Domestication process of *korati, Setaria pumila* (Poaceae), in the Indian subcontinent on the basis of cluster analysis of morphological characteristics and AFLP markers

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The endemic landraces and related weeds were collected in field surveys around the Deccan in India since 1983, to explain the domestication process of *Setaria pumila* (Poir.) Roem. et Schult. (Poaceae) through its mimicry of other grain crops. The domestication process of *Setaria pumila* in relation to the weed-crop complex was comparatively investigated using statistical and AFLP analyses. It was clear on the basis of these results that the domestication process had progressed through the four stages according to geographical trends in morphological (artificial selection) and genetic variation (neutral DNA markers). Under the complex process, the 4 stages were as follows: weed, companion weed, mimic companion weed and domesticated type to the secondary crop. The paddy rice had dispersed from Assam, the humid east toward Deccan, the dry south in the Indian subcontinent. Several species of Indian millet were domesticated by local farmers as the secondary crop of rice along the climatic trend and dispersal route. In South India, one domesticated type of **S. pumila** was cultivated only in mixed stands mostly along *Panicum sumatrense*. Around Orissa, the other types and the related weeds were grown in the sympatric fields with *Paspalum scrobiculatum*, *Eleusine coracana*, and upland rice (*Oryza sativa*) in diverse agro-ecological niches. Therefore, *S. pumila* became exactly a tertiary crop to the other Indian millet (secondary crop to rice).

Keywords: AFLP markers, artificial selection, domestication process, mimicry, mixed stand, tertiary crop

Introduction

Humans have domesticated more than 30 grass species as grain crops in several parts of the world, possibly as long as 12,000 years ago. However, several species are threatened and, despite their potential food value in their native habitats, have disappeared or have not been extensively cultivated. This is because the yield and production of the three major crops: wheat, rice, and maize, have rapidly increased due to technological innovations in crop-improvement programs. Cultivation of other grain crops (e.g., millets) has decreased gradually during the 20th century, resulting in loss of genetic diversity of local varieties. It is currently necessary to recognize the value of these neglected species as exploitable and underutilized genetic resources that exhibit adaptability to stress-prone environments. In this paper, we focus on millet species, which are mostly C_4 plants, are early to mature, and can be cultivated under conditions of severe drought and harsh sunlight.

Small-scale farmers continue to cultivate a few useful local varieties of millet. These indigenous varieties are excellent materials for investigating crop evolution, particularly the origin and dispersal routes of domesticated plants. In the Indian subcontinent, a few millet species are still undergoing domestication (Kimata et al. 2000; Singh and Arora 1972). While crop evolution can be reconstructed mostly from botanical data, details on geographic origin and dispersal will become clear from information on the basic agricultural complex offered by local farmers. This basic agricultural complex consists mainly of cultivation, processing and cooking such as biocultural diversity.

Vavilov (1926) illustrated the domestication process from companion weeds associated with wheat to secondary crops in two genera, *Avena* and *Secale*. For example, *Secale cereale* L. acquired strong resistance to cold in high altitude or latitude areas, and subsequently was able to grow under more severe conditions than those under which wheat can grow. Kobayashi (1987, 1989) proposed an integrating model of the domestication of Indian millet (e.g. *P. sumatrense*, *Echinochloa frumentacea*) as a secondary crop from mimic companion weeds associated with *Oryza sativa* L. Farmers have manipulated the domestication process by selecting for desired growth, visual, and palatability traits, e.g. yield, early maturation, color, sugar content. However, natural selection and hybridization have occurred among closely related weeds during domestication.

The growing area of *O. sativa* expanded from wetlands to establish secondarily in uplands in the Indian subcontinent. In turn, weedy ancestral plants invaded paddy and upland rice fields. Local farmers subsequently domesticated *Panicum sumatrense* Roth. (little millet), *Paspalum scrobiculatum* L. (kodo millet), and *Echinochloa frumentacea* Link (Indian barnyard millet), as secondary crops, because these species demonstrated stronger resistance to drought than upland rice in Eastern India. Several additional species of millet were domesticated in this region: *Brachiaria ramosa* (L.) Stapf. (*korne*, browntop millet), *Digitaria cruciata* [Nees] A. Camus (*raishan*), and *Setaria pumila* (Poir.) Roem. & Schult. (*korati*, yellow foxtail millet; syn. *Setaria* glauca [L.] P. Beauv.) (Chandra and Koppar 1990; de Wet et al. 1983a, b, c).

Recently, archeological studies in the Indian subcontinent have provided useful data on the ancient history of various grains. Millet materials were identified from two archaeological levels in the Southern Neolithic chronology: Phase II (2300–1800 cal BC) and Phase III (1800-1200 cal BC). These materials were identified primarily as two species, *B. ramosa* and *Setaria verticillata* (bristly foxtail millet-grass). *S. pumila* was present in limited quantity, possibly gathered from the wild (Fuller et al. 2001). The first known occurrences of various cereals in the Harrappan Civilization are reported as wheat, barley, and oats in the Early phase (before 2600 BC); *Eleusine* sp. (problematic, *E. coracana*), *Setaria* sp., *Panicum* sp. in the Mature phase (2600-2000 BC); and *Paspalum* sp., *Echinochloa* sp., *Sorghum* sp., and *Pennisetum* sp. in the Late phase (more recent than 2000 BC) (Fuller and Madella 2000; Weber 1992).

Many new techniques using DNA markers, including SSR (simple sequence repeat), RAPD (random amplified polymorphic DNA), RFLP(restriction fragment length polymorphism), and AFLP (amplified fragment length polymorphism analysis), have been conducted for the genus *Setaria* (Benabdelmouna et al. 2001; d'Ennequin et al. 2000; Fukunaga et al. 2002; Lin et al. 2012). Intraspecific polymorphic variability revealed with RAPD and RFLP marker systems was negligible. AFLP has gained wide acceptance for enabling a high degree of resolution and reproducibility in genetic analysis (Lakshmi et al. 2002). AFLP has a number of other relevant applications and advantages for analysis of plant genomes in general. A large number of DNA loci can be assayed in each reaction, and a large number of fragments can be assayed with a relatively small number of primers. Intergeneric polymorphism revealed by AFLP markers was very high (94.4%). At inter-specific level, it was not significant enough AFLP analysis recorded a higher level of variation, 66.5%, between *Panicum miliaceum* and *P. sumatrense* (Bai et al. 1999). Information on intraspecific diversity and species relationships could form the basic foundation for further research on crop-improvement programs (Lakshmi et al. 2002).

GISH (Genomic in situ hybridization) patterns revealed that two diploid species (2n = 18), *S. viridis* and *S. italica*, bore genome AA and a tetraploid species (2n = 36), *S. verticillata*, had genome AABB. The genomic composition of *S. pumila* (polyploid species, 2n = 18, 36, 72) was unknown (Benabdelmouna et al. 2001).

