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A linkage map for *Cucurbita maxima* based on Randomly Amplified Polymorphic DNA (RAPD) markers

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ABSTRACT

Linkage information is needed for marker-assisted selection in squash and pumpkin breeding programs involving *Cucurbita ecuadorensis*, a source of resistance to many diseases. A mapping population was produced by backcrossing an inter-specific cross of *C. maxima* x *C. ecuadorensis* to *C. maxima*. One hundred RAPD primers were used to survey the parental polymorphism, and 42 of these found to be polymorphic between the parents were used for construction of a linkage map. The 42 primers generated 122 distinct and reproducible bands for 55 plants of the BC_1F_1 population. A linkage map consisting of 102 markers on 22 linkage groups was produced with an approximate length of approximately 1195.2 cM. The segregation of most of these markers did not deviate from the expected 1:1 ratio. Some terminal markers on several linkage groups displayed significant deviation from a 1:1 ratio. Only two linkage groups (12 and 22) had a majority of markers that exhibited significant segregation distortion. A total of 20 RAPDs remained unmapped, and nine of these displayed highly distorted (P< 0.01) segregation ratios, suggesting that they were not single sequences segregating genetically. Thus this first linkage map for *C. maxima* appears to give good coverage of the genome. Predominant normal segregation of most markers suggests significant homology between *C. maxima* and *C. ecuadorensis* genome, and that much of the useful traits of *C. ecuadorensis* could be introgressed into *C. maxima*.

Key words: *Cucurbita* sp., *C. ecuadorensis*, *C. maxima*, RAPD, molecular markers, linkage map.

INTRODUCTION

The genus *Cucurbita* L. is native to the Americas and contains 13 species (Nee, 12). *Cucurbita* is considered to be of polyploid origin and all species have n = 20 chromosomes (Weeden and Robbinson, 23). *Cucurbita* species are highly polymorphic and several approaches have been followed for their classification. *Cucurbita* species were divided into seven groups based on isozyme similarities ( ). One of these groups includes *C. maxima* Duch. ex Lam., *C. andreana* Naud., and *C. ecuadorensis* Cutler and Whitaker. *Cucurbita maxima* and *C. andreana* are very closely related, sharing many common allozymes and cross freely. *Cucurbita maximum* are native to South America (Puchalski and Robinson, 17) and is cultivated as winter squash. The species *C. andreana* is considered to be its wild progenitor, and is from Uruguay and Argentina. *Cucurbita ecuadorensis* is from Ecuador and is a source of resistance to multiple diseases and environmental stresses (Provvidenti *et al.*, 16; Andres and Robbinson, 1). It was included in the group with *C. maxima* and *C. andreana*, although it has some differences at the allozyme level. *Cucurbita maxima* cross with *C. ecuadorensis*, but there are post-zygotic barriers leading to partial sterility in the F_2 and backcross generations (Culter and Whitaker, 5). Furthermore, investigation based on isozyme patterns corroborated the distinctiveness between *C. ecuadorensis* and *C. maxima* (Weeden and Robbinson, 23; Weeden and Robbinson, 25). Some of the isozyme loci were found to be linked to one another (Weeden and Robbinson, 25).

Randomly Amplified Polymorphic DNA (RAPD) markers have been used to create linkage maps for other *Cucurbita* species, but not for *C. maxima*. The maps for *C. moschata* (Brown and Myers, 3) and for *C. pepo* (Zraidi and Leelley, 27) cannot be easily compared as they were constructed using different type of mapping populations involving different parental species and different RAPD markers. Both maps contain morphological markers, either simply inherited or quantitative trait loci (QTLs) that have been linked to RAPD markers (Paris and Brown, 14). The map of *C. moschata* contains 148 RAPD markers, while the map of *C. pepo* contains 254 RAPD markers (Brown and Myers, 3; Zraidi and Leelley, 27).

DNA markers tightly linked to the trait of interest can be used for map-based gene cloning, diagnostics, and molecular marker assisted selection of associated traits. RAPDs are commonly used molecular markers and have several advantages: i) a universal set of primers can be used for genome analysis in a wide variety of species, ii) no primary work such as isolation

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of DNA clones, preparation of probes for hybridization, and nucleotide sequencing is required, and iii) each RAPD marker is the equivalent of a sequence tagged site, which can greatly simplify information transfer in collaborative research programs. In addition, using these markers genotyping can be automated and can be produced more efficiently and simply than with other markers. The present investigation was an attempt to produce a RAPD linkage map for *C. maxima* covering most of the genome.

