Genetic relationships among *Hystrix patula*, *H. duthiei* and *H. longearistata* according to meiotic studies and genome-specific RAPD assay

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Abstract

Hybrids including *Hystrix patula*, *H. duthiei* and *H. longearistata* were obtained and genetic relationships among them were studied. Meiotic pairing in hybrids of *H. duthiei* × *Psathyrostachys juncea* (Ns), *H. longearistata* × *Psa. juncea* (Ns), *Leymus multicaulis* (NsXm) × *H. duthiei*, *L. multicaulis* (NsXm) × *H. longearistata*, *Elymus sibiricus* (StH) × *H. patula*, *Roegneria ciliaris* (StY) × *H. patula*, *R. ciliaris* (StY) × *H. duthiei* and *R. ciliaris* (StY) × *H. longearistata* averaged 5.76, 5.44, 11.94, 10.88, 10.08, 3.57, 0.46 and 0.90 bivalents per cell, respectively. The results indicated that *H. duthiei* and *H. longearistata* had the NsXm genomes of *Leymus*, while *H. patula* contained the StH genomes and had a low genome affinity with the StY genomes of *Roegneria*. Results of genome-specific RAPD assay were comparable with the chromosome pairing data. According to the genomic system of classification in *Triticeae*, *H. patula* should be considered as *Elymus hystrix* L., while *H. duthiei* and *H. longearistata* as *Leymus duthiei* and *Leymus duthiei* ssp. *longearistata*, respectively.

Additional key words: chromosome pairing, *Leymus*.

Introduction

Hystrix, originally named *Asperella*, is a small perennial genus of the tribe *Triticeae* (*Poaceae*). Moench (1794) established the genus *Hystrix* according to the distinct morphological character of strongly reduced or lacking glumes, with *H. patula* Moench as the type. Since then, about eleven species have been included in *Hystrix* (Hitchcock 1951, Bor 1960, Tzvelev 1976, Kuo 1987, Osada 1993, Baden *et al.* 1997), two species from North America [*H. patula* and *H. californica* (Bol.) Kuntze] and the remainder from central and eastern Asia (Löve 1984, Baden *et al.* 1997). All of them are tetraploids (2n=4x=28) except *H. californica*, which is an octoploid (2n=8x=56). However, the definition of *Hystrix* and its precise taxonomic rank are still under discussion. Some authors included the species in genus *Hystrix* (Sakamoto 1973, Kuo 1987, Baden *et al.* 1997) or in *Asperella* (Keng 1959, Baum 1983, Ohwi 1984, Koyama 1987), while others regarded them as a part of genus *Elymus* (Dewey 1982, 1984, Löve 1984, Jensen and Wang 1997).

The intergeneric hybridizations of *H. patula*, *H. duthiei* and *H. longearistata* were employed in the present study to verify their genetic relationships. The random amplified polymorphic DNA (RAPD) is a simple, less costly, and less labour than other DNA marker methodologies (Chakrabarti *et al.* 2006, Dikshit *et al.* 2007). Some genome-specific RAPD markers have been identified and used in the genome analysis of the *Triticeae* species (Wei and Wang 1995). Five genome-specific RAPD markers representing St, H, Ns, E^e and E^b genome were utilized in this study to determine if these genome-specific markers were present in the three *Hystrix* species. Based on these results and the cyto-genetic evidence in our previous studies, the taxonomic classification of the three species of *Hystrix* was suggested.

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Abbreviations: CTAB - cetyltrimethylammonium bromide; PMC - pollen mother cells; RAPD - random amplified polymorphic DNA.
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Materials and methods

Plants: Eight species including *H. patula*, *H. duthiei*, *H. longearistata*, *Elymus sibiricus* L. (StH), *Roegneria ciliaris* (Trin.) Nevski (StY), *Psathyrostachys juncea* (Fischer) Nevski (Ns), *Leymus multicaulis* (Kar. & Kir.) Tzvel. (NsXm) and *Lophopyrum elongatum* Á. Löve (E^e) were used in the cross program for genome analysis. Besides these eight species, other eight species with different genomes were used for RAPD assay (Table 1). The accessions with PI numbers were kindly provided by American National Plant Germplasm System (Pullman, Washington, USA) and *H. longearistata* was kindly provided by Dr. S. Sakamoto (Kyoto University, Japan). The others were collected by authors of this paper. Voucher specimens have been deposited at Herbarium of Triticeae Research Institute, Sichuan Agricultural University, China (SAUTI).

