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Genetic diversity and relationships in wild species of *Brassica* and allied genera as revealed by cross-transferable genomic STMS marker assays

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Abstract

One hundred and sixty one genomic STMS markers were tested from four different *Brassica* species (*Brassica napus* L., *Brassica nigra* (L.) Koch, *Brassica rapa* L. and *Brassica oleracea* L.) for cross-transferability to eleven different species of *Brassica* and allied genera. In total, 70 (43.5%) STMS markers showed cross-transferability to at least one of the eleven species which were considered for the study and it revealed a significant effect of taxonomic group on the proportion of markers amplified. Out of the 70 cross-transferable STMS markers, 32 (45.7%) markers were polymorphic and distinguished the studied taxa. The 'B' genome derived STMS markers showed lower level of cross-transferability as compared to those derived from 'A' and 'C' genomes of *Brassica* species. The cross-transferable STMS markers were applied to characterize the genetic variability and to evaluate the genetic relationships among the species using un-weighted pair group method average (UPGMA) analysis. The results indicated a high level of congruence with earlier reports and are in agreement with their recognized taxonomy.

Keywords: Cross-transferability, genetic relationships, genetic variability, sequence tagged microsatellite sites.
Abbreviations: ITS - Internal transcribed spacer; SAHN - Sequential agglomerative hierarchical and nested; STMS - Sequence tagged microsatellite sites; UPGMA - Un-weighted pair group method average.

Introduction

The family *Brassicaceae* includes 338 genera and 3709 species (Warwick et al., 2006) comprising of crops, weeds and ornamental plants (Love et al., 2005). Amongst crops, the genus *Brassica* is of key importance and it includes major sources of edible oil, vegetables and condiments. *Brassica napus* L. and *Brassica rapa* L. are categorized as rapeseed and these are cultivated mainly for the production of canola oil. *Brassica juncea* (L.) Czern & Coss., *B. carinata* Braun and *Brassica nigra* (L.) Koch. are placed in the mustard group and these play a major role in the production of mustard oil and commercial spice (Labana and Gupta, 1993). *Brassica oleracea* L., probably the most diverse of all crop species, is known for its use as vegetable and fodder crops, under the common names of cabbage, cauliflower, broccoli, Brussels-sprout, etc (Balkaya et al., 2005). Some of the other economically important genera of the family *Brassicaceae* are *Eruca*, *Sinapis*, *Lepidium*, *Camelina* etc. These are also cultivated as oilseed and fodder crops, particularly on marginal agricultural locations with poor soils or sub-optimal climatic conditions (Duhoon and Koppar, 1998). Modern plant breeding and the intensive selection over an extended period have drastically reduced the genetic diversity in these crops, which are showing consequences both on the vulnerability of crops to pests and on their ability to respond to changes in climate or agricultural practice (Cowling, 2007; Ananga et al., 2008). Besides, it has also jeopardized the crop improvement programmes. However, reduction in genetic diversity caused by the intensive selection can be counterbalanced by introgression of novel germplasm (Miflin, 2000). Fortunately, *Brassica* coenospecies has been bestowed with nearly 100 species and genera of wild and weedy relatives which has largely been unexplored and can be used as valuable gene sources for improvement of crop Brassicas (Kirti et al., 2003). Therefore, attempts have become extremely essential to analyze possible untapped genetic diversity in wild species of *Brassica* and allied genera and develop tools to introduce them into cultivated crops for breeding and crop improvement. The analysis of DNA sequence variation is of major importance in genetic studies. In this context, molecular markers are useful tools, and these have greatly enhanced the genetic analysis of crop plants in recent years (Varshney et al., 2005). Among the variety of molecular marker techniques available, STMS have been the markers of choice in modern plant breeding. These are useful for a variety of applications in plant genetics and breeding because of their reproducibility, multi-allelic nature, co-dominant inheritance, relative abundance and good genome coverage (Gupta and Varshney, 2000). Unfortunately, development of STMS markers would be impractical for wild species of *Brassica* and allied genera, mainly due to the lack of genomic information and involvement of high cost. However, if markers developed in related species can be used; genetic

