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Introgression from cultivated rice influences genetic differentiation of weedy rice populations at a local spatial scale

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Abstract Hybridization and introgression can play an important role in genetic differentiation and adaptive evolution of plant species. For example, a conspecific feral species may frequently acquire new alleles from its coexisting crops via introgression. However, little is known about this process. We analyzed 24 weedy rice (*Oryza sativa* f. *spontanea*) populations and their coexisting rice cultivars from northern Italy to study their genetic differentiation, outcrossing, and introgression based on microsatellite polymorphisms. A total of 576 maternal plants representing 24 weedy populations were used to estimate their genetic differentiation, and 5,395 progeny (seedlings) derived from 299 families of 15 selected populations were included to measure outcrossing rates. Considerable genetic differentiation ($F_{st} = 0.26$) was detected among weedy rice populations, although the differentiation was not associated with the spatial pattern of the populations. Private alleles (28%) were identified in most populations that exhibited a multiple cluster assignments, indicating stronger genetic affinities of some weedy populations. Outcrossing rates were greatly variable and positively correlated ($R^2 = 0.34$, $P = 0.02$) with the private alleles of the corresponding populations. Paternity analysis suggested that ~15% of paternal specific alleles, a considerable portion of which was found to be crop-specific, were acquired from the introgression of the coexisting rice cultivars. Frequent allelic introgression into weedy populations resulting from outcrossing with nearby cultivars determines the private alleles of local feral populations, possibly leading to their genetic differentiation. Introgression from a crop may play an important role in the adaptive evolution of feral populations.

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Introduction

Hybridization and subsequent introgression are important biological processes that occur naturally between related plant species. They may play an essential role in the genetic divergence and adaptive evolution of these species (Anderson and Stebbins 1954; Hamrick and Godt 1996; Barton 2001; Charlesworth 2003). These processes can create a substantial number of new genotypes via genetic recombination (Stebbins 1959, 1969; Abbott 1992; Arnold 1997; Rieseberg et al. 2003). Some of these genotypes can enhance the fitness of the resultant hybrids and their offspring, potentially driving the adaptive evolution of introgressed populations (Ellstrand et al. 1999; Ellstrand and Schierenbeck 2000; Barton 2001). In addition, hybridization and introgression may encourage the formation of novel alleles (Rieseberg and Carney 1998), although this phenomenon needs further confirmation.

The evolutionary impact of hybridization and introgression would be particularly significant for feral populations that have originated either directly from domesticated ancestors (endoferality) or through hybridization of domesticates with wild relatives (exoferality) (Gressel 2005). Feral populations that inter-mate with their adjacent crop populations have an intimate evolutionary

relationship. Consequently, feral species can acquire and incorporate new alleles via hybridization and introgression from their coexisting crop populations under human management (Ellstrand et al. 1999; Jarvis and Hodgkin 1999). Introgression may result in genetic differentiation among feral populations as they accumulate varietal-specific alleles acquired from crops through time (Xia et al. 2011), possibly explaining why introgression from crops increases adaptability and invasiveness of weedy or wild relatives in agro-ecosystems (Ellstrand and Schierenbeck 2000; Ellstrand 2003; Song et al. 2004), altering the genetic structure of these species (Song et al. 2006; Xia et al. 2011). One study indicates that introgression from cultivated radish (*Raphanus sativus*) has led to an increase in the fecundity of jointed charlock (*R. raphanistrum*), enhancing its competitiveness (Campbell et al. 2006). However, knowledge on how introgression from a crop can influence the population genetic structure of the feral populations across a given geographic region is still limited.

Feral plants have evolved from the best-studied organisms and provide a good system for studying how hybridization and introgression influence their population genetics and adaptive evolution (Ellstrand et al. 2010). One well-known example is weedy rice (*Oryza sativa* f. *spontanea*), a feral taxon distributed worldwide (Olofsdotter et al. 2000; Delouche et al. 2007). As a notorious weed, weedy rice co-occurs with conspecific cultivated rice (*Oryza sativa*), competing for resources such as nutrients, water, and light (Kwon et al. 1992; Burgos et al. 2006). Consequently, this weed causes significant losses in grain yield and quality of rice (Delouche et al. 2007). Interestingly, weedy rice can also serve as an important germplasm source for rice genetic improvement (Heu et al. 1990; Suh 2008). Conspecific with the crop, weedy rice can hybridize freely with cultivated rice under natural conditions and produce normal offspring (Gealy et al. 2003; Zhang et al. 2003; Chen et al. 2004; Messeguer et al. 2004). Introgression from cultivated rice to weedy rice is frequently reported (Oka and Chang 1959; Langevin et al. 1990; Xia et al. 2011), and may have evolutionary impacts on weedy rice populations. Previous studies demonstrated that weedy rice tends to converge morphologically with rice varieties grown in the same field after a few generations (Oka and Chang 1959; Oka and Morishima 1971; Langevin et al. 1990). Such rapid evolution of "crop mimicry" (Barrett 1983) of weedy rice is most likely originated via introgression of alleles from cultivated rice, facilitating the adaptive evolution of weedy rice under natural and human selection (Delouche et al. 2007). In addition, studies based on molecular markers indicated that weedy rice populations have differentiated into "*indica*" or "*japonica*" types (Cho et al. 1995; Tang and Morishima 1996; Suh et al. 1997; Ishikawa et al. 2005). Such genetic differentiation of weedy rice populations is likely associated with the introgression from their two locally corresponding subspecies of rice (*indica* and *japonica*) through time.

Recently, the issue of introgression from crops into their weedy and wild relatives has aroused worldwide attentions because transgene introgression from genetically engineered (GE) crops may create undesirable ecological impacts (for reviews, see Ellstrand et al. 1999; Stewart et al. 2003). For example, the introgression of fitness-enhancing transgenes (e.g., insect- and herbicide-resistance) into weedy or wild populations may significantly increase their weediness. Rice is an important crop worldwide and has been the target of genetic engineering research and development. Many GE rice lines with insect-resistant, herbicide-resistant, and stress-tolerant traits have been produced (for reviews, see Lu and Snow 2005; Lu and Yang 2009). Two insect-resistant GE rice varieties (*Bt*) have received biosafety certificates for commercialization in China, stimulating tremendous biosafety debate. One of the key questions is whether introgressed transgenes from GE rice will enhance the fitness of wild and weedy rice and result in ecological problems (Ellstrand 2003; Lu and Yang 2009). Therefore, understanding the extent of outcrossing in weedy rice will help us to predict the probability of transgene introgression into weedy rice populations and to serve as the first step to assess the potential ecological impacts caused by such introgression.