Meiotic analysis: Hybrids in this study were listed in Table 1. The procedures of hybridization, fixation and staining of cytological materials and meiotic preparation followed Zhang and Zhou (2006). The account of mean pairing frequency (c-value: the mean frequency with which two related chromosome arms pair) was calculated according to Alonso and Kimber (1981). When the mean c-value exceeded 0.50, the two genomes in the hybrids were given the same basic symbol (Wang 1992). Pollen grains from mature anthers were stained in an I_2-IK solution for pollen fertility study.

Random amplification of genomic DNA: DNA was extracted from young leaves by the cetyltrimethylammonium bromide (CTAB) extraction procedure (Sharp *et al.* 1988). Five genome specific RAPD markers, OPC14-450, OPW5-700, OPC9-548, OPR5-700 and OPF3-1200 for the St, H, Ns, E^e and E^b genomes, respectively, were used as single primers (Wei and Wang 1995). PCR reaction and analysis of amplified DNA were performed as described in Wei and Wang (1995).

Results

Crosses, germination and pollen fertility: *H. patula*, *H. duthiei* and *H. longearistata* were crossed with *Psa. juncea* (Ns), *L. multicaulis* (NsXm), *E. sibiricus* (StH), *R. ciliaris* (StY) and *Lo. elongatum* (E^e), respectively. Among the fifteen crosses, eight produced seeds without utilizing the aid of embryo rescue and resulted in mature hybrid plants (Table 1). Crosses of *H. patula* × *Psa. juncea*, *L. multicaulis* × *H. patula*, *E. sibiricus* × *H. duthiei*, *E. sibiricus* × *H. longearistata*, and the combinations of the three *Hystrix* species with *Lo. elongatum* died before flowering (Table 1).

The F_1 plants were vigorous and morphologically intermediate between their parental species. Their pollen grains were shrivelled and could not be stained in I_2-IK solution. No seeds were produced by any of the hybrids under open-pollinated conditions.

Chromosome pairing: Meiotic configurations at metaphase I (MI) in pollen mother cells (PMCs) of the parental species and the hybrids were listed in Table 2. The meiosis of the parental species was quite regular, with seven bivalents in diploid species and fourteen in the tetraploids.

Meiotic pairing in the two triploid hybrids of *H. duthiei* × *Psa. juncea* and *H. longearistata* × *Psa. juncea* were comparatively high, with average values of 5.76 and 5.44 bivalents per cell with c-values of 0.53 and 0.50, respectively (Fig. 1*A*,*C*). Complete pairing (7 I + 7 II) was observed in 22 and 17 % of the cells in the two hybrids (Fig. 1*B*,*D*). A low frequency of trivalents was found in both of the combinations (Fig. 1*E*).

In tetraploid hybrids of *L. multicaulis* × *H. duthiei* and *L. multicaulis* × *H. longearistata*, an average of 11.94 and 10.88 bivalents per cell were observed, with a c-value of 0.68 and 0.60, respectively (Fig. 1*F*,*G*,*H*). 88 and 64 % of the cells formed more than 10 bivalents in the two hybrids, respectively. A low frequency of trivalents and quadrivalents was observed in both of the tetraploid hybrids.

Chromosome pairing in tetraploid hybrid of *E. sibiricus* × *H. patula* was quite regular with an average of 10.08 bivalents per cell and a c-value of 0.57. Twelve to fourteen bivalents were observed in 21 % of the MI cells (Fig. 1*I*). Trivalents and quadrivalents were also observed in this hybrid.