S. pumila is a cosmopolitan weed distributed worldwide. This weed grows sympatrically on roadsides,

uplands, and the levees of lowlands; four intraspecific types of *S. pumila* have been identified based on ecological habit: weed type (W), companion weed type accompanied by crops (Wx), mimic companion weed type accompanied by crops (Mx), and domesticated type mixed with crops (Dx). Kimata et al. (2000 and 2015a) have shown the biocultural diversity of morphological and ecological characteristics in *S. pumila*, and the intraspecific differentiation of vernacular names (linguistic data). The present paper concerns the domestication process of *S. pumila*, which is related ecologically to weeds and several grain crops in the Indian subcontinent, based on cluster analysis of morphological characteristics and AFLP markers.

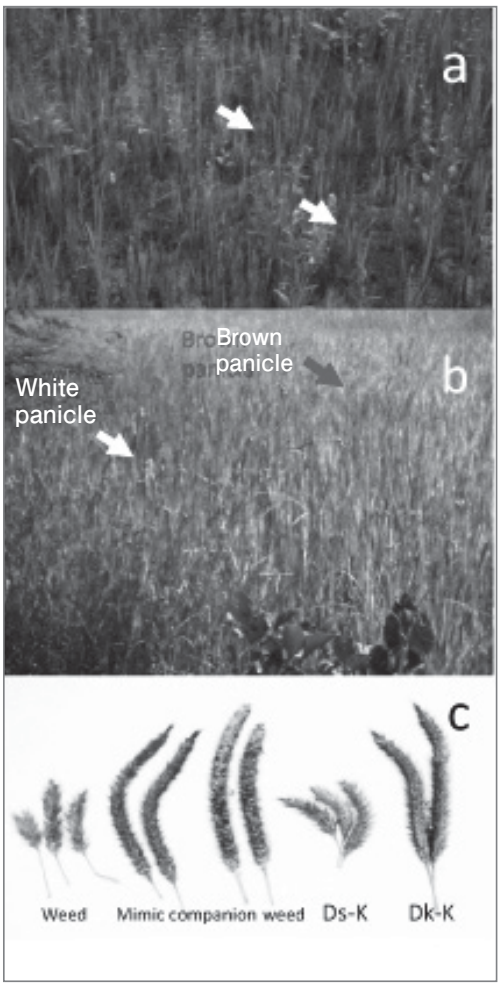

Fig. 1. Fields of *Setaria pumila* mixed with *Pas. scrobiculatum* (a) and with *P. sumatrense* (b) in South India, and spikes of *S. pumila* (c): weed; mimic companion weeds; Ds-K, domesticated type mixed with *P. sumatrense*, and Dk-K mixed with *Pas. scrobiculatum* in Karnataka.

Materials and Methods

Many local varieties and relative weeds of *Setaria pumila* (Poir.) Roem. & Schult. (syn. *S. glauca* [L.] P. Beauv.) have been collected from the Indian subcontinent since 1983 in field surveys (Fig. 1). Concentrated surveys were conducted in Karnataka, Andhra Pradesh, and Orissa (Fig. 2). At the same time, accompanying millet and weed species were examined in five plots (1 m^2) in each of four typical cropping fields (sites) using the quadrat method. Voucher herbarium specimens and grain samples were collected along the survey route and deposited at Tokyo Gakugei University (Tokyo, Japan) and University of Agricultural Sciences (Bangalore, India). Information on agricultural practices, grain processing, food preparation, and vernacular plant names was gathered from local farmers.

The experimental strains (n = 78, Table 1) were selected from these accessions and grown in the greenhouse at Tokyo Gakugei University to compare their morphological and ecological characteristics . In addition, three relative species of *S. pumila*: *S. italica* (n = 6, from Japan), *S. viridis* (n = 2, from Kazakhstan and Uzbekistan), and *S. verticillata* (n = 3, from India) were grown using the same methods.

Ten grains each of 60 strains were sown in a seeding box with row spacing of 8 cm and seed spacing of 2 cm on early June 6, 2002. Two weeks after sowing, germinated plants were transplanted into the greenhouse, with 30-cm row spacing and 15 cm between plants. Chemical fertilizer (N:P:K = 8:8:5) was supplied at 100 $g \cdot m^{-2}$. The following parameters of five types of *S. pumila* were measured at the each full-ripe stage: number of tillers, plant height , length and width of spike, length and width of flag leaf, last internode diameter, and duration to flowering. These types were three weed types; W, Wx, Mx associated with other grain crops, and two domesticated types; Dx mixed with *Paspalum scrobiculatum* and *Panicum sumatrense*. The lowercase character "x" indicates a main crop mimic of *S. pumila* as follows: "p" (paddy, *O. sativa* L.), "k" (*kodora*, *P. scrobiculatum*), "s" (*samai*, *P. sumatrense*),

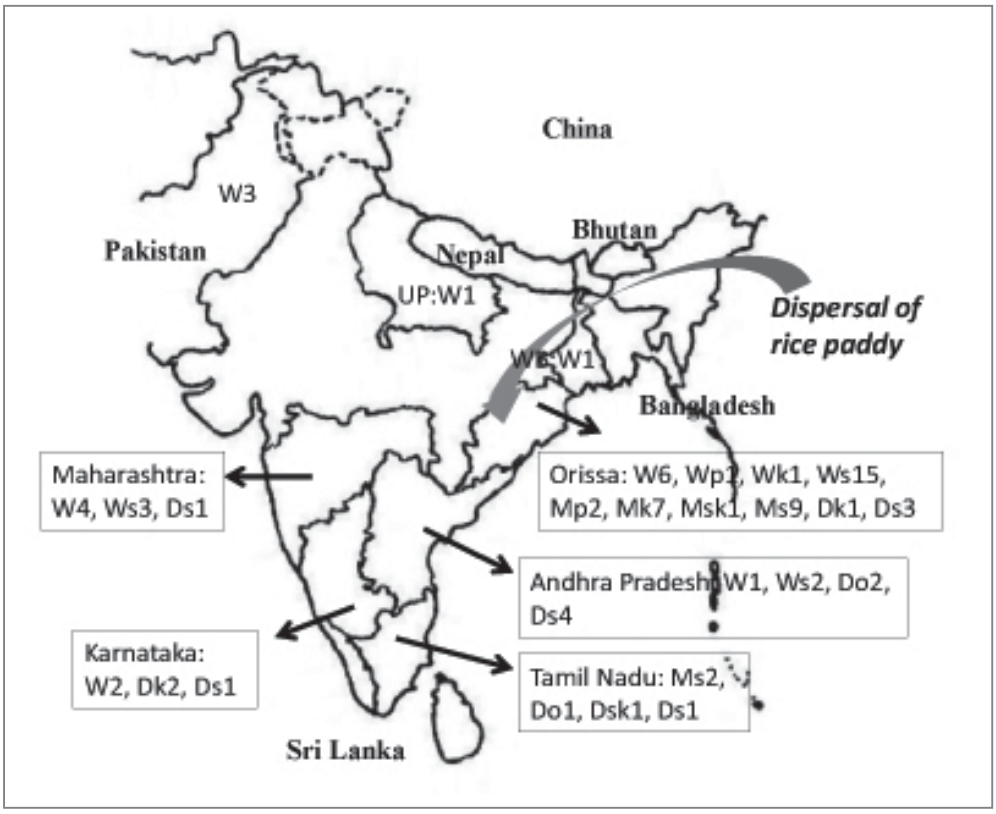

Fig. 2. Sites from which *S. pumila* and the related species were collected in the Indian subcontinent.

and "o" (others, e.g., *Eleusine coracana*). These data were analyzed statistically using partial correlation coefficients and hierarchical cluster analysis (Ward method) by SPSS version 21 (IBM Corp).