Despite the wide spread use of molecular markers in plant breeding, *Cucurbita* spp. have received only limited attention. An isozyme linked with watermelon mosaic virus resistance transferred from *C. ecuadorensis* into *C. maxima* has been identified (Weeden *et al*., 26). Resistance of *C. ecuadorensis* to Zucchini Yellow Mosaic Virus (ZYMV) has been introgressed into squash (Robinson, 18) but could not be associated with any isozyme marker (Robinson *et al*., 19). In addition, progeny from the interspecific hybrid *C. maxima* x *C. ecuadorensis* are known to show severe reduction in fertility despite the good fertility of the hybrid (Culter and Whitaker, 5). This phenomenon of hybrid infertility represents a significant obstacle for cucurbit breeders, yet its cause is poorly understood (Weeden and Robbinson, 25). The present study was undertaken to improve our understanding regarding the *C. maxima* genome. It describes a genetic linkage map produced for *C. maxima* using a BC_1F_1 population from *C. maxima* x *C. ecuadorensis*.

MATERIALS AND METHODS

Hybridization was made between *C. maxima* cv. Buttercup and *C. ecuadorensis*, and the interspecific hybrid was backcrossed to *C. maxima*. RAPD markers were used for development of a linkage map using the BC_1F_1 population. DNA was extracted from the two parents and 55 individuals of the BC_1F_1 backcross population and was screened for RAPD markers. The plants used in the present study were grown in field plots under the normal summer conditions at the New York State Agricultural Experiment Station, Geneva, NY. Newly expanded young leaves were harvested from the parents and 55 individual BC_1F_1 plants. DNA was extracted from freeze-dried tissue using modified CTAB method of Doyle and Doyle (6). Estimation of DNA concentration from individual plant samples was measured using spectrophotometer, where each sample was diluted in ratio of 1:500 µl and quantified at wavelength of 260. Each sample was diluted to a uniform concentration of 5ng/µl and was screened with 100 RAPD primers.

PCR amplification was performed in a 25 µl reaction volume, containing 1X reaction buffer (10mM Tris-HCl pH 8), 1.5mM $MgCl_2$, 200µM of each dNTP, 1 unit of *Taq* polymerase, 30µM of primer, and approximately 25ng genomic DNA template. The thermocycler (Perkin Elmer 480) was programmed for an initial denaturation step of 4 min. at 94°C followed by 45 cycles of 1 min. denaturation at 94°C, 1 min. annealing at 37°C and 2 min extension at 72 ^{0}C, and a final extension step at 72°C for 7 minutes. The amplified products were stored at 4°C until electrophoresis. The amplification products were separated on 1.4% agarose gels with 1X TAE buffer (40 mM Tris-acetate and 1 mM EDTA pH 8.0) at 3 V/cm during 4 hours. A 100 bp DNA ladder (MBI, Fermentas, USA) was used as a molecular size standard. Agarose gel was stained with 0.5 µg/ml ethidium bromide solution, visualized under ultraviolet light, and photographed with a gel documentation system.

Each RAPD fragment was designated by a unique number. For example, if three polymorphic bands were produced by primer OPD16 (Operon), they were designated as $OD16_{1200}$, $OD16_{800}$ and $OD16_{650}$ in increasing order of electrophoretic mobility. The same pattern was followed and designated for primers of UBC (University of British Columbia), for example, BC-323_{1200}, BC-323_{700} and BC-323_{800}. Both parents were screened for a total of 100 RAPD primers (including Operon and UBC sets) and a set of 42 polymorphic primers were identified. The parents and 55 BC_1F_1 individuals were evaluated for these polymorphic primers. Segregation of the markers among the BC_1F_1 progeny was analyzed by the χ^2 test. Badly skewed markers having p<0.01 for a 1:1 ratio were not used to generate the initial map. An analysis of the data was made using the EXCEL macro, QUIKMAP (Weeden and Bernad, 22). This initial analysis permitted the construction of linkage groups and confirms that each mapped marker was unambiguously associated with a single linkage group. Multipoint software version 1.1 (Mester *et al*., 11) was used to generate the final linkage map. Markers were assigned to linkage groups using a cut off recombination value (q) of 0.35 with a threshold of LOD = 3.0. A linkage map was generated with the "Map" command using the "Kosambi" function.