Introgression of a plant species is largely associated with its ability to outcross, usually measured by its outcrossing rates (Rieseberg and Carney 1998; Xia et al. 2011). In a study of modeling pollen-mediated gene flow (PMGF) in rice, Rong et al. (2010) identified the outcrossing rates of pollen recipients to be a key factor that determines the PMGF frequency. Empirical data also indicate that PMGF significantly varies with the mating systems of populations and species (e.g., Holtsford and Ellstrand 1989; Messeguer 2003), along with climatic conditions such as wind speed and humidity. For example, the PMGF frequencies in maize with high level of outcrossing rates are as high as 46% (Byrne and Fromherz 2003; Sanvido et al. 2008), whereas those in rice with low level of outcrossing rates are $<1\%$ (Rong et al. 2005, 2007). PMGF frequencies vary significantly between different rice species depending on the outcrossing rates of pollen recipients. Experimental data indicate that PMGF frequencies from cultivated rice to its wild ancestor *O. rufipogon* with relatively high-outcrossing rates (Oka 1988) ranged between ca. 1 and 20% (Song et al. 2003; Wang et al. 2006). On the contrary, PMGF frequencies from cultivated rice to its weedy counterpart with low outcrossing rates (Xia et al. 2011) varied between ca. 0 and 1% (Gealy et al. 2003; Zhang et al. 2003; Chen et al. 2004; Shivrain et al. 2007).

Therefore, measuring the outcrossing rate is important for understanding the relationships of outcrossing, introgression, and differentiation.

Using microsatellites (simple sequence repeat, SSR) fingerprints, we characterized the population genetic structure of weedy rice populations collected from a relatively small rice cultivation area in northern Italy, including the coexisting rice cultivars in the corresponding fields for comparison. We also measured the outcrossing rates of the randomly selected weedy rice populations. The primary objectives of this study were to answer the following questions: (1) What is the population genetic structure of these weedy rice populations at this small spatial scale? (2) What is the level of outcrossing rates in weedy rice populations, and is there evident introgression from rice cultivars? (3) What is the role of introgression from rice cultivars in the differentiation of these weedy rice populations?

Materials and methods

Sampling of weedy rice populations and rice varieties

A total of 24 weedy rice populations distributed in rice fields of Lombardia and Piemonte Regions of northern Italy (Fig. 1) were included in this study. The two regions are the primary rice production areas of Italy, where direct seeding of various rice varieties is practiced (Faivre-Rampant et al. 2011). Weedy rice infestation in this area is severe, with up to 90% of rice fields infested in a bad year (M. Tabacchi, personal communication). The flowering time of weedy rice populations is usually protracted for ~10–18 days. In these regions, the high-pick flowering time of weedy rice is usually about 7–10 days earlier than that of the corresponding cultivated rice varieties, from July 15th to August 15th, depending on the time of seed sowing and variety of cultivated rice. The average duration of these rice varieties from sowing to flowering ranged between 80 and 100 days (Table 1). The phenology overlaps sufficiently to permit gene flow between the weedy rice plants and the proximal rice varieties. Detailed information about sample size and locality of both the weedy rice populations and their coexisting rice varieties was indicated in Table 1.

To estimate the population genetic structure, leaf samples of each 19–25 maternal plants from the 24 populations were randomly collected (Table 1). Collecting sites for the sampled weedy rice populations are mapped in Fig. 1. A total of 576 leaf samples from the 24 weedy rice populations were included for analyses. One to two young leaves were collected from each individual and stored in a zip-lock bag containing silica gel desiccant. To estimate the population outcrossing rates, seed samples from each three mature panicles of the corresponding maternal weedy rice plants of 15 randomly selected weedy rice populations were selected (Table 1). Nineteen to 20 maternal weedy rice plants (as families) in each population, and 15–20 mature seeds (as progeny) from each family were randomly selected. As a result, a total of 299 families and 5,395 progeny from the 15 populations were included for analysis. In addition, five seed samples from different plants for each of the 23 rice varieties coexisting with corresponding weedy rice populations were included to determine the crop-specific alleles and evidence for crop-weed introgression (Table 1). A crop-specific allele in a weedy

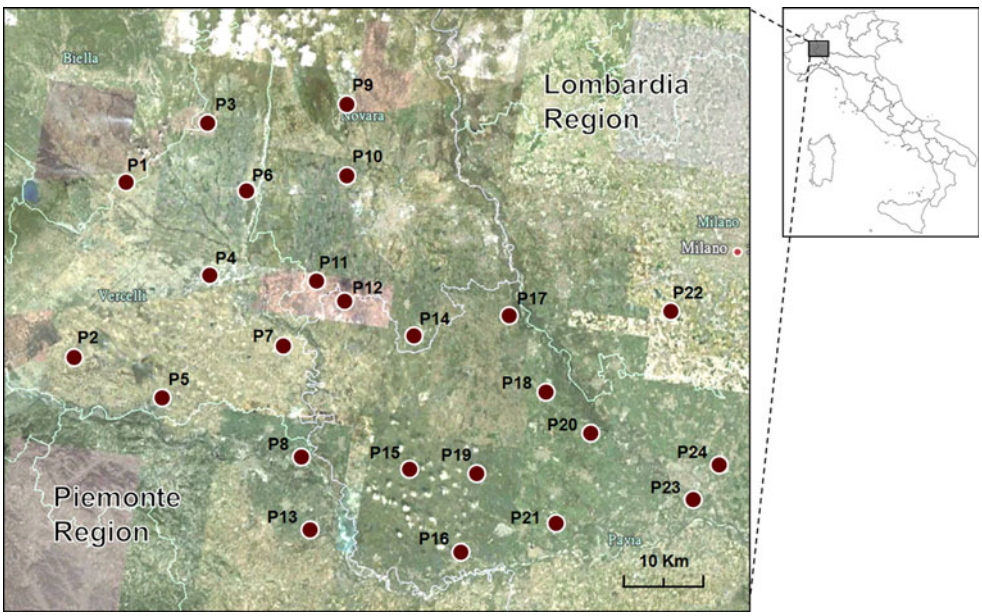

Fig. 1 Spatial location of 24 weedy rice populations (P1–P24, as indicated by *dark-red dots*) collected from Lombardia and Piemonte Regions of northern Italy. Twenty-three rice varieties in the same fields with the corresponding weedy populations (except for P4) were also sampled. See Table 1 for detailed information regarding the weedy rice populations and rice varieties sampled

Table 1 Samples and location of the 24 studied weedy rice populations and their coexisting rice varieties from northern Italy