In the *R. ciliaris* × *H. patula* hybrid, an average of 3.57 bivalents per cell was observed with a c-value of 0.17 (Fig. 1*J*). A low frequency of trivalents was observed in this hybrid (Fig. 1*K*). However, in the hybrids of *R. ciliaris* × *H. duthiei* and *R. ciliaris* × *H. longearistata*, many univalents were observed at MI, with an average of 27.09 and 26.21 per cell and a c-value of 0.02 and 0.03, respectively (Fig. 1*M*,*O*). 61 and 44 % of the cells produced 28 univalents in these two hybrids (Fig. 1*L*,*N*). The number of bivalents was quite low with an average of 0.46 and 0.90 per cell, respectively.

Genome-specific RAPD assays: $OPC14_{450}$, specific to the St genome, was amplified only with template DNAs of species containing the St genome, *Pse. spicata* (St), *E. sibiricus* (StH), *H. patula* (StH), *R. caucasica* (StY) and *R. ciliaris* (StY) (Fig. 2*A*). $OPW5_{700}$, specific to

Table 1. Species and hybrids used in this study. [a] - Plants were weak and died before maturity; [b] - seeds failed to germinate and no hybrid plant was obtained; [c] - data from Zhang *et al.* (2006); [d] - Data from Zhang and Zhou (2007).

No.	Species	2n	Genomes	Accession No.	Origin	Hybrids	Number of emascula-ted florets	Number of seeds	Number of plants
1	*Pseudoroegneria spicata* (Pursh) Á. Löve	14	St	PI 232138	Idaho, USA	*H. patula* × *Psa. juncea*	30	9	1[a]
2	*Hordeum bogdanii* Wilensky	14	H	Y 0829	Xinjiang, China	*H. duthiei* × *Psa. juncea*	26	11	5
3	*Psathyrostachys juncea* (Fischer) Nevski	14	Ns	PI 406468	Russian Federation	*H. longearistata* × *Psa. juncea*	22	10	3
4	*Psathyrostachys huashanica* Keng ex Kuo	14	Ns^h	ZY 3157	Shanxi, China	*L. multicaulis* × *H. patula*	48	6	0[b]
5	*Lophopyrum elongatum* Á. Löve	14	E^e	PI 531719	St. Angulf, France	*L. multicaulis* × *H. duthiei*	36	17	15
6	*Thinopyrum bessarabicum* (Savul. & Rayss) Á. Löve	14	E^b	PI 531711	Crimea, Ukraine	*L. multicaulis* × *H. longearistata*	35	21	19[c]
7	*Elymus sibiricus* L.	28	StH	ZY 1005	Gansu, China	*E. sibiricus* × *H. patula*	24	3	3[d]
8	*Hystrix patula* Moench	28	StH	PI 372546	Ottawa, Canada	*E. sibiricus* × *H. duthiei*	30	0	0
9	*Roegneria caucasica* C. Koch	28	StY	PI 531753	Dilidjan, Armenia	*E. sibiricus* × *H. longearistata*	22	0	0
10	*Roegneria ciliaris* (Trin.) Nevski	28	StY	88-89-236	Sichuan, China	*R. ciliaris* × *H. patula*	28	5	4[d]
11	*Leymus arenarius* (L.) Hochst.	28	NsXm	PI 272126	Alma-Ata, Kazakhstan	*R. ciliaris* × *H. duthiei*	28	15	2
12	*Leymus ramosus* (Trin.) Tzvel.	28	NsXm	PI 499653	Xinjiang, China	*R. ciliaris* × *H. longearistata*	32	14	2
13	*Leymus secalinus* (Georgi) Tzvel.	28	NsXm	PI 499535	Xinjiang, China	*Lo. elongatum* × *H. patula*	26	1	0[b]
14	*Leymus multicaulis* (Kar. & Kir.) Tzvel.	28	NsXm	PI 499520	Xinjiang, China	*H. duthiei* × *Lo. elongatum*	48	6	1[a]
15	*Hystrix duthiei* (Stapf) Bor	28	Ns-	ZY 2004	Sichuan, China	*H. longearistata* × *Lo. elongatum*	18	3	0[b]
16	*Hystrix longearistata* (Hackel) Honda	28	Ns-	ZY 2005	Tokyo, Japan				