RESULTS AND DISCUSSION

One hundred RAPD primers were used for the parental polymorphism survey and 42 of these primers generated DNA bands that were polymorphic between the parents (Table 1). The 42 primers generated 122 distinct and reproducible polymorphic bands. The number of polymorphic bands generated per primer ranged from one to six. Primers OB-12 and P-314 generated the maximum of six polymorphic bands. Primers BC-423, BC-419, BC-533 and OD-11 generated single polymorphic band (Table 1). An average of 2.90 markers was generated per primer.

Table 1. Marker ID, primer sequence and No. of polymorphic bands generated.

S. No.	Maker ID	Primer Sequence (5'---3')	No. of polymorphic bands produced
1	OPD-16	AGGGCGTAAG	3
2	OPB-12	CCTTGACGCA	6
3	UBC-323	GACATCTCGC	3
4	UBC-359	AGGCAGACCT	3
5	UBC-379	GGGCTAGGGT	4
6	UBC-357	AGGCCAAATG	2
7	UBC-405	CTCTCGTGCG	3
8	UBC-411	GAGGCCCGTT	4
9	UBC-374	GGTCAACCCT	3
10	UBC-421	ACGGCCCACC	2
11	UBC-425	CGTCGGGCCT	3
12	UBC-430	AGTCGGCACC	3
13	UBC-432	AGCGTCGACT	3
14	UBC-447	CAGGCTCTAG	4
15	UBC-489	CGCACGCACA	3
16	OPK-11	AATGCCCCAG	2
17	OPK-6	CACCTTTCCC	4
18	OPD-11	AGCGCCATTG	1
19	UBC-530	AATAACCGCC	2
20	UBC-533	GCATCTACGC	1
21	UBC-546	CCCGCAGAGT	2
22	P-21	CCCTGTCTCT	2
23	P-197	GGTCGATCAC	2
24	UBC-577	GTCTGATGTG	4
25	UBC-589	GACGGAGGTC	3
26	UBC-597	TGGTTCCCGA	3
27	UBC-592	GGCCGAGTGC	3
28	P-143	GCCTCATACC	4
29	P-166	GTGACGGACT	3
30	P-206	TGAAGGAGAG	4
31	P-217	CGCTCATTCG	4
32	P-105	CAGTCGCGTG	2
33	P-245	CTAGGTCTCC	2
34	P-312	GTGCGCCTAA	3
35	P-300	ATTGCAGCCA	4
36	S-24	GCGGCATTGT	4
37	S-35	AGTCGCTCAT	3
38	P-314	TGTGATATCG	6
39	UBC-423	GGGTCTCGAA	1
40	UBC-419	TACGTGCCCG	1
41	S-34	GATAGCCGAC	2
42	UBC-418	GAGGAAGCTT	1

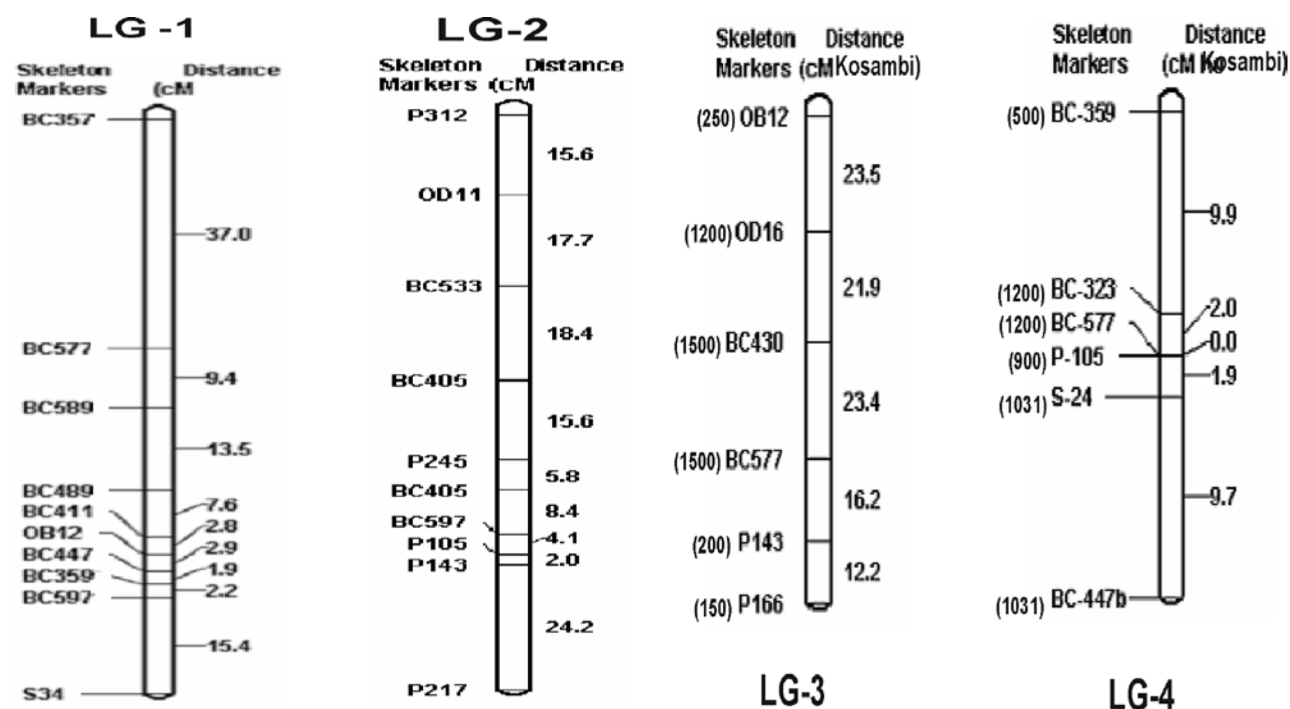
The presence versus absence of a fragment is usually treated as a dominant trait, although in some cases an alternative allele can be recognized and a pair of bands may then be scored as co-dominant genes as demonstrated by Perin *et al.* (15). We observed only one primer (P245) that appeared to generate allelic fragments, and these were treated as a single locus.