Population code	No. of plants sampled	Location (municipality/province)	Longitude (E), latitude (N)	Coexisting rice variety	Days from sowing to flowering of the rice varieties
P1	25	Salussola/Biella	8°11′05″, 45°27′07″	Flipper	85
P2	25	Livorno Ferraris/Vercelli	8°06′54″, 45°15′34″	Creso	95
P3	23	Rovasenda/Vercelli	8°17′51″, 45°31′26″	Gladio	90
P4	25	Olcenengo/Vercelli	8°20′14″, 45°21′11″	Nembo[a]	85
P5	25	Trino Vercellese/Vercelli	8°17′34″, 45°12′11″	Selenio	95
P6	23	Greggio/Vercelli	8°22′35″, 45°27′09″	Nembo	85
P7	25	Prarolo/Vercelli	8°15′32″, 45°75′13″	Gladio	90
P8	25	Casale Monferrato/Alessandria	8°32′07″, 45°10′10″	Loto	80
P9	24	Momo/Novara	8°31′40″, 45°33′45″	Balilla	100
P10	25	San Pietro Mosezzo/Novara	8°32′30″, 45°28′55″	Nembo	85
P11	19	Casalino/Novara	8°30′46″, 45°21′30″	Augusto	85
P12	24	Confienza/Pavia	8°33′48″, 45°21′03″	CRLB1	80
P13	23	Pomaro Monferrato/Alessandria	8°33′17″, 45°04′24″	Centauro	90
P14	25	Borgolavezzaro/Novara	8°41′00″, 45°18′23″	Apollo	87
P15	25	Valle Lomellina/Pavia	8°41′29″, 45°09′03″	Nembo	85
P16	25	Pieve del Cairo/Pavia	8°46′58″, 45°02′52″	Brio	91
P17	25	Cassolnovo/Pavia	8°50′15″, 45°21′13″	Augusto	85
P18	22	Borgo San Siro/Pavia	8°53′56″, 45°15′03″	Balilla	100
P19	19	Ottobiano/Pavia	8°47′54″, 45°08′38″	Balilla	100
P20	25	Gropello Cairoli/Pavia	8°59′34″, 45°12′12″	Aleramo	100
P21	24	Pieve Albignola/Pavia	8°56′55″, 45°06′40″	Creso	95
P22	25	Lacchiarella/Milano	9°07′55″, 45°20′31″	Selenio	95
P23	25	Travacò Siccomario/Pavia	9°10′38″, 45°09′11″	Volano	100
P24	25	Pavia/Pavia	9°12′59″, 45°11′40″	Volano	100

The 15 weedy populations indicated by bold, underlined letters were used for outcrossing analysis

[a] No rice sample was collected due to the severe infestation of weedy rice in the field

population was defined as "the paternal specific allele that could only come from the crop by examining alleles in cultivated rice coexisting in the same fields" (Xia et al. 2011) in weedy rice progeny.

DNA extraction, amplification, electrophoresis and SSR fragment score

The total genomic DNA was extracted from dry leaf samples and seedlings following a modified cetyltrimethyl ammonium bromide (CTAB) protocol (Saghai-Maroof et al. 1984). About 1 g of dry leaf tissue was used for DNA extraction. Seeds of collected weedy rice and cultivated rice were germinated in an illuminated incubator (25°C) with a light/dark cycle of 16/8 h. DNA was extracted from the fresh leaf tissue of individual 10-day-old seedlings.

The 24 SSR primer pairs for assaying population genetic structure were selected from the Database of Rice Markers (http://www.gramene.org) to be distributed on both arms of each of the 12 rice chromosomes (Table 2). Of these, six highly polymorphic SSR loci (RM219, RM220, RM228, RM234, RM241, and RM276) were selected for estimating of outcrossing rates and paternity (Table 2). All forward primers were fluorescently labeled by either FAM (blue), ROX (red) or JOE (green), respectively.

The polymerase chain reaction (PCR) was performed with a 2720 Thermal Cycler (Applied Biosystems Inc., Foster, USA). Reactions were carried out in a volume of 10 μL containing 1× buffer (with Mg^{2+}), 0.2 mM each of dNTPs, 0.2 μM of SSR primer, 20 ng of genomic DNA and 0.3 U of Taq polymerase (Takara Bio Inc., Otsu, Japan). The reaction procedure was programed as follows: a denaturation period of 4 min at 94°C followed by 28 cycles of 30 s at 94°C, 30 s at 55°C and 40 s at 72°C and then 7 min at 72°C for the final extension.

After amplification, each of the DNA fragments was labeled with a proprietary fluorophore (Invitrogen Inc., Shanghai, China). According to the size of fragments, labeled fragments of 3–5 SSR loci were added to the mixture of 9 μL Hi-Di Formamide (to denature the dsDNA) and internal lane size standard (GeneScan™ -500 LIZ) for denaturing at 94°C for 5 min and then cooled at

Table 2 The DNA sequences of 24 SSR primer pairs used for genetic diversity and differentiation analyses of weedy rice populations

Primer ID	Location on chromosome	Forward primer (5′ → 3′)	Reverse primer (5′ → 3′)	SSR motif
RM220	1	GGAAGGTAACTGTTTCCAAC	GAAATGCTTCCCACATGTCT	$(CT)_{17}$
RM24	1	GAAGTGTGATCACTGTAACC	TACAGTGGACGGCGAAGTCG	$(GA)_{29}$
RM208	2	TCTGCAAGCCTTGTCTGATG	TAAGTCGATCATTGTGTGGACC	$(CT)_{17}$
RM263	2	CCCAGGCTAGCTCATGAACC	GCTACGTTTGAGCTACCACG	$(CT)_{34}$
RM218	3	TGGTCAAACCAAGGTCCTTC	GACATACATTCTACCCCCGG	$(TC)_{24}$
RM55	3	CCGTCGCCGTAGTAGAGAAG	TCCCGGTTATTTTAAGGCG	$(GA)_{17}$
RM241	4	GAGCCAAATAAGATCGCTGA	TGCAAGCAGCAGATTTAGTG	$(CT)_{31}$
RM280	4	ACACGATCCACTTTGCGC	TGTGTCTTGAGCAGCCAGG	$(GA)_{16}$
RM249	5	GGCGTAAAGGTTTTGCATGT	ATGATGCCATGAAGGTCAGC	$(AG)_5A_2(AG)_{14}$
RM289	5	TTCCATGGCACACAAGCC	CTGTGCACGAACTTCCAAAG	$G_{11}(GA)_{16}$
RM253	6	TCCTTCAAGAGTGCAAAACC	GCATTGTCATGTCGAAGCC	$(GA)_{25}$
RM276	6	CTCAACGTTGACACCTCGTG	TCCTCCATCGAGCAGTATCA	$(GA)_{33}$
RM11	7	TCTCCTCTTCCCCCGATC	ATAGCGGGCGAGGCTTAG	$(GA)_{17}$
RM234	7	ACAGTATCCAAGGCCCTGG	CACGTGAGACAAAGACGGAG	$(CT)_{25}$
RM149	8	GCTGACCAACGAACCTAGGCCG	GTTGGAAGCCTTTCCTCGTAACACG	$(AT)_{10}$
RM44	8	ACGGGCAATCCGAACAACC	TCGGGAAAACCTACCCTACC	$(GA)_{16}$
RM215	9	CAAAATGGAGCAGCAAGAGC	TGAGCACCTCCTTCTCTGTAG	$(GA)_{16}$
RM219	9	CGTCGGATGATGTAAAGCCT	CATATCGGCATTCGCCTG	$(GA)_{17}$
RM228	10	CTGGCCATTAGTCCTTGG	GCTTGCGGCTCTGCTTAC	$(GA)_{18}$
RM258	10	TGCTGTATGTAGCTCGCACC	TGGCCTTTAAAGCTGTCGC	$(GA)_{21}(GGA)_3$
RM202	11	CAGATTGGAGATGAAGTCCTCC	CCAGCAAGCATGTCAATGTA	$(CT)_{30}$
RM21	11	ACAGTATTCCGTAGGCACGG	GCTCCATGAGGGTGGTAGAG	$(GA)_{21}$
RM19	12	CAAAAACAGAGCAGATGAC	CTCAAGATGGACGCCAAGA	$(ATC)_{10}$
RM235	12	AGAAGCTAGGGCTAACGAAC	TCACCTGGTCAGCCTCTTTC	$(CT)_{24}$