the H genome, was presented only in species containing the H genome, *i.e*., *H. bogbanii* (H), *E. sibiricus* (StH) and *H. patula* (StH) (Fig. 2*B*). $OPC9_{548}$, the Ns genome-specific maker, was prominently presented in *Psa. juncea* (Ns) and *Psa. huashanica* (Ns^h), and a lightly stained band of the same length was present in *H. duthiei*, *H. longearistata* and the four *Leymus* species (NsXm) (Fig. 2*C*). $OPR5_{700}$ and $OPF3_{1200}$, specific to the E^e and E^b genomes, were amplified only from *Lo. elongatum* (E^e) and *Th. bessarabicum* (E^b), respectively (Fig. 2*D*,*E*).

Discussion

Genome analysis is considered an important tool and has been widely utilized in determining relationships in the tribe *Triticeae* (Dewey 1984, Wang 1985, Zhou *et al.* 1999). Genome affinity is usually determined by the observation of chromosome pairing behaviour at meiotic metaphase-I of interspecific or intergeneric hybrids. However, chromosome pairing is known to be influenced by a number of environmental and genetic factors, and some authors have raised theoretical objections (Sears 1976, Seberg and Petersen 1998). But reliable conclusions of genome analysis could be drawn if they are based on several sources of information, including pairing in the parental species and in a network of interrelated hybrids (Dewey 1982).

Table 2. Meiotic associations at metaphase I in pollen mother cells of the parental species and their hybrids. [a] - Data from Zhang *et al.* (2006); [b] - data from Zhang and Zhou (2007).

Species and hybrids	2n	Number of cells	Chromosome associations						Chiasmata [cell^{-1}]	c-value
			I	II			III	IV		
				total	ring	rod				
Hystrix patula	28	50	-	14.00	13.70	0.30	-	-	27.70	0.99
				14	12-14	0-2			25-28	
Hystrix duthiei	28	50	-	14.00	13.70	0.30	-	-	27.70	0.99
				14	12-14	0-2			26-28	
Hystrix longearistata	28	50	-	14.00	13.60	0.40	-	-	27.60	0.99
				14	11-14	0-3			25-28	
Elymus sibiricus	28	50	-	14.00	13.43	0.57	-	-	27.43	0.98
				14	12-14	0-2			26-28	
Roegneria ciliaris	28	50	-	14.00	13.64	0.36	-	-	27.64	0.99
				14	13-14	0-1			27-28	
Psathyrostachys juncea	14	50	-	7.00	6.12	0.88	-	-	13.12	0.94
				7	4-7	0-3			11-14	
Leymus multicaulis	28	57	0.58	13.70	11.20	2.50	-	-	24.90	0.89
			0-4	12-14	8-14	0-6			22-28	
H. duthiei × *Psa. juncea*	21	54	9.06	5.76	1.39	4.37	0.15	-	7.44	0.53
			4-13	4-8	0-6	1-7	0-1		5-13	
H. longearistata × *Psa. juncea*	21	71	9.85	5.44	1.37	4.07	0.09	-	6.99	0.50
			4-15	2-8	0-4	1-8	0-2		3-10	
L. multicaulis × *H. duthiei*	28	50	4.03	11.94	6.91	5.03	0.03	-	18.91	0.68
			0-10	9-14	4-10	3-8	0-1		13-23	
L. multicaulis × *H. longearistata*[a]	28	66	5.55	10.88	5.41	5.47	0.21	0.02	16.76	0.60
			0-16	6-14	2-10	1-10	0-2	0-1	11-24	
E. sibiricus × *H. patula*[b]	28	50	5.83	10.08	4.52	5.56	0.33	0.25	16.02	0.57
			0-12	5-14	0-12	1-10	0-2	0-2	11-26	
R. ciliaris × *H. patula*[b]	28	50	20.43	3.57	0.79	2.79	0.14	-	4.64	0.17
			14-28	0-7	0-3	0-4	0-1		0-10	
R. ciliaris × *H. duthiei*	28	101	27.09	0.46	-	0.46	-	-	0.46	0.02
			22-28	0-3		0-3			0-3	
R. ciliaris × *H. longearistata*	28	58	26.21	0.90	-	0.90	-	-	0.90	0.03
			22-28	0-3		0-3			0-3	