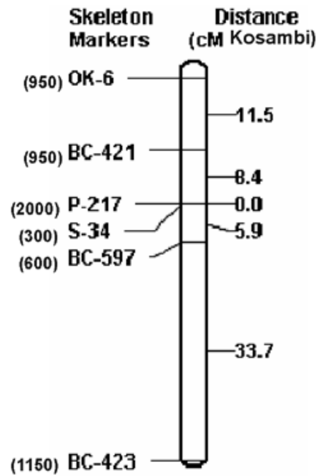
A total of 102 markers (about 84%) of the 122 markers analyzed could be placed on linkage groups. Only nine bands showed a major distortion (P<0.01) from the expected 1:1 segregation ratio. The segregation ratios for several of these fragments did not differ significantly from 3:1, and these RAPDs may have represented two overlapping bands. The lower two thirds of linkage group 12, and the two markers on linkage group 22 were the only regions on the map to have several markers deviating significantly from a 1:1 ratio (Table 2). Some of these markers were highly distorted (P<0.01). In other sections of the map, particularly the terminal marker on several linkage groups displayed distorted segregation. In most cases, the *C. maxima* (A) allele was favored, but on linkage groups 2, 3 and 16 the *C. ecuadorensis* (B) allele was in excess. Figure 1 shows the distribution of the 102 markers and the genetic distances (cM) for all the markers placed on 22 linkage groups. The map covers a total distance of 1195.2 cM. Table 2 presents a summary of the linkage groups and identifies regions where markers displayed significant deviation from the expected 1:1 segregation ratio.

Mapping of predominant number of 102 RAPD markers, grouped into 22 linkage groups indicates near normal segregation of most markers, which should have resulted because of predominant normal meiotic cycle in BC_1F_1 plants, with normal pairing of most chromosomes. This has facilitated a good coverage of the chromosomes of the two parents (n = 20). It is the most extensive map for *C. maxima* currently available. This initial map can be used for identifying loci around the genes of interest, particularly for disease resistance and stress tolerance. Additionally, this reflects, that the genome of two parent species is predominantly homologous; corroborating the inferences of Oris *et al.,* (13) based on mitochondrial genes; therefore, introgression of genes from one to other would be easy. A number of the markers that remained unlinked display distorted segregation. However, the segregation pattern of these unlinked markers differed from those observed on either linkage group-12 or 22, and did not match each other in segregation pattern. This may either be because of a problem in the scoring of these markers or they represent the regions of the genome that are segregating abnormally. Weeden and Robinson (25) also noted significant skewed ratios at isozyme loci in segregating generations from *C.*

Table 2. Linkage groups produced along with the number of markers and total length of each linkage group.