analysis of these species could be rapidly advanced (Yadava et al., 2009). In the present study, we have tested the cross-transferability of 161 genomic STMS markers derived from four different species of *Brassica* (*B. napus*, *B. nigra*, *B. rapa* and *B. oleracea*) to *Brassica tournefortii* Gouan., *Crambe abyssinica* Hochst. ex R. E. Fries, *Brassica fruticulosa* Cyr. subsp. *fruticulosa*, *Brassica spinescens* Pomel, *Diplotaxis assurgens* (Del.) Gren., *Diplotaxis siettiana* Maire, *Diplotaxis tenuisiliqua* Del. subsp. *tenuisiliqua*, *Erucastrum canariense* Webb and Berth., *Capsella bursa-pastoris* L., *Camelina sativa* (L.) Crantz. and *Lepidium sativum* L., Medik, representing eleven species from seven different genera of the family *Brassicaceae*. The cross-transferable STMS markers were used to evaluate the genetic diversity and establish inter-specific as well as inter-generic genetic relationships among the studied taxa. To our knowledge, this is the first comprehensive evaluation of cross-transferability of genomic STMS markers to the wild species of *Brassica* and allied genera.

Results and discussion

Cross-transferability of STMS markers

In the present study, a total of 161 STMS markers derived from *B. napus*, *B. nigra*, *B. rapa* and *B. oleracea* were used to search orthologous match in other genera/species of family *Brassicaceae*. Among the 161 STMS markers, 70 (43.5%) showed cross-transferability to at least one of the eleven species considered for the study (Table 1). However, only 32 (45.7%) of the cross-transferable markers showed polymorphism and distinguished the studied taxa. Polymorphism revealed by Na12-E02 (*B. napus* derived) and Ol10-A09 (*B. oleracea* derived) STMS markers is shown in Fig 1. The monomorphic nature of the STMS markers in related genera/species reveals low genetic diversity and high level of conservation of these loci and their flanking regions. The possibility of high degree of conservation across the genera and potentially among species of the same genera has already been reported (Kresovich et al., 1995; Dayanandan et al., 1997). Lower level of polymorphism at these loci may also be possible due to 'ascertainment bias' whereby a microsatellite chosen to be maximally long in the source species is then likely to be shorter in the new target species (Ellegren et al., 1995). It is thought that ascertainment operates in part via a restriction in microsatellite length, such that occasional deletions or internal point mutation lead to shorter and less polymorphic loci upon cross-species transfer (Vowles and Amos, 2006). Among the 70 cross-transferable STMS markers which were obtained in the study, (42) 60.0% amplified in *B. tournefortii*, (34) 48.6% in *C. abyssinica*, (47) 64.1% in *B. fruticulosa*, (48) 68.6% in *B. spinescens*, (37) 52.9% in *D. assurgens*, (38) 54.3% in *D. siettiana*, (34) 48.6% in *D. tenuisiliqua*, (40) 57.1% in *E. canariense*, (18) 25.7% in *C. bursa-pastoris*, (17) 24.3% in *C. sativa* and (13) 18.6% in *L. sativum*. The extent of cross-transferability of STMS markers derived from *B. napus*, *B. nigra*, *B. rapa* and *B. oleracea* to different species of *Brassica* and allied genera is indicated in Fig 2. The study indicates medium to high level of cross-transferability of genomic STMS markers among *Brassica* and allied genera. Maximum (72.7%) cross-transferability was exhibited by *B. napus* having genomic composition (AACC), followed by 52.6% by *B. rapa* (AA) and 41.1% by *B. oleracea* (CC) derived STMS markers. *B. nigra* (BB) derived genomic