The six highly polymorphic SSR primer pairs used for mating analyses are indicated by bold, underlined letters

4°C. The mixture was separated on a capillary electrophoresis genotyper, ABI 3130xl (Applied Biosystems Inc., Foster, USA). The separated SSR fragments were scored using the software Genemapper ver. 3.7 (Applied Biosystems Inc., Foster, USA).

Data analysis

Genetic diversity and differentiation of weedy rice populations

The genotypic data matrix from 576 samples of 24 weedy rice populations generated by the 24 SSR loci was analyzed to estimate the genetic diversity. The following parameters were calculated: (1) percentage of polymorphic loci (P); (2) average number of alleles per locus (N_a); (3) observed heterozygosity (H_o); (4) Nei's expected heterozygosity (H_e) (Nei 1978); (5) fixation index (F); and (6) the frequency of private alleles (F_{pa}). Private alleles were defined as the alleles that were only detected in a particular weedy rice population, similar to the concept of Slatkin (1985). Wright's F statistics, including global and pairwise F_{st}, were calculated to measure the differentiation among weedy rice populations. Analysis of molecular variance (AMOVA) was also performed to estimate the differentiation by partitioning genetic diversity within and among populations. The above analyses were performed using the program GenAlEx ver. 6.2 (Peakall and Smouse 2006).

A UPGMA dendrogram was constructed based on pairwise F_{st} to estimate the relationships of weedy rice populations, using the software NTSYS ver. 2.02 (Rohlf 1998). The Mantel test was conducted to examine the correlation of genetic differentiation estimated by pairwise F_{st} with spatial distances among weedy rice populations based on an isolation-by-distance model, using the software GenAlEx ver. 6.2.

STRUCTURE analysis was conducted to examine the clustering of plants from weedy rice populations and rice varieties, within a hypothetical number of populations (K) (Pritchard et al. 2000). The genotypic data matrix of 24 weedy rice populations and rice varieties (as a bulk) generated from 24 SSR primer pairs was analyzed using the software STRUCTURE ver. 2.2 (Pritchard et al. 2000). The running parameters were set as follows: an initial burn-in

run of 50,000 followed by a run length of 200,000 and a model allowing for admixture and correlated allele frequencies between populations. The appropriate K value was determined by running the data matrix in STRUCTURE for ten times following the principle of Evanno et al. (2005).

Outcrossing rate of weedy rice and introgression from cultivated rice

Outcrossing rate of weedy rice populations was calculated using the multilocus mixed-mating model as implemented in the software MLTR ver. 3.0 (Ritland 2002). The following parameters were computed: multilocus outcrossing rate (t_m); single-locus outcrossing rate (t_s); the proportion of the biparental inbreeding to the total outcrossing rate $[(t_m - t_s)/t_m]$. These parameters were estimated using maximum likelihood procedures. The Newton–Raphson method was used to solve the likelihood equation for the maximum likelihood estimates. Results were subject to 1,000 bootstraps using families as the resampling unit.

Paternity (paternal specific alleles) in weedy rice progeny was analyzed by comparing maternal alleles with paternal alleles to estimate the possible introgression from cultivated rice. The following procedures were performed to obtain paternity: (1) estimating frequency of alleles from paternal (pollen) and maternal (ovule) plants by MLTR software (Ritland 2002) and (2) determining paternal-specific alleles by comparing alleles from both parents, alleles that only appear in pollen donors were defined as paternal-specific allele. As a consequence, crop-specific alleles (defined above) in weedy rice progeny were determined by comparing paternal alleles with alleles actually detected in and specific to the coexisting rice varieties.

Correlations between single-locus outcrossing rates (t_s) and private allele frequencies of the 15 weedy rice populations were analyzed using the linear regression model in the SPSS software ver. 17.0 (http://www.spss.com/).

Results

Genetic diversity of weedy rice populations

In general, the 24 weedy rice populations sampled from northern Italy demonstrated relatively high genetic diversity, despite the limited area of our sample collection (ca. 5,000 km^2; Fig. 1). Using the selected 24 SSR primer pairs, we detected a total of 178 alleles with an average of ca. 7.5 per locus. All SSR loci were polymorphic. The overall Nei's unbiased genetic diversity was nearly 0.5, although substantial variation was detected among different populations. Population 13 had the highest level of genetic diversity ($H_e = 0.48$), whereas population 16 (P16) had the lowest level of genetic diversity (H_e close to 0.2). The observed heterozygosity and fixation index of the 24 weedy rice populations varied between 0.005 and 0.024 and 0.80 and 0.99, respectively (Table 3), indicating the self-pollinating nature of these weedy rice populations. Private alleles were found in 20 weedy rice populations, accounting for about 28% of the total number of alleles in all weedy populations. The private alleles were unevenly distributed among populations, varying from 0.1 to 2.2% (Table 3).

Genetic differentiation of weedy rice populations

Analyses showed generally moderate genetic differentiation among the weedy rice populations. The overall F_{st} value for the weedy rice populations was about 0.26, indicating about 26% of genetic variation occurred among the populations. Genetic differentiation was not evenly distributed across populations, reflected by the nearly ten-fold variation in the pairwise F_{st} values, ranging from about 0.04 to 0.32 (data not shown). AMOVA analysis revealed partitioning of genetic variation similar to overall F_{st}, with about 75% of genetic variance occurring within weedy rice populations and 25% occurring among weedy rice populations (Table 4). The results from the AMOVA agreed with that indicated by the overall F_{st} value showing the similar differentiation pattern of weedy rice populations.