Ten grains of 78 strains were sown by the same method above-mentioned on Oct. 4, 2007. DNA extraction was performed on young leaf tissue ground in liquid nitrogen and incubated in 1.5-ml tubes containing 0.5 ml of buffer A for 10 min at 60 °C using CTAB (hexadecyl-trimethyl-ammonium bromide) methods (Murray and Thompson 1980). Buffer A contained 2% CTAB, 1% PVP (polyvinylpyrrolidone), 1.4 M NaCl, 0.25 M sucrose, 0.2% 2-mercaptoethanol, 20 mM EDTA, and 100 mM TRIS-HCl (pH 8.0); ultra pure water was added to bring the solution to 100 ml. We then added 0.5 ml of buffer B into the tube, suspended the mixture and then centrifuged it for 10 min at 10,000 g. Buffer B contained 2% CTAB, 1% PVP, 1.4 M NaCl, 1.1 M sucrose, 0.2% 2-mercaptoethanol, 20 mM EDTA, and 100 mM TRIS-HCl; water was added to bring the solution to 100 ml. The supernatant (approximately 700 µl) was transferred to a new tube containing 700 µl of chloroform/isoamyl alcohol (24/1), and the tubes were shaken gently for 5 min and then centrifuged for 10 min at 10,000 g. The supernatant (approximately 650 µl) was transferred to a new tube containing 700 µl of chloroform/isoamyl alcohol, and the tubes were shaken for 5 min and then centrifuged for 10 min at 10,000 g. The supernatant (approximately 600 µl) was transferred to a new tube containing 700 µl of isopropanol to precipitate DNA for 20 min. DNA pellets were collected by centrifugation at 14,000 g for 10 min, and then were washed in 70% ethanol, dried twice, and resuspended in 30 µl TE buffer (1×) containing RNAse at 4 °C for 3 to 4 d, followed by storage at –20 °C. TE buffer (800 µl) was added to 16 µl of RNAse (10 mg/ml).

AFLP procedure

The AFLP procedure was performed according to Applied Biosystems (2005), Bai et al. (1999), and Suyama (2001) with some modifications. Briefly, 1 µl of

