajno povezane sa visokom tolerantnošću (75%) na belu trulež stabla kod *H. rigidus*, *H. mollis*, kao i kod svoh testiranih populacija. Utvrđeno je da je lokus C15-600 bp značajno povezan sa visokom tolerantnošću (75%) kod svih ispitivanih populacija i srednjom tolerantnošću (50%) na belu trulež stabla kod *H. mollis*.

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