In our studies, hybrids involving *H. patula*, *H. duthiei* and *H. longearistata* had been obtained. Meiotic pairing in triploid hybrids *H. duthiei* × *Psa. huashanica* and *H. longearistata* × *Psa. huashanica* averaged 5.18 and 5.11 bivalents per cell, respectively (Zhang and Zhou 2006). In the present study, similar meiotic pairing was observed in triploid hybrids *H. duthiei* × *Psa. juncea* and *H. longearistata* × *Psa. juncea*, with average values of 5.67 and 5.44 bivalents per cell. These results indicated that one of the two genomes in *H. duthiei* and *H. longearistata* was homologous to the Ns genome of *Psathyrostachys*. In tetraploid hybrid *L. multicaulis* (NsXm) × *H. longearistata*, an average of 10.15 bivalents per cell was observed at MI (Zhang *et al.* 2006). In this study, chromosome pairing in hybrid of *L. multicaulis* × *H. duthiei* was similar to that of *L. multicaulis* × *H. longearistata*, with an average of 11.94 bivalents per cell. The results suggested that there was considerable chromosome homology between genomes of *H. duthiei* and *H. longearistata* and those of *L. multicaulis*. Therefore, *H. duthiei* and *H. longearistata* had the NsXm genome of *Leymus*.

However, *H. patula* had different genomic constitution from those of *H. duthiei* and *H. longearistata*. Meiotic pairing in triploid *H. patula* × *Psa. huashanica* and interspecific hybrid *H. patula* × *H. longearistata* characterized by a large number of univalents, with average values of 20.43 and 25.36 univalents per cell (Zhang and Zhou 2006). Church (1967) reported that *H. patula* had a close affinity to species of the *Elymus canadensis* based on hybridization studies. When *H. patula* was crossed with tetraploids *E. sibiricus* and *E. wawawaiensis*, average bivalents of 10.08 and 12.83 per cell were observed at MI, respectively (Zhang and Zhou 2007). The results suggested that genome of *H. patula* was homology with those of the two *Elymus* species. Genomic *in situ* hybridization (GISH) analysis of *H. patula* revealed the same result as the genome analysis

(Zhang *et al.* 2006). Thus, *H. patula* contained the StH genome of *Elymus*.

The different genome constitution between *H. patula* and *H. duthiei* and *H. longearistata* was also verified by the different meiotic pairing association in crosses with *R. ciliaris*. In hybrid *R. ciliaris* × *H. patula*, an average of 3.57 bivalents per cell was observed at MI (Zhang and Zhou 2007). However, only average values of 0.46 and 0.90 bivalents per cell were found in hybrids *R. ciliaris* × *H. duthiei* and *R. ciliaris* × *H. longearistata* in the present study. The results indicated a lower homology between the StH genome of *H. patula* and the StY genome of *Roegneria*, but non-homology between the NsXm genome of *H. duthiei* (or *H. longearistata*) and the StY genome.