Table 1. Materials used of *Setaria pumila*

Sample no. & Status	Main crop and remarks	Collection no.	Locality
1Ds-A	*Panicum sumatrense* mixed with *Eleusine coracana*	85-10-31-3-12	Duggamvapalli, Andhra Pradesh
2Ws-M	*P. sumatrense*	k87-9-28-9-4	Kumbharoshi (800m), Maharashtra
3Ws-M	*P. sumatrense*	k87-9-28-9-6	
4Ds-M	*P. sumatrense*	k87-10-1-7-8	16km from Lanja (200m), Maharashtra
5W-M	none	k87-10-3-3-1	Gabi (650m), Maharashutra
6Ws-M	*P. sumatrense*	k87-10-3-5-7	Nadagao village (541m), Maharashtra
7W-M	*Oryza sativa*	k87-10-4-6-7	8km W from Kolhapur (600m), Maharashtra
8W-M	*Setaria italica*	k87-10-5-10-5	Udtare village (652m), Maharashtra
9W-M	*S. italica*	k87-10-5-10-6	Udtare village (653m), Maharashtra
10Ms-O	*P. sumatrense*	k87-10-9-1-1	Sunabeda (895m), Orissa
11Ms-O	*P. sumatrense*	k87-10-9-1-6	
12Ms-O	*P. sumatrense*	k87-10-9-1-7	
13Ms-O	*P. sumatrense*	k87-10-9-1-8	
14Wsk-O	*P. sumatrense* mixed with *Paspalum scrobiculatum*	k87-10-9-2-2	Kundali village (875m), Orissa
15Ws-O	*P. sumatrense*	k87-10-9-5-6	Potang (895m), Orissa
16W-O	none	k87-10-10-2-1	7km from Sunabeda (900m), Orissa
17Ws-O	*P. sumatrense*	k87-10-10-5-5b	2km of Boiparigurha (608m), Orissa
18Ws-O	*P. sumatrense*	k87-10-10-5-6b	
19Ws-O	*P. sumatrense*	k87-10-10-5-10d	
20Ws-O	*P. sumatrense*	k87-10-10-5-13A	
21Ws-O	*P. sumatrense*	k87-10-10-5-13B	
22Ds-O	*P. sumatrense*	k87-10-10-5-14e	
23Ds-O	*P. sumatrense*	K87-10-10-5-16A	
24Ds-O	*P. sumatrense*	k87-10-10-5-16B	
25Ws-O	*P. sumatrense*	k87-10-10-6-8	Beragaon, 12km of Koraput (605m), Orissa
26Mk-O	*Pas. scrobiculatum*	k87-10-11-2-2	Anchalguda village, 20km of Kolaput (870m), Orissa
27Dk-O	*Pas. scrobiculatum*	k87-10-11-2-3	
28Mk-O	*Pas. scrobiculatum*	k87-10-11-2-5	
29Ws-O	*P. sumatrense*	k87-10-11-6-7	Damaniganda village (728m), Orissa
30Ms-O	*P. sumatrense*	k87-10-11-6-8	
31W-O	none	k87-10-12-2-3	Sagada village (240m), Orissa
32W-O	none	k87-10-12-2-7	
33Ws-O	*P. sumatrense*	k87-10-12-5-4	47km NW of Bhawanapatna (690m), Orissa
34Ws-O	*P. sumatrense*	k87-10-12-5-5	
35Ms-O	*P. sumatrense*	k87-10-12-5-7	
36Ws-O	*P. sumatrense*	k87-10-12-5-8	
37Wp-O	*Oryza sativa* mixed with *Pas. scrobiculatum*	k87-10-12-6-2	Balsora village (690m), Orissa
38Mp-O	*O. sativa* mixed with *Pas. scrobiculatum*	k87-10-12-6-3	
39Mp-O	*O. sativa* mixed with *Pas. scrobiculatum*	k87-10-12-6-4	
40Ws-O	*P. sumatrense*	k87-10-12-7-4	Duliguda village,11km of Gopalpur(922m), Orissa
41Ws-O	*P. sumatrense*	k87-10-12-7-5	
42Ws-O	*P. sumatrense*	k87-10-12-8-4	Dakuta (937m), Orissa
43Wk-O	*Pas. scrobiculatum*	k87-10-13-4-14	Puda Pali village (269m), Orissa
44Mk-O	*Pas. scrobiculatum*	k87-10-13-5-6	12km of Kharhiar (272m), Orissa
45Mk-O	*Pas. scrobiculatum*	k87-10-13-5-11	
46W-O	none	k87-10-14-2-1	Mandiapadar village (139m), Orissa
47W-O	none	k87-10-14-2-3	
48W-O	none	k87-10-14-2-4	
49Mk-O	*Pas. scrobiculatum*	k87-10-14-4-3	Budhitadar village (146m), Orissa
50Mk-O	*Pas. scrobiculatum*	k87-10-15-1-6	Ramisarda Tilemal (149m), Orissa
51Ms-O	*P. sumatrense*	k87-10-16-2-3	Kolarapaju village (766m), Orissa
52Ms-O	*P. sumatrense*	k87-10-16-2-4	
53Mk-O	*Pas. scrobiculatum*	k87-10-16-3-4	Bekarakhol village, 30km of Phulabani (522m), Orissa
54Ms-O	*P. sumatrense* mixed with *E. coracana*	k87-10-16-5-4	4km from Tikaball (569m), Orissa
55W-W	none	k87-11-7-0-26	Kalimpong, West Bengal
56Dk-K	*Domesticated type, a few mixed in Pas. scrobiculatum*	96-11-5-1a-2	Kalidevapura, Karnataka
57Ds-K	*A few mixed with P. sumatrense*	96-11-5-2b-6	Madhagiri, Karnataka
58Dk-K	*A little shattering, only one plant mixed with Pas.*	96-11-5-7-2	
59Ds-A	*P. sumatrense*	97-4-12-2-2	Jalaripalli, Andhara Pradesh
60Ds-A	*P. sumatrense*	97-4-12-2-3	
61W-U	weed mixed with *Echinochloa frumentasea*	96-11-17-0-1	Ranichauri, Uttar Pradesh
63Ws-A	*P. sumatrense*	01-10-8-1-5	Mulbagal, Andhra Pradesh
64Ws-A	*P. sumatrense*	01-10-8-2-5	Palmaner, Andhra Pradesh
66Ds-A	*P. sumatrense*	01-10-9-2-4	Dombarpally, Andhra Pradesh
69Ws-O	*P. sumatrense*	01-10-19-2a-3	Polehorebrdle, Orissa
70Ds-T	*P. sumatrense*	85-10-28-1-1	Morumu, Tamil Nadu
71Do-A	mixed stand	85-11-10-1-11	Gandrajupalli, Andhra Pradesh
72Do-A	mixed stand	85-11-10-1-16	
73W-A	mixed stand	85-11-10-1-18	
75W-P	*Vigna mungo*	85-9-15-5-2	39km from Abbottabad to Hazara, Pakistan
76W-P	mixed stand	89-9-29-3-3-5	47km from Muzafabad, Pakistan
762W-P	mixed stand	89-9-29-3-3-6	
77Dsk-T	*P. sumatrense* and *Pas. scrobiculatum*	89-10-25-3-7	Bawalia village, Mandia, Tamil Nadu
81W-K	*S. pumila* ssp. *pallide-fusca*, mixed stand	85-10-16-3-2	Namanahalli, Karnataka
82W-K	*S. pumila* ssp. *pallide-fusca*, mixed stand	85-10-17-3-3	Honnavara, Karnataka
84Do-T	mixed stand	85-10-27-3-6	Vellakadai (Goundar tribe), Tamil Nadu
85Ms-T	*P. sumatrense*	85-10-23-2-15	Kollimalai (Kotha tribe), Tamil Nadu
86Ms-T	*P. sumatrense*	85-10-23-2-7	

Sample number and status: W, weed type; M, mimic weedy medium type; D, domesticated type. Main crop: s, *samai* (*Panicum sumatrense*); k, *kodo* (*Paspalum scrobiculatum*); p, paddy (*Oryza sativa*); o, other species. Locality: A, Andhara Pradesh; K, Karnataka; M, Maharashtra; O, Orissa; P, Pakistan; T, Tamil Nadu; U, Uttar Pradesh; W, West Bengal

Table 2. Partial correlation coefficients of morphological characters in *Setaria pumila*

characteristics	tillers	plant height	spike length	spike width	sl/sw	flag leaf length	flag leaf width	fll/flw	first node diameter	dulation to flowering
tillers	1	−0.142	−0.055	−0.410*	0.221	0.166	−0.289	0.301	−0.239	−0.095
plant height	−0.142	1	0.256	−0.001	0.086	−0.224	0.404*	−0.517**	0.388*	0.211
spike length	−0.055	0.256	1	0.151	0.739**	0.664**	0.584**	0.166	0.716**	−0.242
spike width	−0.410*	−0.001	0.151	1	−0.455*	−0.132	0.254	−0.251	0.227	−0.091
sl/sw	0.221	0.086	0.739**	−0.455*	1	0.704**	0.172	0.488*	0.292	−0.227
flag leaf length	0.166	−0.224	0.664**	−0.132	0.704**	1	0.194	0.720**	0.311	−0.544**
flag leaf width	−0.289	0.404*	0.584**	0.254	0.172	0.194	1	−0.508**	0.882**	0.122
fll/flw	0.301	−0.517**	0.166	−0.251	0.488*	0.720**	−0.508**	1	−0.35	−0.561**
first node diameter	−0.239	0.388*	0.716**	0.227	0.292	0.311	0.882**	−0.35	1	0.171
dulation to flowering	−0.095	0.211	−0.242	−0.091	−0.227	−0.544**	0.122	−0.561**	0.171	1

Control variables : grain size、shattering

each genomic DNA (was digested with 0.3 µl of *Eco*RI adapter (TAKARA), 0.3 µl of *Mse*I adapter (New England), 2.0 µl of 10× reaction buffer H, and 7.4 µl of H_2O in a final volume of 20 µl for 3 h at 37 °C. After incubation, ligation of adapters corresponding to the sticky ends of both enzymes was performed. Adapter mix (*Eco*RI, *Mse*I, 2.0 µl), 0.5 µl of T4 DNA ligase (TAKARA), 1.5 µl of 10× reaction buffer, and 3.5 µl H_2O were added to 7.5 µl of the digested DNA. The resulting reaction mix was incubated for 5 min at 95 °C.