Linkage group	No. of markers	Length (cM)	Distortion from 1:1
1	10	92.7	none
2	10	111.8	P217d
3	6	97.2	P166c
4	6	23.5	P359c
5	6	59.5	none
6	6	79.0	P166a
7	7	107.3	none
8	6	74.9	none
9	4	45.4	none
10	4	53.2	none
11	4	23.2	none
12	6	81.5	BC489a, BC405b, P314e, P217c, P300a
13	5	78.9	OK11b
14	4	52.8	none
15	2	19.4	none
16	3	33.2	BC357a
17	3	39.6	none
18	2	18.4	BC323b
19	2	26.5	none
20	2	17.7	P206d
21	2	31.3	P312b
22	2	28.2	OK11a, BC432c
Total	102	1195.2	





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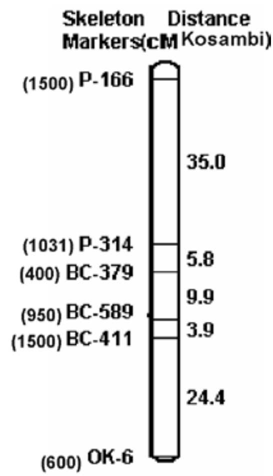

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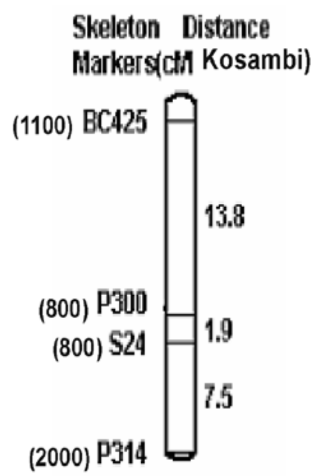

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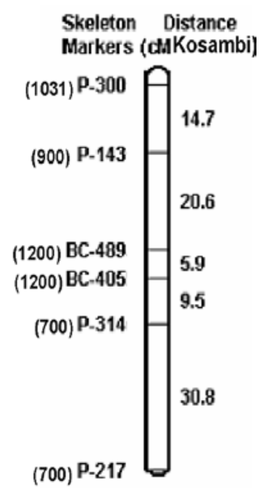

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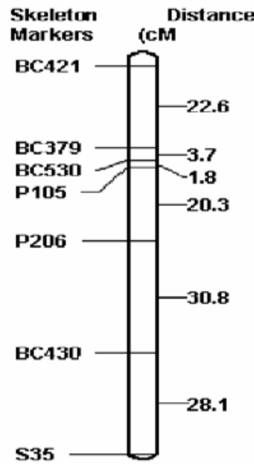

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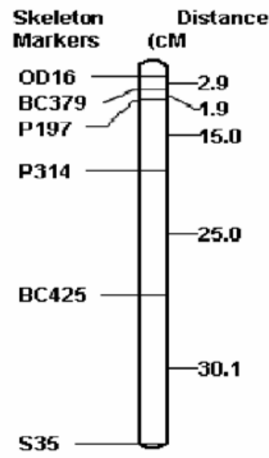

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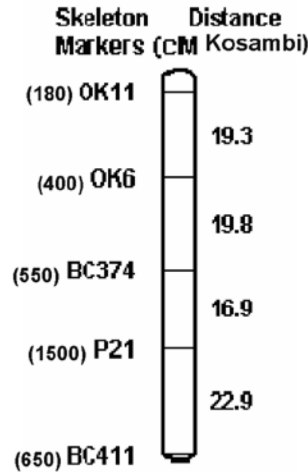

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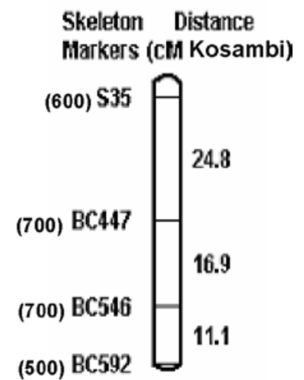

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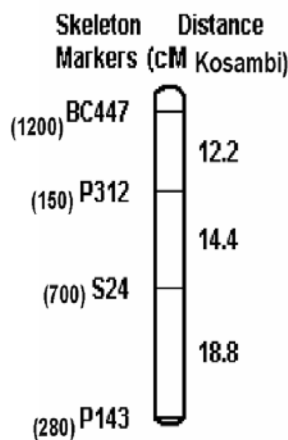

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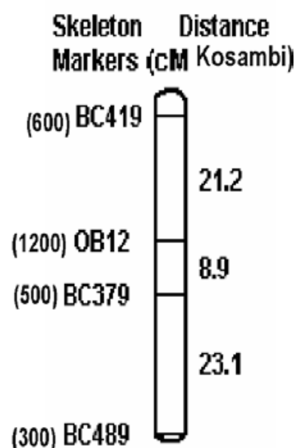