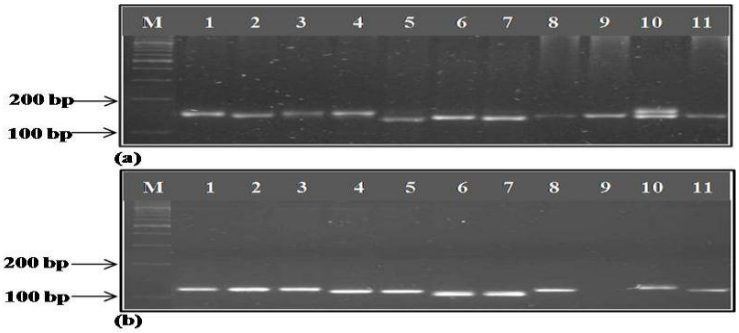

Fig 1. Cross-transferability and polymorphism of STMS markers. (**a)** *B. napus* derived marker Na12-E02 (**b)** *B. oleracea* derived marker Ol10-A09. Lane M - 100 bp DNA ladder, Lane 1- *B. tournefortii*, Lane 2- *C. abyssinica*, Lane 3- *B. fruticulosa*, Lane 4- *B. spinescens*, Lane 5- *D. assurgens*, Lane 6- *D. siettiana*, Lane 7- *D. tenuisiliqua*, Lane 8- *E. canariense*, Lane 9- *C. bursa-pastoris*, Lane 10- *C. sativa*, Lane 11- *L. sativum.*

STMS markers showed minimum (36.1%) cross-transferability. These results substantiate the earlier report that 'B' genome always altered less than the 'A' or 'C' genomes, and was relatively conserved in the evolution of *Brassica* polyploids (Liu and Wang, 2006). A high degree of conservation of 'B' genome was presumably due to selection pressure in view of it containing the important disease resistance genes (Plieske et al., 1998; Dixelius and Wahlberg, 1999). It is also reported that 'B' genome diploid species might have existed as an isolated relative during inter-specific breeding involving the three diploid ancesteral 'A', 'B' and 'C' genome *Brassica* species for creation of allotetraploids (Lowe et al., 2004). Moreover, low cross-transferability of STMS markers could also be indicative of the rapid evolution of the genomic sequences harboring the microsatellite loci.

Molecular diversity and genetic relationships

The dendrogram obtained by the UPGMA analysis of 70 cross-transferable STMS markers clearly distinguished the studied taxa and it resulted in a definitive grouping among genera and species, corresponding well with their recognized taxonomy (Fig 3). The estimated similarity coefficients, using the Jaccard index amongst the eleven species which were considered for the study, ranged between 0.133 (*L. sativum*/*D. tenuisiliqua*) and 0.785 (*D. tenuisiliqua*/*D. assurgens*) (Table 2). As indicated in Fig 3, the major Group I was represented by the tribe *Brassiceae* while Group II contained the members of two closely related tribes *Lepidieae* and *Camelineae.* Sub-group IA was constituted by *Brassica* species including *B. fruticulosa*, *B. spinescens* and *B. tournefortii*. *B. fruticulosa* and *B. spinescens* showed highest similarity and clustered at a similarity level of 0.767; and *B. tournefortii* joined the cluster at 0.465. These three *Brassica* species belong to the Nigra lineage (Warwick and Black, 1993) and in the recent taxonomical classification *B. fruticulosa* and *B. spinescens* have been placed in the section *Micropodium* while *B. tournefortii* is included in section *Sinapistrum* (Go´mez-Campo, 1999). A high level of genetic similarity between *B. fruticulosa* and *B. spinescens* support the

Table 1. List of cross-transferable STMS markers of *B. napus*, *B. nigra*, *B. rapa* and *B. oleracea.*