A preselective amplification step using non-selective primers was then performed in a total volume of 20 µl containing 15 µl of the AFLP Amplification Core Mix, 1 µl of preselective primer pair (Eco A/Mse C), and 4 µl of ligation mixture (diluted 5 times in 10 mM Tris buffer, pH 8.0). The core mix contains all of the components necessary to amplify modified target sequences, e.g. buffer, nucleotides, and AmplTaq DNA polymerase. The polymerase chain reaction (PCR) program was the same as that described by AFLP Plant Mapping Protocol (Applied Biosystems 2005) using a PCR thermal cycler (TAKARA TP3000). Selective amplifications were performed in a 20-µl final volume containing 3 µl of pre-amplification products (diluted in 10 mM Tris buffer) with primers having three additional 3′ nucleotides. Amplification reactions were performed according to the same protocol. Five primers associated with *Eco*RI (E+AAC; E+AAG; E+AGG; E+ACT; E+ACA) were used in combination with 5 primers associated with *Mse*I (M+CAG; M+CTG; M+CTA; M+CAT; M+CAA). Five microliters of amplification products were loaded onto a 5.75% denaturing polyacrylamide gel (LONZA) and electrophoresed in 1× TBE for 1 h. Bands were detected using the silver staining protocol described by Cho et al. (1996).

Data analysis

The bands were detected on the gel at the finest level of sensitivity by Lane Analyzer (ATTO), the raw data were adjusted, and then the visible and reproducible bands were scored for accessions as present (1) or absence (0). The dendrogram of the AFLP markers was constructed using the neighbor-joining method and bootstrap analysis (PAUP* version 4.0) on all data matrices (Nei and Kumar 2000).

Results

Morphological characteristics

Results of statistical analyses of partial correlation coefficients of the ten characteristics (number of tillers, plant height, length (pl) and width (pw) of spike, the ratio of pl/pw, length (fll) and width (flw) of flag leaf, the ratio of fll/flw, last internode diameter, and duration to flowering, are shown in Table 2. Those characteristics have been strongly affected by artificial selection during the domestication process. The controlled variables were seed size and seed shattering in this analyses. Statistical significance at the 1% level was found for the following

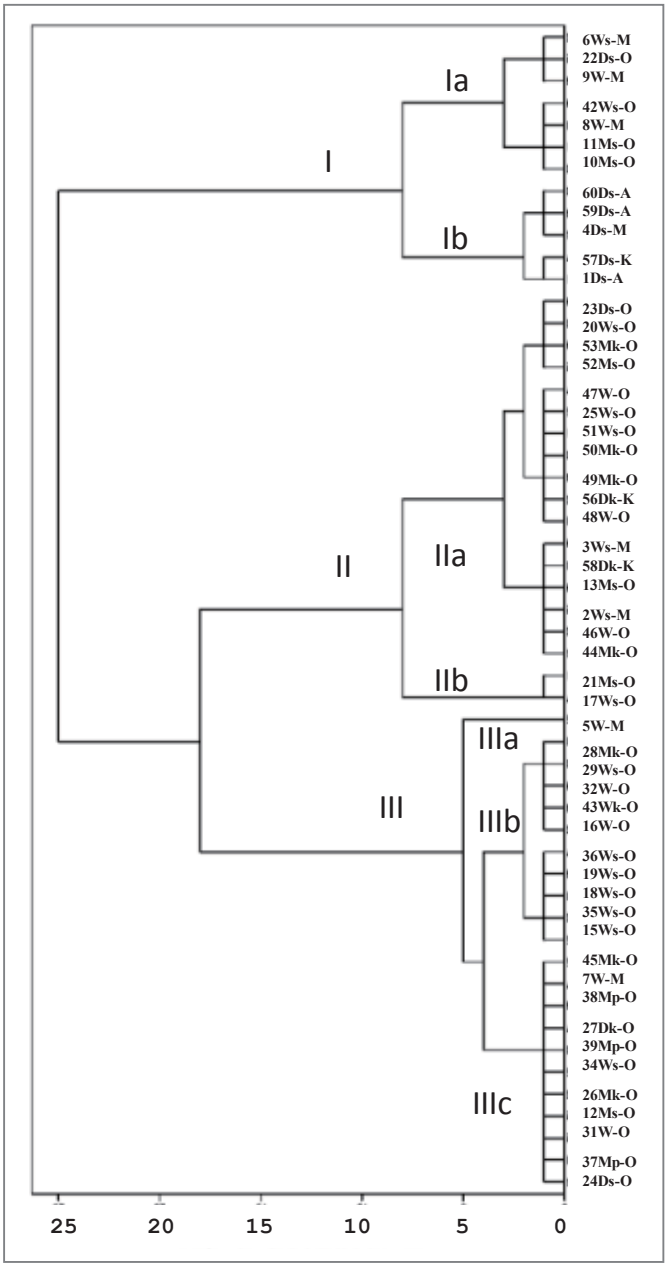

Fig. 3. Dendrogram of cluster analysis based on morphological characteristics.

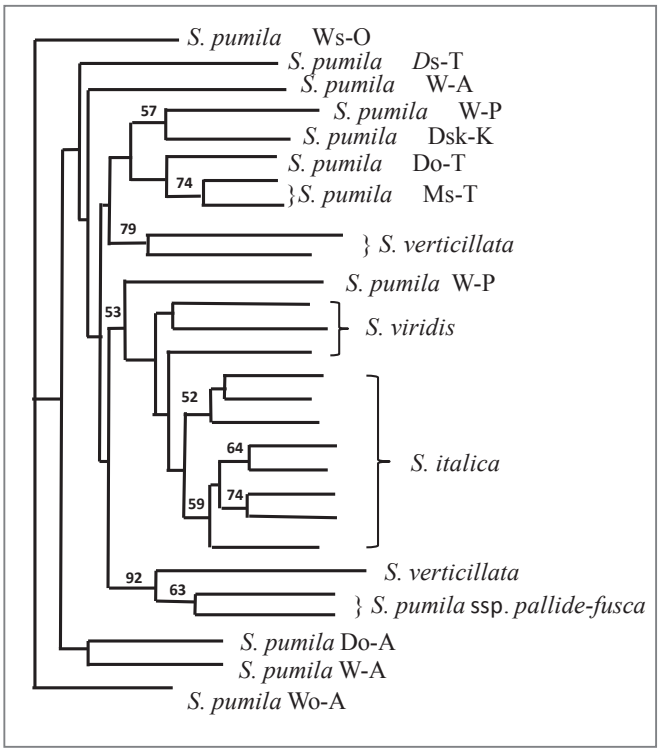

Fig. 4. Dendrogram of neighbor-joining method based on AFLP markers of genus *Setaria*

results: ratio of length/width of flag leaf to plant height (-0.517); ratio of spike length/width (0.739), length of flag leaf (0.664), width of flag leaf (0.584), and diameter of last internode (0.716) to spike length; spike length (0.739) and length of flag leaf (0.704) to the ratio of spike length/width; spike length (0.664), the ratio of spike length/width (0.704), the ratio of length/width of the flag leaf (0.720), and the duration to flowering (-0.544) to length of flag leaf; spike length (0.584), the ratio of length/width of the flag leaf (-0.508), and the last internode diameter (0.882) to width of flag leaf; plant height (-0.517), length of flag leaf (0.720), width of flag leaf (-0.508), and the duration to flowering (-0.561) to the ratio of length/width of the flag leaf; spike length (0.716) and width of flag leaf (0.882) to the last internode diameter; and length of flag leaf (-0.544) and the ratio of length/width of the flag leaf (-0.561) to the duration to flowering. There were no significant ($p < 0.01$) correlations between the number of tillers and the last internode diameter.