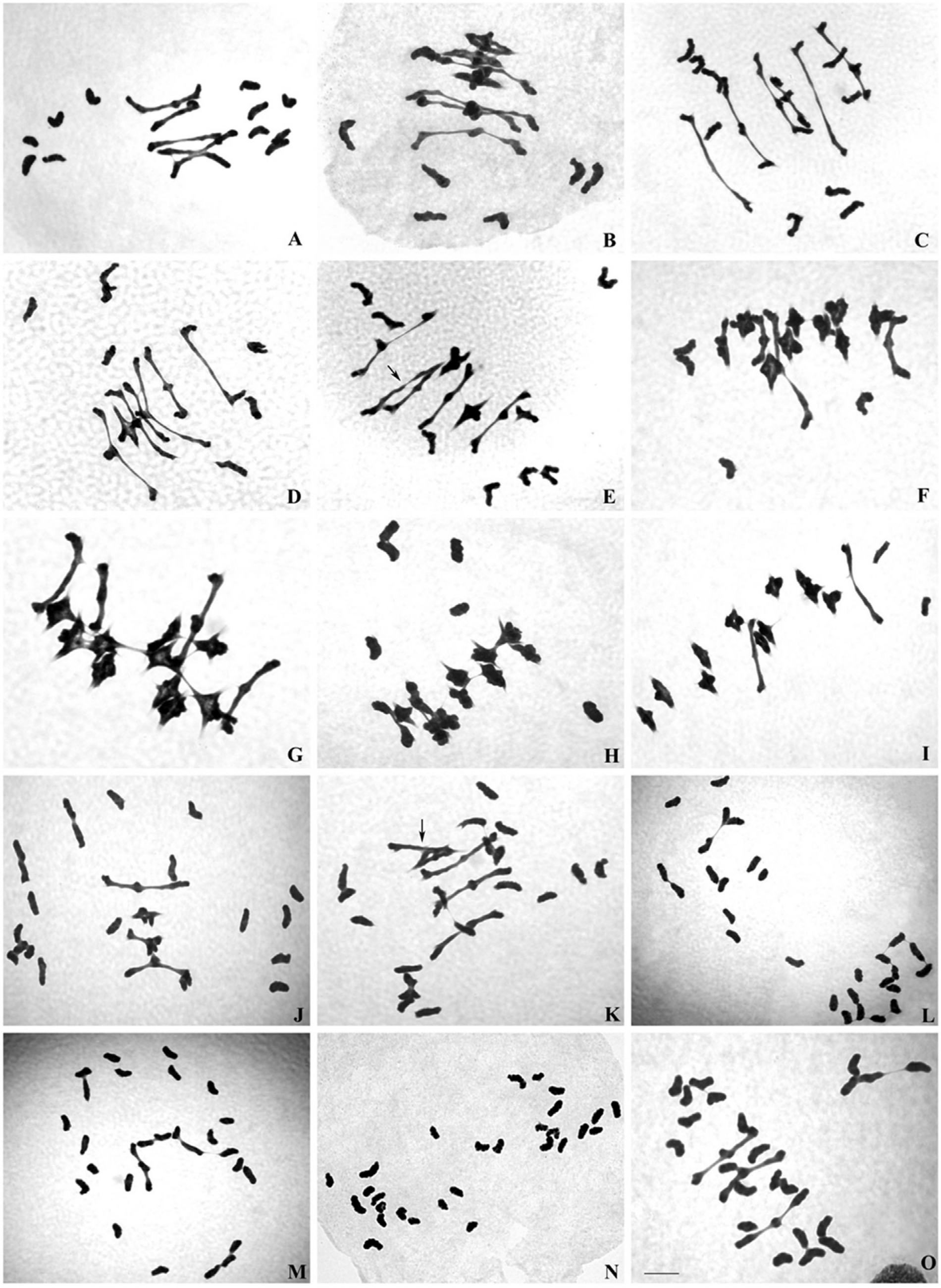

Fig. 1. Meiotic chromosome associations at MI of meiosis in intergenetic hybrids. *A,B*: *H. duthiei* × *Psa. juncea* (Ns), *A* - 11 I + 5 II, *B* - 7 I + 7 II; *C-E*: *H. longearistata* × *Psa. juncea* (Ns), *C* - 9 I + 6 II, *D* - 7 I + 7 II, *E* - 8 I + 5 II + 1 III (*arrow*); *F,G*: *H. duthiei* × *L. multicaulis* (NsXm), *F* - 4 I + 12 II, *G* - 14 II; *H*: *H. longearistata* × *L. multicaulis* (NsXm) with 6 I + 11 II; *I*: *E. sibiricus* (StH) × *H. patula* with 2 I + 13 II; *J,K*: *R. ciliaris* (StY) × *H. patula*, *J* - 20 I + 4 II, *K* - 19 I + 3 II + 1 III (*arrow*); *L,M*: *R. ciliaris* (StY) × *H. duthiei*, *L* - 28 I, *M* - 26 I + 1 II; *N,O*: *R. ciliaris* (StY) × *H. longearistata*, *N* - 28 I, *O* - 22 I + 3 II. *Bar* represents 10 μm.