SI. No.	Locus code	*B. tournefortii*	*C. abyssinica*	*B. fruticulosa*	*B. spinescens*	*D. assurgens*	*D. siettiana*	*D. tenuisiliqua*	*E. canariense*	*C. bursa-pastoris*	*C. sativa*	*L. sativum*	SI. No.	Locus code	*B. tournefortii*	*C. abyssinica*	*B. fruticulosa*	*B. spinescens*	*D. assurgens*	*D. siettiana*	*D. tenuisiliqua*	*E. canariense*	*C. bursa-pastoris*	*C. sativa*	*L. sativum*
1	Na10-A08	+	+	+	+	+	+	+	+		+		36	Ol10-B02			+	+							
2	Na10-A09		+	+	+	+			+				37	Ol10-B08	+		+	+	+	+	+				
3	Na12-A02	+	+	+	+	+	+	+	+	+	+		38	Ol10-C01			+	+		+		+			
4	Na12-A07	+		+	+					+		+	39	Ol10-C10	+	+	+	+		+		+	+	+	+
5	Na12-E02	+	+	+	+	+	+	+	+	+	+	+	40	Ol10-D02	+	+	+	+	+	+	+	+	+	+	+
6	Na12-E05	+	+	+	+	+	+		+	+	+	+	41	Ol10-D03		+	+	+	+	+	+	+	+	+	+
7	Na14-F11	+	+	+	+	+	+	+	+				42	Ol10-D10	+	+	+	+	+	+	+	+	+	+	+
8	Na14-G06	+	+	+	+	+	+	+	+	+	+	+	43	Ol10-D11	+	+	+	+				+			
9	Ni2-A08								+				44	Ol10-E05								+	+	+	
10	Ni2-A12	+				+	+	+	+				45	Ol10-E06			+	+							
11	Ni2-B02	+		+	+								46	Ol10-E12		+									
12	Ni2-B07								+			+	47	Ol10-F06				+							
13	Ni2-E05	+	+	+	+	+	+	+	+	+	+		48	Ol10-F07		+	+	+	+	+	+			+	
14	Ni4-A03	+										+	49	Ol10-F09		+	+	+	+	+	+	+	+		
15	Ni4-A10		+			+	+	+	+				50	Ol10-F11	+		+	+							
16	Ni4-B06	+		+	+	+	+	+	+				51	Ol10-F12			+	+	+	+	+	+			
17	Ni4-E03	+	+	+	+	+	+						52	Ol10-G05	+	+	+	+							
18	Ni4-F06			+	+		+	+	+				53	Ol10-H02		+			+	+	+				
19	Ni4-G08	+	+	+	+	+	+	+	+	+	+		54	Ol10-H04	+	+	+	+							
20	Ni4-H03		+			+	+	+	+	+			55	Ol11-B07		+									
21	Ni4-B10	+		+	+	+	+	+	+			+	56	Ol11-C02	+	+	+	+				+			
22	Ra2-A01	+		+	+		+		+				57	Ol11-E03	+				+	+	+	+			
23	Ra2-B07	+							+				58	Ol11-F12		+	+	+	+	+	+				
24	Ra2-E03	+							+				59	Ol11-G11		+		+				+			
25	Ra2-E04	+		+	+	+	+	+	+	+	+		60	Ol12-A01									+		
26	Ra2-E11	+	+	+	+	+	+	+					61	Ol12-A04	+										
27	BRMS-037	+		+	+	+	+	+		+	+		62	Ol12-B03					+	+	+				
28	BRMS-040					+	+	+					63	Ol12-D03			+	+	+	+	+	+			
29	BRMS-042					+	+	+	+				64	Ol12-D09	+		+	+	+	+	+				
30	BRMS-042-2			+	+				+				65	Ol12-E03		+									
31	BRMS-050	+		+	+								66	Ol12-F03	+		+	+	+	+	+				
32	Ol09-A02	+		+	+						+	+	67	Ol13-A10		+									
33	Ol09-A06	+		+	+	+			+				68	Ol13-C10	+	+	+	+							
34	Ol10-A09	+	+	+	+	+	+	+	+		+	+	69	Ol13-C12	+										
35	Ol10-A11	+	+	+	+	+			+	+			70	Ol13-G05	+	+	+				+	+			

1-8: *B. napus* derived; **9-21**: *B. nigra* derived; **22-31**: *B. rapa* derived; **32-70**: *B. oleracea* derived; '+' indicates successful amplification of the STMS marker loci in respective taxa.