The UPGMA dendrogram based on pairwise F_{st} indicated that the 24 weedy rice populations were clustered into two major groups differentiated from each other by an F_{st} value of about 0.23, with 22 populations in a large group and two populations (P10 and P21) in another group (Fig. 2). The large group was further divided into a few clusters at different F_{st} values. The Mantel test showed no correlation ($R^2 = 0.004$, $P = 0.276$) between genetic differentiation (F_{st}) and spatial distance, indicating essentially no isolation-by-distance pattern among the weedy rice populations.

The appropriate K value for STRUCTURE analysis was determined to be 5 (Fig. 3); the analysis revealed a clear differentiation between the 24 weedy rice populations and the rice varieties (as a whole group). The majority of weedy rice plants exhibited a multiple cluster assignments, implying an admixed genetic constitution of these plants. Consequently, some weedy rice populations shared a closer genetic affinity than others, which was in agreement with the results from UPGMA analysis (e.g., P10 and P21). Nearly all rice varieties were assigned into one unique cluster that was rarely represented by weedy rice populations. In contrast, some weedy rice plants showed highly admixed genetic constitutions. Some weedy rice plants had

Table 3 Parameters of genetic diversity in 24 weedy rice populations from northern Italy based on 24 SSR primer pairs

Population code	*P* (%)	N_a	H_o	H_e	*F*	F_{pa}
P1	91.67	2.750 (0.202)	0.012 (0.005)	0.375 (0.040)	0.962 (0.018)	0.004
P2	95.83	3.042 (0.266)	0.020 (0.006)	0.410 (0.048)	0.886 (0.050)	0.003
P3	83.33	2.667 (0.317)	0.007 (0.003)	0.336 (0.049)	0.920 (0.048)	0.007
P4	91.67	2.792 (0.233)	0.008 (0.004)	0.341 (0.040)	0.930 (0.046)	0.007
P5	100.00	3.458 (0.269)	0.013 (0.005)	0.399 (0.049)	0.896 (0.058)	0.006
P6	91.67	2.875 (0.220)	0.007 (0.003)	0.400 (0.047)	0.942 (0.044)	0.006
P7	87.50	2.208 (0.147)	0.005 (0.004)	0.264 (0.043)	0.987 (0.008)	0.002
P8	95.83	3.042 (0.185)	0.010 (0.004)	0.395 (0.044)	0.933 (0.044)	0.003
P9	79.17	2.333 (0.206)	0.005 (0.003)	0.332 (0.047)	0.986 (0.007)	0.000
P10	87.50	2.500 (0.170)	0.017 (0.005)	0.278 (0.034)	0.929 (0.023)	0.003
P11	95.83	2.667 (0.167)	0.024 (0.010)	0.277 (0.029)	0.885 (0.052)	0.001
P12	66.67	2.167 (0.246)	0.021 (0.008)	0.217 (0.042)	0.812 (0.071)	0.002
P13	87.50	3.292 (0.229)	0.009 (0.005)	0.481 (0.043)	0.982 (0.009)	0.008
P14	100.00	3.167 (0.231)	0.009 (0.004)	0.398 (0.037)	0.970 (0.016)	0.004
P15	87.50	2.417 (0.180)	0.018 (0.005)	0.343 (0.045)	0.902 (0.047)	0.000
P16	95.83	2.458 (0.147)	0.020 (0.004)	0.199 (0.027)	0.797 (0.069)	0.000
P17	87.50	2.750 (0.211)	0.010 (0.004)	0.326 (0.037)	0.960 (0.017)	0.000
P18	75.00	2.333 (0.206)	0.008 (0.004)	0.270 (0.049)	0.928 (0.049)	0.002
P19	87.50	3.125 (0.320)	0.011 (0.007)	0.443 (0.044)	0.980 (0.012)	0.011
P20	87.50	2.792 (0.208)	0.007 (0.005)	0.425 (0.047)	0.985 (0.010)	0.007
P21	79.17	2.583 (0.240)	0.016 (0.006)	0.247 (0.036)	0.909 (0.029)	0.022
P22	91.67	3.167 (0.214)	0.009 (0.004)	0.441 (0.048)	0.982 (0.008)	0.003
P23	95.83	2.833 (0.214)	0.007 (0.003)	0.378 (0.042)	0.944 (0.043)	0.004
P24	91.67	2.833 (0.206)	0.013 (0.005)	0.396 (0.049)	0.926 (0.044)	0.003
Overall	100.00	7.417 (0.801)	0.012 (0.002)	0.478 (0.042)	0.948 (0.019)	0.004

Numbers in parentheses indicate standard error (SE)

P percentage of polymorphic loci, N_a average number of alleles per locus, H_o observed heterozygosity, H_e unbiased expected heterozygosity, *F* fixation index, F_{pa} frequency of private alleles

Table 4 Analysis of molecular variance (AMOVA) of 24 weedy rice populations based on 24 SSR primer pairs

Source of variation	df	SS	Est. var.	Total (%)
Among populations	23	3697.89	5.96	25
Within populations	552	9803.91	17.76	75
Total	575	13501.80	23.72	100

P value (<0.001) estimates are based on 9,999 permutations

df degree of freedom, *SS* sum of squared deviations, *Est. var.* variance component estimates, *% total* percentage of total variation

a high probability of assignment to the cultivated rice cluster (Fig. 3), suggesting introgression from cultivated rice.

Outcrossing rate and introgression from cultivated rice to weedy rice

MLTR analysis revealed low outcrossing rates for all weedy rice populations examined (Table 5). The average outcrossing rate estimated by a single locus was 0.7 (t_s) and 1.8% (t_m) as estimated by all six loci, consistent with weedy rice's well-known self-pollinating nature. Outcrossing rate estimates varied significantly among populations with t_s ranging from 0.1 to 1.6%, and t_m from 0.2 to 6.7%. The average proportion of the biparental inbreeding to the total outcrossing rate was about 61%, implying that a considerable amount of outcrossing occurred between related plants in the same field. Correlation analysis detected a positive correlation ($R^2 = 0.337$, $P = 0.023$) between the outcrossing rates (t_s) and the frequencies of private alleles among the weedy rice populations (Fig. 4).