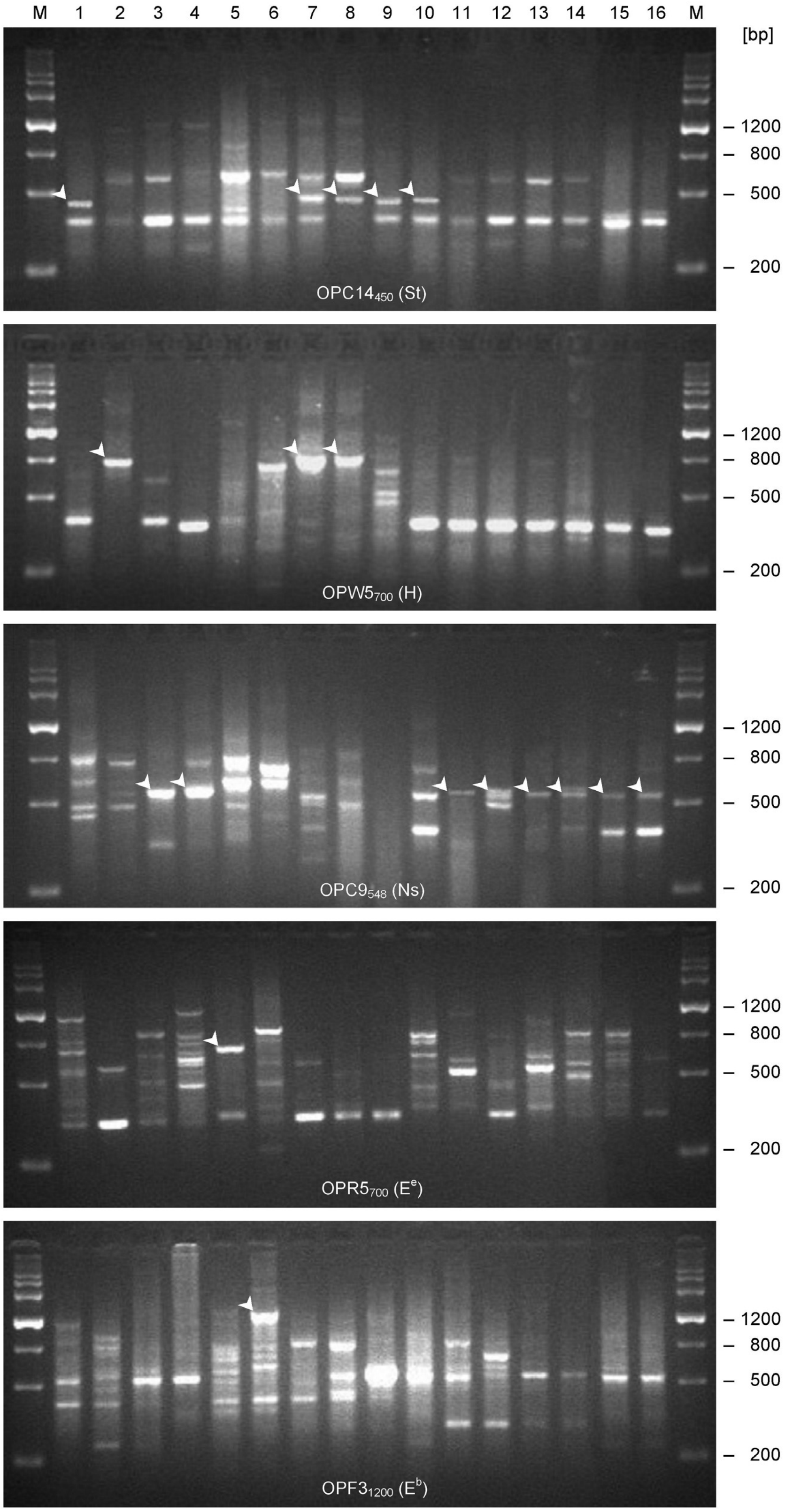

Fig. 2. Results of RAPD assay amplified by genome-specific primers: $OPC14_{450}$, St-genome-specific marker (*arrow*); $OPW5_{700}$, H-genome-specific marker (*arrow*); $OPC9_{548}$, Ns-genome-specific marker (*arrow*); $OPR5_{700}$; E^e - genome-specific marker (*arrow*); $OPF3_{1200}$, E^b - genome-specific marker (*arrow*). The number of 1 - 16 refers to the species listed in Table 1. M is DNA molecular mass marker.

Baden *et al.* (1997) divided *H. duthiei* into three subspecies, *H. duthiei* ssp. *duthiei*, *H. duthiei* ssp. *longearistata* and *H. duthiei* ssp. *japonica* based on morphology and geography analysis. Zhou *et al.* (1999) analyzed meiotic pairing in the hybrid *H. duthiei* × *H. longearistata* and observed mainly bivalent pairing (averaged 13.98), suggesting that they were closely related and shared two highly homologous genomes. In our studies, similar results from patterns of meiotic pairing, GISH and genomic-specific RAPD assay were observed in *H. duthiei* and *H. longearistata* (Zhang *et al.* 2006 and present study), confirming the close relationship between *H. duthiei* and *H. longearistata*.

There are minor morphological variations between *H. duthiei* and *H. longearistata* in the width of leaf blade, length of lemma awn and number of florets per spikelet (Zhou *et al.* 1999). *H. duthiei* has a disjunct distribution, from Northern India, Western Nepal to Southwest of China and Korea, while *H. longearistata* is endemic to Japan, from Kyushu to Hokkaido (Baden *et al.* 1997). Based on morphology, cytology and distribution, it is reasonable to consider *H. longearistata* as a subspecies of *H. duthiei*, *H. duthiei* ssp. *longearistata*.