earlier findings which considered that these two species are originally identical but later differentiated ecologically and morphologically into different species. *B. fruticulosa* and *B. spinescens* have been found to be considerably similar at the cytological level also; and hence they have been placed in a single 'cytodeme' (Harberd, 1976). Besides, distinct similarity is also reported in their chloroplast genomes (Warwick and Black, 1993), seed proteins (Sa´nchez-Ye´lamo, 1992) and flavonoids (Sa´nchez-Ye´lamo 2002, 2004). The detection of significant similarity among *B. fruticulosa*, *B. spinescens* and *B. tournefortii* substantiate the findings of Warwick and Black (1991) and Pradhan et al. (1992) who suggested that *B. tournefortii* evolved from *B. fruticulosa* - like ancestor. Sub-group IB was shared by five species belonging to three different genera. Amongst them, maximum similarity was detected between *D. assurgens* and *D. tenuisiliqua* which clustered together at a similarity level 0.785; and *D. siettiana* was observed to be the most distant among these three taxa, joining the cluster at 0.646. These species of *Diplotaxis* belong to Nigra lineage (Warwick et al., 1992). *D. siettiana* has been placed in section *Heterocarpum* and is morphologically quite distinct from *D. assurgens* and *D. tenuisiliqua* which constitute a morphologically related group included in section *Rynchocarpum* (Go´mez-Campo, 1999). Moreover, *D. assurgens* and *D. tenuisiliqua* find positions in the same cytodeme (Takahata and Hinata, 1986) and share a unique morphological trait: a purple spot on the top of the anther. The results of the present study indicate significant genetic closeness between *D. assurgens*, *D. tenuisiliqua* and *E. canariense*, similar to the morphological based studies of Takhata and Hinata (1986). They have already indicated that, although belonging to different genera, they are as close as if they are within the same genus. It is also reported that the degree of similarity in the vegetative and floral characters of these two genera is so high that it often leads to taxonomic confusion (Go´mez-Campo, 1984). In our study, *C. abyssinica*, which is considered to be the most distinct within the tribe *Brassiceae* (Go´mez-Campo, 1980), exhibited significant genomic similarity to the *Diplotaxis - Erucastrum - Brassica* complex and joined the *Diplotaxis - Erucastrum* cluster at a similarity level 0.409. It indicates that, despite so distinctive, *C. abyssinica* still share a significant part of its genome with these taxa. The Group II contained *L. sativum*, *C. bursa-pastoris* and *C. sativa.* The taxa *L. sativum* belonging to the tribe *Lepidieae* was distinctly separated from *C. bursa-pastoris* and *C. sativa* of tribe *Camelineae.* Both of these tribes have been placed in the "core group" of lineage I by German et al. (2009), based on their studies using nuclear ribosomal ITS region, and share a considerable level of morphological and molecular similarity (Koch and Al-Shehbaz, 2009). In summary, the present study detected 8.1 - 29.8% cross-species/genera transferability of genomic STMS markers, developed earlier in different *Brassica* species. However, only 19.9% markers were polymorphic among the studied taxa. The extent of cross-transferability of markers corresponded to the recognized taxonomic relationships which revealed a significant effect of taxonomic group on the proportion of markers amplification and polymorphism. The cross-transferable polymorphic STMS markers identified in the study have significant bearing on the genetic analysis of wild species of *Brassica* and allied genera. However, due to low level of transferability and polymorphism detected in some of the species, it is imperative to employ a