Paternity analysis showed 40 paternal alleles (of 276 total alleles) that were only detected in weedy rice progeny, but not in families (maternal plants) (Table 6). We compared the paternity of weedy rice progeny and the alleles actually detected from the coexisting rice varieties; five alleles were found to be shared by weedy rice (P2, P3, P5, P10, and P21) and certain cultivars (Creso, Gladio, Selenio, and Nembo). This was detected through comparing the

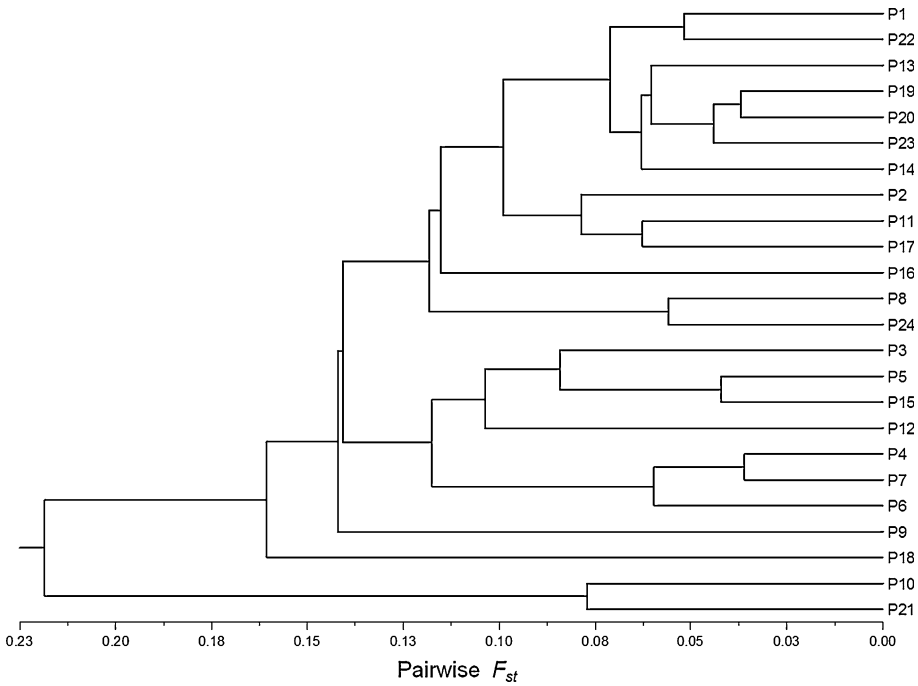

Fig. 2 UPGMA dendrogram constructed based on the pairwise F_{st} of 24 weedy rice populations from northern Italy estimated by 24 SSR loci in 576 maternal plants

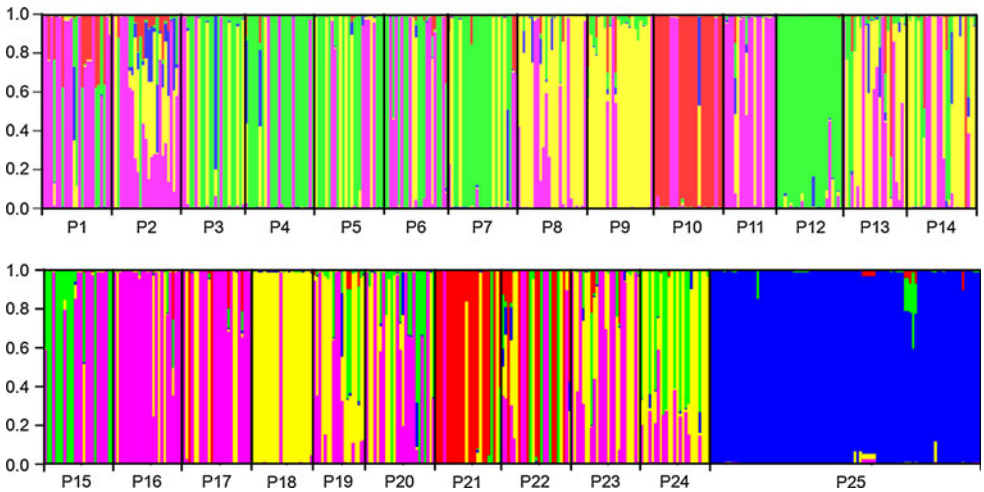

Fig. 3 Genetic relationships of 24 weedy rice populations (P1–P24) and their coexisting rice varieties (as a bulk, P25) as indicated by the *barplots* based on STRUCTURE analyses. Each individual is represented by a *single vertical line* (*bar*) assigned into five *colored* segments, with lengths proportional to each of the five *inferred clusters*. The numbers of *vertical axis* represents probability of assignment

paternity of weedy rice progeny and the alleles actually detected from the coexisting rice varieties. Among these shared alleles, three had allelic frequency as high as 100% in the cultivars Gladio, Selenio, and Creso.

Discussion

Our results based on allelic polymorphisms of 24 SSR loci indicated a relatively high level ($H_e = 0.48$) of overall genetic variation in weedy rice populations collected from a relatively small rice planting area in northern Italy. This estimation is made based on a relatively large number of weedy rice samples (576 plants) scattered in a relatively small rice planting area (ca. 5,000 km^2). The level of genetic diversity in the weedy rice populations is substantially higher than that found in other studies of weedy rice populations from northern and northeastern China, where the expected heterozygosity (H_e) ranging from 0.05 to 0.31 was estimated (Yu et al. 2005; Cao et al. 2006). The difference in genetic diversity between this study and other studies in China is possibly due to the higher level of genetic diversity in northern Italy; but it could be due to differences in methodologies because our study used high-resolution florescent SSR electrophoresis. Other studies of weedy rice populations from southern USA estimated relatively high genetic diversity (H_e) ranging from ca. 0.27 to 0.48 (Londo and Schaal 2007; Gealy et al. 2009; Shivrain et al. 2010). The genetic diversity detected in the weedy rice populations from northern Italy provides a good

Table 5 Outcrossing rate of 15 weedy rice populations based on six SSR loci using the multilocus mixed-mating model (MLTR, Ritland 2002)

Population code	No. of families	No. of progeny	t_s	t_m	$(t_m - t_s)/t_m$ (%)
P1	20	308	0.005 (0.003)	0.014 (0.008)	64.29
P2	20	391	0.010 (0.004)	0.027 (0.148)	59.26
P3	20	299	0.005 (0.003)	0.013 (0.246)	61.54
P5	20	397	0.013 (0.004)	0.037 (0.012)	64.86
P7	20	398	0.009 (0.004)	0.024 (0.156)	62.50
P9	20	397	0.007 (0.003)	0.002 (0.059)	~0.00
P10	20	380	0.005 (0.003)	0.006 (0.552)	16.67
P11	19	284	0.001 (0.001)	0.067 (0.523)	98.51
P13	20	295	0.007 (0.003)	0.002 (0.114)	~0.00
P16	20	389	0.002 (0.002)	0.002 (0.111)	~0.00
P18	20	395	0.003 (0.002)	0.006 (0.004)	66.67
P20	20	297	0.001 (0.001)	0.022 (0.021)	95.45
P21	20	380	0.016 (0.007)	0.021 (0.184)	23.81
P22	20	390	0.010 (0.005)	0.024 (0.285)	58.33
P24	20	395	0.005 (0.002)	0.002 (0.393)	~0.00