Cluster analysis of six morphological characteristics (number of tillers, plant height, spike length, length and width of flag leaf, and flag leaf length/width ratio) and the duration to flowering are illustrated in Fig. 3. Using the Ward method, 60 accessions were categorized into three clusters and several sub-clusters. Cluster I contained sub-clusters Ia and Ib. Subcluster Ia (7 accessions) included weed type (W2); companion weed type (Ws1) from Maharashtra; companion weed type (Ws1); mimic companion weed type (medium, Ms2); and domestication type mixed with *samai* (*P. sumatrense*, Ds1) from Orissa. Sub-cluster Ib

Table 3. AFLP analysis of 72 accessions of *Setaria pumila* using 4 combinations of promer pairs

EcoRI/MseI	Total no. of bands	No. of polymorphic	Percent polymorphism
AAC/CAG	59	52	88.1
ACT/CAT	60	49	81.7
AGG/CTA	68	64	94.1
AAG/CTG	76	71	93.4
Total	263	236	89.7

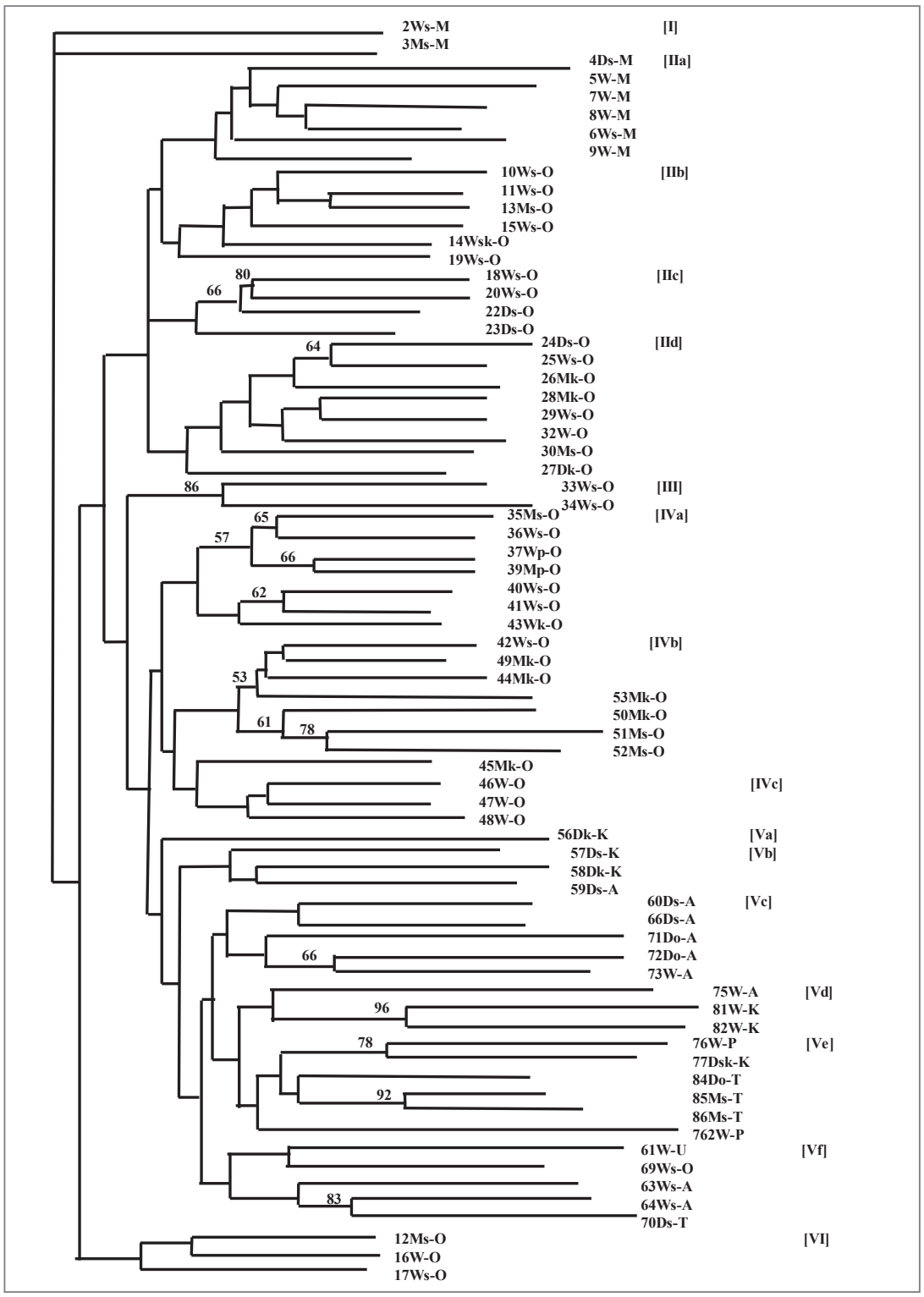

Fig. 5. Dendrogram of neighbor-joining method based on AFLP markers of *S. pumila*.

(5 accessions) included Ds5 from Andhra Pradesh (3), Karnataka (1), and Maharashtra (1). Cluster II contained sub-clusters IIa and IIb. Sub-cluster IIa (17 accessions) included: Ds1, Ms2, Mk4, Ws3, and W3 from Orissa; Dk2 from Karnataka; and Ws2 from Maharashtra. Sub-cluster IIb (2 accessions) included Ms1 and Ws1 from Orissa. Cluster III contained sub-clusters IIIa–c. Sub-cluster IIIa (1 accession) comprised W1 from Maharashtra. Sub-cluster IIIb (10 accessions) included Mk1, Ws6, Wk1, and W2 from Orissa. Sub-cluster IIIc (11 accessions) included Ds1, Dk1, Ms1, Mk2, Mp3, Ws1, and W1 from Orissa, and W1 from Maharashtra. The "W" type of *S. pumila* was distributed around the Indian subcontinent as a cosmopolitan weed.