A large number of univalents (averaged 25.36 per cell) formed in interspecific hybrid *H. patula* × *H. longearistata*, indicating that they are genetically distinct from one another (Zhang and Zhou 2006). Results from genomic-specific RAPD assay in this study and GISH further confirmed this meiotic analysis (Zhang *et al.* 2006). Owing to the close relationship between *H. longearistata* and *H. duthiei*, the genetic relationship between *H. duthiei* and *H. patula* were distant too.

Morphologically, plants of *Hystrix* are loosely tufted caespitose, relatively tall, with broadly lanceolate leaves, large anthers and reduced glumes. All of them are found in moist places, *i.e.*, on the edges thickets hillsides or along rocky river banks (Hitchcock 1951, Baden *et al.* 1997). However, from a cytological point of view, different genome constitutions of *Hystrix* species have been reported (Church 1967, Jensen and Wang 1997, Zhang and Zhou 2006, Zhang et al. 2006, Zhang and Zhou 2007). Based on the cytological and molecular results, *H. patula*, the type species of *Hystrix*, contains the StH genomes, whereas *H. duthiei* and *H. longearistata* contain the NsXm genomes of *Leymus*, and have no genome homology with the genomes of *H. patula* and the StY genomes of *Roegneria*. According to the genomic system of classification in *Triticeae*, *H. patula* should be included in *Elymus*, while *H. duthiei* and *H. longearistata* should be transferred from *Hystrix* to *Leymus*. The taxonomic treatments of the three species should be made as follows:

Elymus hystrix L.: Spec. Pl.: 560. 1753. - *Asperella hystrix* (L.) Humb., Mag. Bot. (Roem. & Usteri) 3: 5, 1790. - *Hystrix patula* Moench, Meth. Pl.: 295. 1794. Type: J.F. Gronovius, described from Virginia, USA.

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Leymus duthiei (Stapf) Y.H. Zhou *et* H.Q. Zhang ssp. *longearistata* (Hackel) Y.H. Zhou *et* H.Q. Zhang, comb. nov.

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