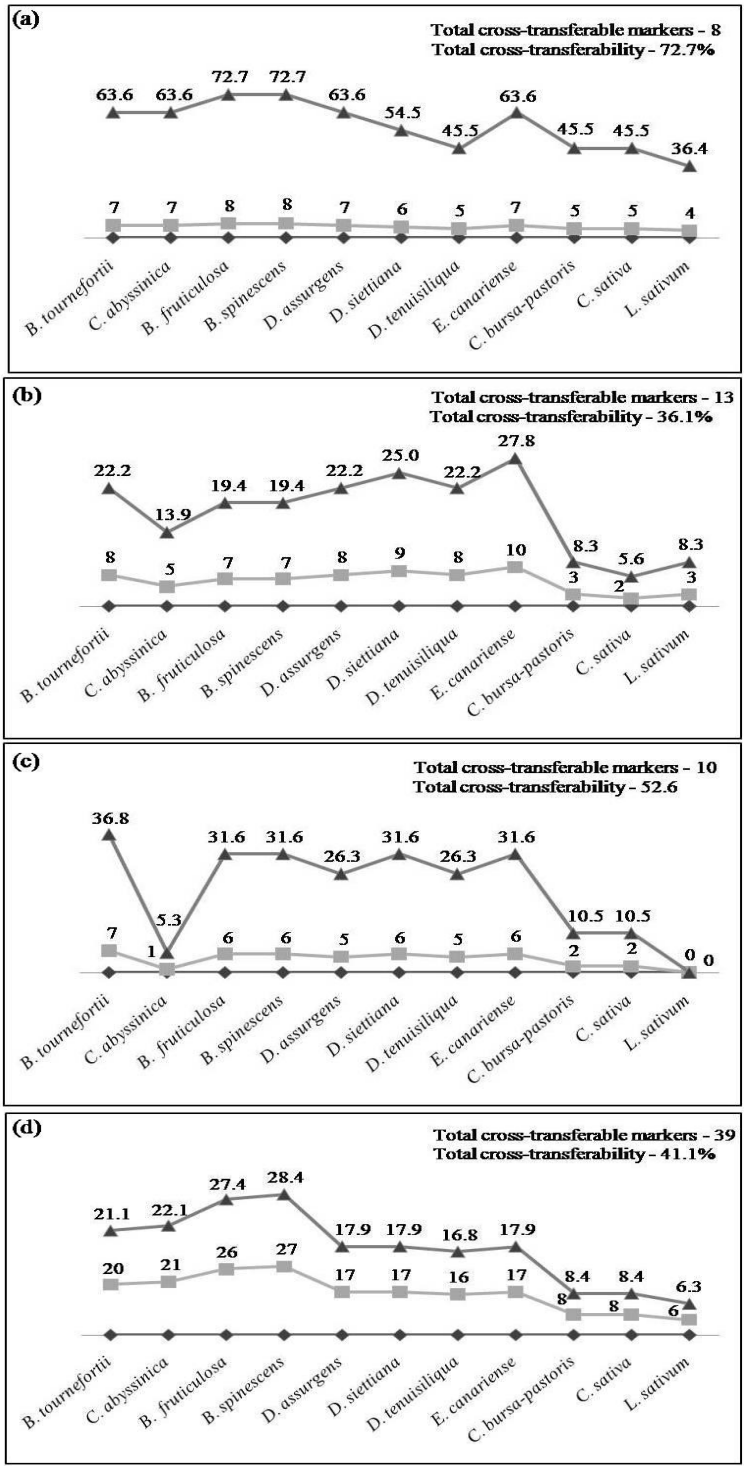

Fig 2. Extent of cross-transferability of STMS markers derived from **(a)** *B. napus*, **(b)** *B. nigra*, **(c)** *B. rapa*, **(d)** *B. oleracea*. Numbers depicted on the dark grey line and the light grey line on the y-axis indicate percent cross-transferability and the number of cross-transferable markers, respectively, to the corresponding taxa indicated on the x-axis.

Table 2. Cross-transferable genomic STMS markers based Jaccard similarity matrix indicating pairwise similarities between every possible pairs of the studied taxa.

Species	1	2	3	4	5	6	7	8	9	10	11
(1) *B. tournefortii*	1.000										
(2) *C. abyssinica*	0.341	1.000									
(3) *B. fruticulosa*	0.487	0.333	1.000								
(4) *B. spinescens*	0.447	0.329	0.767	1.000							
(5) *D. assurgens*	0.382	0.457	0.356	0.400	1.000						
(6) *D. siettiana*	0.373	0.424	0.333	0.344	0.692	1.000					
(7) *D. tenuisiliqua*	0.300	0.382	0.309	0.305	**0.785**	0.593	1.000				
(8) *E. canariense*	0.426	0.371	0.351	0.383	0.547	0.437	0.438	1.000			
(9) *C. bursa-pastoris*	0.246	0.310	0.243	0.256	0.322	0.312	0.233	0.318	1.000		
(10) *C. sativa*	0.276	0.303	0.219	0.216	0.295	0.327	0.224	0.312	0.656	1.000	
(11) *L. sativum*	0.159	0.206	0.162	0.175	0.225	0.218	**0.133**	0.208	0.428	0.516	1.000