Variation of AFLP markers

The results of AFLP on 72 accessions from the Indian subcontinent are shown in Table 3. Most bands showed polymorphic more than 81.7% to 94.1% polymorphisms, excluding the main bands were detected more than 70% of all accessions. Each combination of *Eco*RI and *Mse*I detected from 59 to 76 fragments based on the four primer pairs selected, and were used for analysis as follows: E+AAG/M+CTG, E+AGG/M+CTA, E+ACT/M+CAT, and E+AAC/M+CAG. Those primer combinations revealed 263 visible polymorphic bands. The dendrogram accounted for accessions of related species on one hand (Fig. 4) and the accessions of *S. pumila* on the other (Fig. 5).

The diversity of AFLP markers was compared among relative species (28 accessions) of *S. pumila* (14 including ssp. *pallide-fusca* 2), domesticated *S. italica* (8 from Japan), the ancestral weed *S. viridis* (3 from Central Asia), and the weed *S. verticillata* (3 from India). The dendrogram constructed with the neighboring-joint method is illustrated in Fig. 4. The clusters of *S. pumila* were composed, successively, of Ws1 from Orissa; Wo1, Do1, and W1 from Andhra Pradesh; Ds1 from Tamil Nadu; and W1 from Andhra Pradesh. The other clusters included W1 from Pakistan, Dsk1 from Karnataka, and Do1 and Ms2 from Tamil Nadu. *S. pumila* ssp. *pallide-fusca* (2) from Karnataka and *S. verticillata* (1) from Andhra Pradesh formed a cluster. W1 of *S. pumila* from Pakistan was located as the neighbor of *S. viridis*. *S. verticillata* (2) was located in the cluster of *S. pumila*, but *S. viridis* (3) and *S. italica* (8) were located in the same cluster. The location of species within clusters was not significant at $p \leq 0.05$ based on the bootstrap test, but the species were clearly categorized.

S. pumila (72 accessions) were divided into six clusters including 16 sub-clusters based on AFLP marker data as shown in Fig. 5. Cluster I contained Ws1 and Ms1 from Maharashtra; Cluster III consisted of Ws2; and Cluster VI contained W1, Ws1, and Ms1 from Orissa. These clusters did not containea domesticated type.

Cluster II (4 sub-clusters, 23 accessions) consisted of sub-cluster IIa (6), W4, Ws1, and Ds1 from Maharashtra; sub-cluster IIb (6), Ws (4), Ms1, and Wsk1 from Orissa; sub-cluster IIc (4), Ws2 and Ds2 from Orissa; and sub-cluster IId (8), W1, Ws2, Ms1, Ds1, Mk2, and Dk1 from Orissa. Cluster IV (three sub-clusters, 18 accessions from Orissa) consisted of sub-cluster IVa (7), Wp1, Mp1, Wk1, Ws3, and Ms1; sub-cluster IVb (7), Ws1, Ms2, and Mk4; sub-cluster IVc (4), W3, and Mk1. Cluster V (6 sub-clusters, 23 accessions) consisted of sub-cluster Va (1), Dk1 from Karnataka; Vb (3), Ds1, Dk1 from Karnataka, and Ds1 from Andhra Pradesh; Vc (5), W1, Do2 and Ds2 from Andhra Pradesh; Vd (3), W1 from Andhra Pradesh and W2 from Karnataka; Ve (6), W2 from Pakistan, Ms2, Dsk1, and Do1 from Tamil Nadu; Vf (5), W1 from Utter Pradesh, Ws1 from Orissa, Ws2 from Andhra Pradesh, and Ds1 from Tamil Nadu.

Discussion

The domestication process for each species was a complex combination of natural and artificial selection, mimicry, hybridization, and polyploidy. Pioneer farmers required plants some to have some degree of tolerance to conditions (e.g., cold, hot, drought, harsh sunlight). Farmers continue to gather wild cereals in dry areas of Africa and the Indian subcontinent. For example,

Secale cereale L. has acquired strong resistance to cold in high altitude or latitude areas, and farmers have been able to grow *S. cereale* mixed with wheat as a secondary crop as a companion weed under severe conditions (Vavilov 1926). Kobayashi (1987, 1989) proposed an integrated model of the domestication process of several millet species as secondary crops derived from weeds by mimicking companion weeds associated with *Oryza sativa* in the Indian subcontinent.

Increasing the size and shattering resistance in seeds are important factors in the domestication process. The partial correlation coefficients that describe control of seed size and shattering (Table 2) explain that the cylindrical spike has become longer, the last internode diameter of the main culm has thickened, and the flag leaf has widened for effective photosynthesis as a result of artificial selection by farmers.

The low coefficient between the number of tillers and the other characteristics reveals that the number of tillers in Dk has decreased during domestication by processes such as mimicry of *Pas. scrobiculatum*, while the number of tillers of Ds has increased as the mimicry of *P. sumatrense*. Separate selection processes functioned to both decrease and increase the number of tillers (Kimata 2015a). The low coefficients for length of flag leaf and ratio of length/width of the flag leaf to duration of flowering indicate that artificial selection has operated on the flag leaf, causing it to become narrower and to mature early under domestication.

The negative correlation between ratio of length/width of the flag leaf to plant height demonstrated that the flag leaf has become longer and narrower, while plant height has increased, as in Ds. The Ds of *S. pumila* matures early and has a relatively long and narrow flag leaf due to artificial selection, reflected in the significant negative correlations between length of flag leaf and the ratio of length/width of the flag leaf in relation to duration to flowering. In addition, Ds has acquired a relatively long and narrow flag leaf as a result of taller plant height, as seen in the significant negative correlation between plant height and length/width of flag leaf.

During the evolutionary process from companion weed to secondary crop, which involved morphological mimicry of other species (Mo), *S. pumila* (Ds) became a slender-type mimic with long-narrow leaves as in *P. sumatrense*, while *S. pumila* (Do) became a thick-type mimic with wide leaves as in *Pas. scrobiculatum* and other species. Based on the PANTONE Formula Guide (Pantone Inc.), it was clear that the leaf, leaf sheath, culm, and glume of *S. pumila* exhibited mimetic coloration among species and demonstrated mimicry of coloration of *P. sumatrense* and *Pas. scrobiculatum*, according to anthocyanin composition revealed by HPLC analysis (Kimata 2015a).

From the cluster analysis, *S. pumila* cluster I clearly showed that the domestication process of *S. pumila* has occurred continuously in fields of *P. sumatrense* and other grain crops around the Deccan. Cluster II consisted of sub-cluster IIa; Ds1, Ms2, Mk4, Ws3 and W3 from Orissa, Dk2 from Karnataka, Ws2 from Maharashtra, and sub-cluster IIb; Ms1 and Ws1 from Orissa. Cluster II gives an example of the domestication process by mimicry, which *S. pumila* has become Dsk mixed with *P. sumatrense* and *Pas. scrobiculatum* in each fields. Cluster III revealed that the W type of *S. pumila* was distributed around the Indian subcontinent as a cosmopolitan weed.