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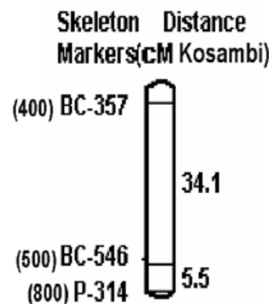

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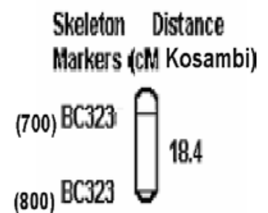

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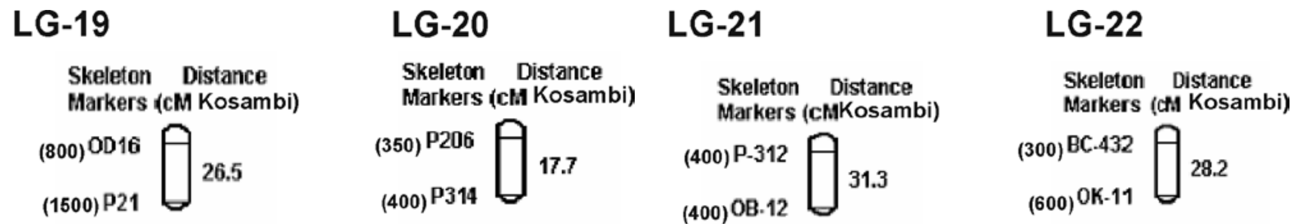

Fig. 1. RAPD–based genetic linkage map of Cucurbita *maxima* derived from a backcross population of *C. maxima* x (*C. maxima* x *C. ecuadorensis*). Marker names are on the left, while distance between the markers (in cM) is on the right of each linkage group.

maxima x *C. ecuadorensis.* This disturbed segregation is perhaps because of the genetic dissimilarity between the specific chromosome pairs of parent species. In populations produced through wide hybridization, particularly interspecific and intergeneric crosses, skewed segregation is common because of abnormal pairing and segregation of some chromosomes leading to abnormal meiotic cycle, thereby, abnormal segregation of traits and markers associated with them (Bonirable *et al.*, 2; Durham *et al.*, 7; Weeden, 24). The genetic distinctiveness between the two species has also been expressed in terms of cross-incompatibility between the two, resulting in post-zygotic crossability barriers. Also, the established hybrid between the two species shows abnormal meiotic cycle and a moderate level of pollen sterility. Further, in such a situation preferential pairing between the chromosomes of the same parent or no pairing could also result in abnormal segregation of chromosomes during the meiotic cycle, thus altering the segregation pattern and producing lesser recombination and greater skewed segregation of markers towards either of the parents. This type of phenomenon has been reported even with two cultivars of the same cucurbit species, *Cucumis melo* L. (Wang *et al.*, 21) and may be the reason for abnormal segregation of certain markers resulting in non-association with any linkage group.

The problem of skewed segregation of polymorphic bands can be minimized by studying more advanced generation populations in the future, these more advanced populations having been reported to be highly fertile (Robinson *et al.*, 19). Using isozyme loci and sequence tagged site (STS) markers, the present map for *C. maxima* can also be extended to and merged with maps of other cucurbits, particularly *Cucurbita* spp., for establishing linkage relationships (Wang *et al.*, 21; Levi *et al.*, 10; Levi *et al.*, 9; Staub and Sequen, 20). Therefore, extension of this map to the maps of other cucurbit species is a high priority for genome analysis. Molecular marker assisted selection is now widely used for several crops but not yet for squash and pumpkin. There are not many cases reported for association between molecular markers of *Cucurbita* spp. and traits of breeding interest. Association/Linkage has been reported for an isozyme marker with watermelon mosaic virus resistance, transferred from *C. ecuadorensis* to *C. maxima* (Weeden *et al.*, 26), but attempts to find linkage of zucchini yellow mosaic virus with an isozyme (Robinson et al., 19) or RAPD (Brown *et al.*, 4) marker were unsuccessful. A gene for naked seed coat, important for oilseed pumpkin breeding, was found to be linked with RAPD markers (Brown *et al.*, 4). Use of the present map for *C. maxima* in identification of certain loci of interest, particularly fragment(s)/loci associated with resistance to ZYMV, would be of significance because once this is achieved the fragment can be isolated, sequenced, and characterized for further use in screening and marker assisted selection. Further analysis is needed for tracking association of a molecular marker with ZYMV resistance and other important traits in segregating populations.

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