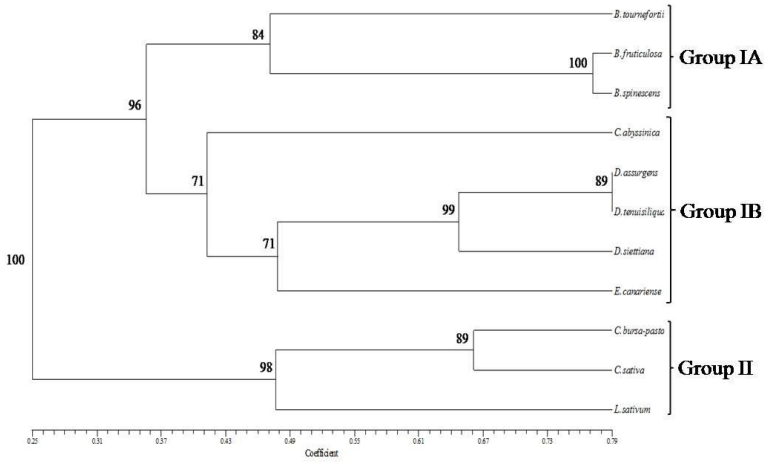

Fig 3. Dendrogram showing genetic relationships among the species of *Brassica* and allied genera based on Jaccard's similarity coefficient using 70 cross-transferable genomic STMS markers. The bootstrap values are indicated at the nodes of each cluster.

larger set of genomic STMS markers available in the public domain.

Materials and methods

Plant material

Eleven species from seven different genera of the family *Brassicaceae* were analyzed in this study, as indicated in Table 2. Five individuals per taxon were used. Seed samples were obtained from National Research Centre on Plant Biotechnology (NRCPB), New Delhi, India.

Molecular analysis

Primer sets for one hundred sixty one STMS markers derived from four different *Brassica* species (*B. napus*, *B. nigra*, *B. rapa*, and *B. oleracea*) were custom synthesized by using sequence information publicly available in the *Brassica* microsatellite information exchange database (www.brassica.info/resourse/markers/ssr-exchange.php) and these primers were used for PCR amplification. The details of the PCR primers including their origin, repeat motif and nucleotide sequence are indicated in supplementary table. The reaction was performed using 50 ng of genomic DNA extracted by the procedure described by Murray and Thompson (1980), in a total volume of 25 µl containing 0.2 µM of each primer, 0.2 mM of each dNTP, 1.0-2.5 mM $MgCl_2$, and 1.5 U Taq polymerase. Touchdown PCR protocol was used with 30 cycles at 94°C for 30 s, 65-56°C for 30 s, and 72°C for 5 s. The annealing temperature started at 65°C and dropped by 0.3°C each cycle, followed by three cycles with annealing at 56°C. The PCR products were visualized in 3.5%, MetaPhor (FMC BioProducts, Rockland, ME, USA) agarose gels containing 0.5 ng/ml of ethidium bromide.

Scoring and analysis of data

Differences in the DNA banding patterns were qualitatively scored from gel photographs for presence (1) and absence (0) of bands assuming that each band represents a unique genetic locus. Homology of bands among samples was based on the distance of migration in gel. Scoring was done for clear, unambiguous amplicons and their sizes were determined by comparing with 100 bp DNA ladder. Based on the presence or absence of amplicons, a binary 1-0 data matrix was created and used to calculate Jaccard's similarity coefficient (Jaccard, 1908). Cluster analysis was carried out among the genotypes based on Jaccard's similarity coefficients using UPGMA (Sneath and Sokal, 1973) and SAHN-clustering

algorithm in NTSYS-pc, version 2.02e (Applied Biostatistics) software. The confidence limits of the UPGMA based dendrogram was determined by bootstrap analysis. One thousand bootstrap replicates were computed and bootstrap of 50% majority rule consensus tree was constructed using the WinBoot software (Yap and Nelson, 1996).

Conclusion

Genomic STMS markers have been extensively used for molecular breeding, genetic diversity analysis and for studying genetic relationships. However, its unavailability in the wild species of *Brassica* and allied genera has severely affected the use of these species in marker assisted introgression breeding programmes. Our study highlights a reliable and efficient way of obtaining genomic STMS markers for wild relatives of *Brassica*, the major oilseed crops of the world. From the present study, a set of 32 polymorphic genomic STMS markers were identified and evaluated to study the genetic diversity and establish relationships among the wild species of *Brassica* and allied genera. The present study demonstrates varying degree of cross-species/genera transferability of genomic STMS markers derived from different *Brassica* species. A significant effect of taxonomic group on the proportion of amplification and polymorphism of genomic STMS markers was also observed. This knowledge can be helpful in designing more extensive studies on determined taxa.

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