The domestication process of *S. pumila*, Dsk, has taken a route from weed type to companion weed and then to mimic companion weed with *O. sativa*, *P. sumatrense*, and *Pas. scrobiculatum* in Orissa. Therefore, the domestication process of *S. pumila* has moved forward as follows. First, the mimic companion weeds (Mks, mainly in Orissa) and second, the domesticated type (Do) evolved and moved south to the Deccan Plateau via Andhra Pradesh. After that, the domesticated type progressed from Dk to Ds in Karnataka and Tamil Nadu.

The natural intraspecific hybrids of *S. pumila* occurred continually in sympatric fields among weeds, companion weeds, mimic companion weeds, and domesticated types, as revealed by a geographic bias in both morphological characteristics and AFLP markers

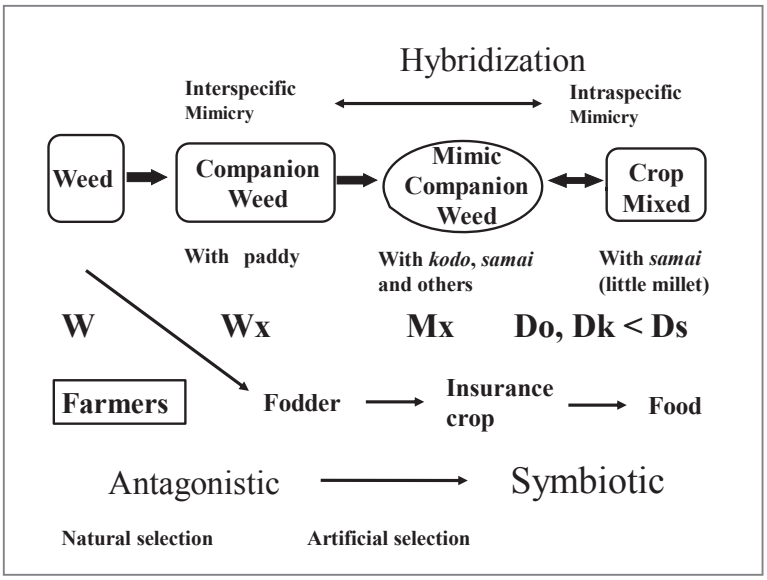

Fig. 6. Domestication process of *S. pumila* in relation to the weed-crop complex.

(Figs. 3 and 5). There were two cases of mimicry, interspecific and intraspecific, in this domestication process. Interspecific mimicry was found in the mimic companion weed with *O. sativa*, *Pas. scrobiculatum*, and *P. sumatrense* and other species. The intraspecific mimicry occurred by continuous natural hybridization between weed and domesticated type and also though natural or artificial selection by farmers. The mimic companion weed type was quite similar to the domesticated type, but farmers were able to differentiate between the two by seed shattering.

The diversity of AFLP markers was compared among relative species of *S. pumila*, domesticated *S. italica*, the ancestral weed *S. viridis*, and another weed, *S. verticillata* (see dendrogram in Fig. 4). Recently, Wang et al. (2009) and Zhao et al. (2013) indicated that the genome constitution of *S. verticillata* had diploid (BB) and tetraploid (AABB) forms based on GISH, while *S. glauca* (syn. *S. pumila*) was identified genome 'D,' but its genomic constitution was not known. Based on the dendrograms by Bayesian analyses for 5s rDNA and kn1 sequences, the A genome included *S. italica*, *S. viridis*, and *S. verticillata*; the B genome comprised *S. verticillata*, and the D genome consisted of *S. glauca* (syn. *S. pumila*). *S. pumila* (W-P) from Pakistan was located the neighbor of *S. viridis* in Fig. 4. *S. pumila* ssp. *pallide-fusca* from Karnataka and *S. verticillata* from Andhra Pradesh made a cluster. The irregular positions in which *S. pumila* was located related to its multiple ploidy levels and obscure genomic constitution.

The AFLP methodology gave highly reproducible bands, and polymorphisms among individuals within accessions were very low (d'Ennequin et al. 2000). Small millet species including *S. pumila* have shown remarkable genetical variation (Lakshmi et al. 2002) because of its polyploidy and natural hybridization. The AFLP variation in *S. pumila* was generally high because of the grouping of many sub-clusters, but the bootstrap values were low in each sub-cluster. Intraspecific morphological differentiation was easily detected, but the variation in AFLP was reduced by natural hybridization. Therefore, based on the AFLP dendrogram, which was not directly influenced by the artificial selection by farmers, it was obvious that there was a regional bias; many accessions of mimic companion weed type were located in sub-cluster IVb, and the most accessions of the domesticated type were in Cluster V with little significance in bootstrap value. Moreover, Cluster IV, from Orissa only, did not include the domesticated type but contained the most accessions of mimic companion weed type. Cluster II from Maharashtra and Orissa indicated that the companion/

mimic companion weeds coexist with the domesticated type mixed with other crops.

The domestication process of *S. pumila* may have passed through four steps as illustrated in Fig. 6. The first step consisted of a weed that had grown along roadsides and other unstable habitats moving to invade upland rice fields. The second step was an evolutionary process of obtaining an agro-ecological niche as use for fodder, to attain companion weed status in upland rice and millet fields. The third step was a process of advancing from mimic companion weed status to a semi-domesticated insurance crop in case of famine, under mixed cropping with *P. scrobiculatum*, *E. coracana*, and *P. sumatrense*. After their invasion into upland rice and millet fields and under the severe weed control measures practiced by farmers, weeds evolved to mimic particular crops and to create a close weed-crop complex. In the third step, farmers reduced the aggressiveness of their weed-control practices. In the fourth step, mimic companion weeds were used as both a fodder source for cattle and as a supplementary grain to the main cereal species. In the case of *S. pumila*, overly strict weeding was avoided as a means of crop insurance in years of extreme drought in the Deccan. This may have led to *S. pumila* growing taller with larger spikes and seeds accompanied by less shattering, and gradually progressing towards domestication. *S. pumila* has obtained mimetic traits such as a long leaves, a few tillers, and tall height, in fields of *P. sumatrense* (Fig. 2 and Table 2). The pigmentation of leaves and leaf sheaths by anthocyanin creates the mimicry among grain crops and closely related weeds in mixed crop stands (Kimata 2015a, Kimata et al. 2000).

S. pumila concurrently diversified its traits entirely through hybridization among the four types under natural and artificial selection in severely arid environments. Mimic companion weeds were harvested together with other (crop) millet, and were sown again involuntarily the following season. Recently, at the fourth step, this situation was followed by mixed cropping. *S. pumila* is termed a “tertiary crop” in relation to its associated plants, secondary crops such as *P. sumatrense* and *Pas. scrobiculatum*, with respect to rice. The domestication process of *S. pumila*, a tertiary crop mixed with other grain crops, proceeds from inter- and intraspecific mimicry by natural and artificial selection in sympatric fields. This process has occurred by adaptation to aridity as a result of the spread of *S. pumila* from the east to the south in the Indian subcontinent.

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