Numbers in the brackets indicate standard deviation

t_m multilocus outcrossing rate, t_s single-locus outcrossing rate, $(t_m - t_s)/t_m$ the proportion of the biparental inbreeding to the total outcrossing rate

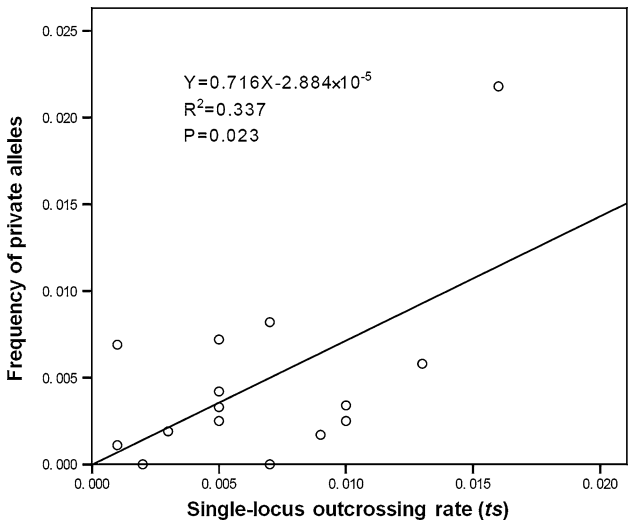

Fig. 4 Positive correlation between the single-locus outcrossing rates (t_s, x-axis) and the frequencies of private alleles (y-axis) of the 15 weedy rice populations included in mixed-mating (MLTR) analysis, using the linear regression model

system for studying whether introgression contributes to the genetic differentiation of these populations.

We found a moderate level (~0.26) of genetic differentiation based on the pairwise F_{st} among the 24 weedy rice populations with substantial variation, despite the limited distribution area of these populations (ca. 5000 km^2). UPGMA cluster analysis based on the pairwise F_{st} showed varying divergence among the weedy rice populations. STRUCTURE analysis also indicated evident genetic differences among the weedy rice populations, with some sets of populations sharing a closer genetic affinity. Some individuals in populations showed strong affinities with the group of rice cultivars. Evolution of genetic differentiation of populations may result from any of the following evolutionary forces: mutation, selection, migration (gene flow), and genetic drift (Hartl and Clark 1997). Which factors are mainly responsible for the observed genetic differentiation of the weedy rice populations in this study?

The natural mutation rate for SSRs is typically too low to be a major factor responsible for population differentiation at the observed levels. Genetic drift is unlikely to have a strong influence on the evolution of inter-population differences because of the large size of the weedy rice we sampled (thousands of individuals). Given the uniformity of environmental conditions and rice cultural practices across our sampled area, selection by local environmental heterogeneity is unlikely to drive the observed inter-population differentiation. Mantel test did not show significant pattern of isolation-by-distance of the weedy rice populations, which supports our conclusions about evolution of differentiation by drift or by clinal environmental selection pressures. Therefore, immigration of different alleles to the respective weedy rice populations would be the major factor causing genetic differentiation. Weedy rice always coexists and it is highly cross-compatible with cultivated rice. The specific local cultivar varies with farmer choice and it is not likely to vary in a systematic way over space. Thus, weedy rice populations have the opportunity to acquire new alleles easily through constant crop-to-weed outcrossing and introgression in different rice fields, driving genetic differentiation based on differences between adjacent cultivars that are the gene flow source. Clearly,

Table 6 Paternal-specific alleles in weedy rice populations as estimated by the multilocus mixed-mating model (MLTR, Ritland 2002) and the alleles that were also detected in the coexisting rice varieties generated by the six highly polymorphic SSR primer pairs

Population code	Locus	Paternal-specific allele[a]	Allele detected in the coexisting rice variety[a]
P1	RM228	105 (0.28)	–
	RM234	133 (0.28), 137 (0.28)	–
P2	RM228	103 (0.20), 105 (0.10)	–
	RM234	127 (0.10), 129 (0.30)	–
	RM276	86 (0.10), 128 (0.10)	86 (0.40)
P3	RM220	104 (0.32)	104 (1.00)
	RM228	115 (0.32)	–
	RM234	125 (0.32)	–
	RM276	124 (0.32)	–
P5	RM219	186 (0.14)	–
	RM220	108 (0.08)	–
	RM276	124 (0.21)	124 (1.00)
P7	RM220	122 (0.16)	–
	RM234	135 (0.47)	–
	RM241	136 (0.16)	–
P9	RM220	120 (0.43)	–
P10	RM220	126 (0.50)	–
	RM228	105 (0.50)	–
	RM241	136 (0.50)	136 (0.40)
P13	RM228	105 (0.14)	–
P16	RM228	105 (1.00)	–
	RM234	133 (1.00)	–
P18	RM219	184 (0.44)	–
	RM234	133 (0.44)	–
P20	RM220	104 (1.00)	–
P21	RM220	118 (0.27)	–
	RM228	101 (0.28)	–
	RM234	135 (0.27)	135 (1.00)
	RM241	114 (0.14)	–
	RM276	118 (0.14)	–
P22	RM276	116 (0.17), 126 (0.17)	–
P24	RM234	125 (0.40), 129 (0.20)	–
	RM241	116 (0.40)	–
	RM276	126 (0.40)	–

Numbers in parentheses indicate allelic frequency

[a] Molecular weight (bp) of alleles

this hypothesis needs further support based on solid experimental evidence.

Interestingly, several private alleles were detected in different weedy rice populations, which are probably responsible for their inter-population differentiation. "Private allele" was defined by Neel (1973) and further developed by Slatkin (1985) as an allele found only in one population but not in others. In other words, if a population has more private alleles, it will share less common alleles with other populations, resulting in differentiation of this population from others by changing its genetic constitution. As a consequence, the number of private alleles is usually used as one measure for assessing genetic differentiation among populations and to estimate how distinct a given population is from other populations (Wagenaar et al. 2003; Kalinowski 2004; Maggs et al. 2008). Therefore, we ask whether the occurrence of the private alleles detected in our weedy rice populations have any association with the outcrossing and introgression from various rice cultivars.

Outcrossing rates are often related to hybridization and introgression. In this study, a variable, but low level of outcrossing, was determined in the sampled weedy rice populations from northern Italy. The level is in accordance with the previous studies that have measured a high level of self-pollination in weedy rice (Delouche et al. 2007), but still sufficiently high for the possibility of its hybridization and introgression from cultivated rice (Lu and Snow 2005; Lu and Yang 2009).

The detected outcrossing rates are not evenly distributed among populations and have considerable variation. Previous studies either by directly measuring transgene flow from cultivated rice (Gealy et al. 2003; Chen et al. 2004; Messeguer et al. 2004; Shivrain et al. 2007) or by assessing gene flow among weedy rice populations by population genetic analyses (Cao et al. 2006; Londo and Schaal 2007) all indicated a relatively low level of hybridization and introgression of weedy rice, ranging from ca. 0.01 to 3%. The variation in gene flow frequencies depended largely on the genotypes of weedy rice used, in addition to environmental conditions. A recent study using SSR loci and the mixed-mating model (Xia et al. 2011) also detected a comparable variation in outcrossing rates in weedy rice from eastern and northeastern China, supporting our observation of possible introgression in weedy rice.

We found a considerable number of paternal-specific alleles in weedy rice progeny (seedlings) that had not been detected in maternal plants, based on paternity analysis in this study. Thus, the pollen donor for these alleles was most likely a rice cultivar. We cannot exclude an unsampled weedy plant with a rare allele, but ca. 12.5% of the total paternal-specific alleles found in the weedy rice progeny were identified as crop-specific alleles that were absent in all of maternal weedy rice plants (results similar to Xia et al. 2011). Evidently, crop-specific alleles are acquired by weedy rice plants through hybridization with the coexisting rice cultivars in the corresponding fields, supporting our viewpoint regarding the role of crop-to-weed introgression in the evolution of differentiation. Similarly, hybridization between cultivated rice and red weedy rice was also found in the USA indicating potential allelic introgression from the crop (Gealy et al. 2009).

Similar to inter-population variation in outcrossing rates, private alleles were not evenly distributed across the weedy rice populations. Interestingly, the frequency of private alleles and outcrossing rates (estimated by t_s) of the corresponding weedy rice populations was positively and significantly correlated. In other words, weedy rice populations with higher outcrossing rates possessed a higher level of private alleles. This phenomenon has not been explored in previous genetic studies of weedy rice populations, but might shed a more meaningful light for understanding the impact of introgression on the evolution of their differentiation. We assume that the constant introgression of crop alleles from different rice varieties through time will accumulatively increase the private alleles in a particular weedy rice population, making it more distinct from other populations. The amount of crop-specific alleles in a weedy rice population depends significantly on its ability to outcross (outcrossing rates). A higher outcrossing rate of a population would promote more opportunity to hybridize with surrounding cross-compatible crop plants that coexist with weedy rice and have much higher density in the crop fields. Subsequent introgression should vary accordingly. If the hypothesis holds true, then explaining why private alleles are positively correlated with outcrossing rates of corresponding weedy rice populations is straightforward.

Spontaneous hybridization and introgression between crops and their close wild relatives are extensively found in nature. Ellstrand (2003) reviewed spontaneous hybridization between the world's 25 most important food crops and their wild relatives, finding that 23 are known to naturally hybridize with their corresponding wild relatives. Such hybridization sometimes may have significant impacts on the evolution and invasiveness of the wild species (Schierenbeck and Ellstrand 2009). Outcrossing and introgression from cultivated rice have been found to play an important role on differentiation and adaptive evolution of wild and weedy rice. For instance, one well-documented example is a study that compared the relative fitness of common wild rice (*O. rufipogon*) and its hybrids obtained by artificial outcrosses (Song et al. 2004). The hybrids showed evident fitness changes in various traits, suggesting the important impacts of crop-to-wild introgression in the evolutionary potential of wild rice. Further population genetic studies including common wild rice in China and typical *indica* and *japonica* rice cultivars from diverse sources demonstrated considerable genetic differentiation of the wild populations into "*indica*" and "*japonica*" types (Song et al. 2006). Collectively, these studies concluded that natural introgression from different types of cultivated rice has played a critical role in such differentiation. Previous population studies also indicated that introgression from different rice varieties through time can considerably affect genetic differentiation and the level of heterozygosity of weedy rice populations (Cao et al. 2006; Gealy et al. 2009; Xia et al. 2011). Hybridization studies involving insect-resistant GE rice and weedy rice strains have demonstrated that introgression of transgenes from GE rice enhances the performance of offspring (F_1–F_3) under environmental conditions with insect pressure (Cao et al. 2009; Yang et al. 2011). Therefore, introgression from cultivated rice, particularly involving alleles that confer a selective advantage, can not only influence the genetic differentiation, but also the adaptive evolution of populations. This is probably true to other crops with conspecific feral taxon and close wild relatives. As an intermediate between crops and wild species, feral taxa provide an excellent system for studying evolution (Ellstrand et al. 2010). Knowledge generated from such studies has significances in genetic conservation in situ and utilization of wild relatives of crops, as well as in predicting potential environmental impacts caused by transgene introgression from a GE crop to the wild relatives.

In conclusion, our population genetic analyses based on 24 polymorphic SSR loci indicated a relatively high level of genetic diversity and considerable genetic differentiation in 24 weedy rice populations from a relatively small region in northern Italy, where direct seeding of rice is the predominant cultivation method. However, the detected genetic differentiation was not associated with the spatial distances among the populations, suggesting that isolation-by-distance did not play a major role in the evolution of inter-population differentiation. Cluster analysis revealed a closer genetic affinity between some of the weedy rice populations, and a closer genetic affinity of a few weed rice populations with rice cultivars. The above observation can probably be explained by the hybridization and introgression between weedy rice and the cultivars. Outcrossing rate of the weedy rice populations as estimated by the mixed-mating model of SSR fingerprints was generally low and varied significantly, confirming the self-pollination nature of weedy rice. Interestingly, outcrossing rate is positively correlated with the private alleles of the corresponding weedy rice populations, which is possibly responsible for the genetic differentiation among populations. Paternity analysis indicated a considerable amount of paternal-specific alleles in weedy rice progeny, a certain portion of which was identified as crop-specific alleles that are most likely acquired through hybridization with the coexisting rice cultivars. These results clearly indicated allelic introgression from rice cultivars. It is apparent that outcrossing and allelic introgression from rice cultivars through time can contribute different private alleles into coexisting weedy rice populations, which may be responsible for the genetic differentiation of weedy populations. Constant allelic introgression from rice cultivars into related weedy relatives can drive their genetic differentiation and serve as a substrate for adaptive evolution; and the process is likely true for feral taxa other than weedy rice that persists within the mating distance with their crop progenitors.

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