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Assessment of Cultivar Distinctness in Alfalfa: A Comparison of Genotyping-by-Sequencing, Simple-Sequence Repeat Marker, and Morpho-physiological Observations

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Abstract

Cultivar registration agencies typically require morphophysiological trait-based distinctness of candidate cultivars. This requirement is difficult to achieve for cultivars of major perennial forages because of their genetic structure and ever-increasing number of registered material, leading to possible rejection of agronomically valuable cultivars. This study aimed to explore the value of molecular markers applied to replicated bulked plants (three bulks of 100 independent plants each per cultivar) to assess alfalfa (*Medicago sativa* L. subsp. *sativa*) cultivar distinctness. We compared genotyping-by-sequencing information based on 2902 polymorphic single-nucleotide polymorphism (SNP) markers (>30 reads per DNA sample) with morphophysiological information based on 11 traits and with simple-sequence repeat (SSR) marker information from 41 polymorphic markers for their ability to distinguish 11 alfalfa landraces representative of the germplasm from northern Italy. Three molecular criteria, one based on cultivar differences for individual SSR bands and two based on overall SNP marker variation assessed either by statistically significant cultivar differences on principal component axes or discriminant analysis, distinctly outperformed the morphophysiological criterion. Combining the morphophysiological criterion with either molecular marker method increased discrimination among cultivars, since morphophysiological diversity was unrelated to SSR marker-based diversity ($r = 0.04$) and poorly related to SNP marker-based diversity ($r = 0.23$, $P < 0.15$). The criterion based on statistically significant SNP allele frequency differences was less discriminating than morphophysiological variation. Marker-based distinctness, which can be assessed at low cost and without interactions with testing conditions, could validly substitute for (or complement) morphophysiological distinctness in alfalfa cultivar registration schemes. It also has interest in sui generis registration systems aimed at marketing alfalfa landraces.

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Core Ideas:

- Morphophysiological distinctness is difficult to achieve in cultivars of major perennial forages
- GBS-generated SNP markers and SSR markers displayed high alfalfa cultivar discrimination
- SNP or SSR markers could substitute (or complement) morphophysiological distinctness in alfalfa

CULTIVAR (i.e., variety) registration according to Union for the Protection of Varieties (UPOV), which is currently adopted in 71 countries to grant Plant Breeders' Rights, requires distinctness, uniformity, and stability (DUS) and value for cultivation and use (VCU) standards to be met by candidate varieties. In the European Union, registration in the national list of one member state according to UPOV criteria is required also for variety marketing. According to the UPOV Act of 1991, a variety is distinct if it is "clearly distinguishable from any

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Abbreviations: AFLP, amplified fragment-length polymorphism; DUS, distinctness, uniformity, and stability; GBS, genotyping-by-sequencing; PCA, principal components analysis; PCR, polymerase chain reaction; RFLP, restriction fragment-length polymorphism; SNP, single-nucleotide polymorphism; SSR, simple-sequence repeat; UPOV, Union for the Protection of Varieties; VCU, value for cultivation and use.

other variety" (www.upov.int/export/sites/upov/upovlex/en/conventions/1991/pdf/act1991.pdf). The distinctness requirement is satisfied if at least one statistically significant difference across a series of morphophysiological characteristics, mostly observed in field experiments, can be observed between the candidate cultivar and each of the registered cultivars (UPOV, 2008). The distinctive traits do not need to be of agronomic value. Cultivar distinctness has been envisaged as the only requirement for sui generis registration systems aimed at the marketing of landrace cultivars (i.e., traditional cultivars selected and multiplied by farmers), or at implementing cultivar Plant Breeders' Rights in developing countries that do not adopt the UPOV convention (Leskien and Flitner, 1997). Variety distinctness is also important for certifying authorities controlling seed production chains and lawsuits regarding illicit seed marketing, variety plagiarism, and essential derivation claims (Roldán-Ruiz et al., 2000).

Satisfying the distinctness requirement according to UPOV criteria is more challenging for synthetic varieties than for pure line or hybrid varieties. This is particularly true for species including a large and ever-increasing number of registered varieties, such as alfalfa or perennial ryegrass (*Lolium perenne* L.). For alfalfa, which is the most grown perennial forage legume worldwide (Annicchiarico et al., 2015a), the EU database lists over 380 registered varieties (http://ec.europa.eu/food/plant/plant_propagation_material/plant_variety_catalogues_databases/search/public/?event=SearchForm&ctl_type=A). Large numbers of registered varieties also imply high costs for DUS testing (Gilliland and Gensollen, 2010). There is evidence that lack of distinctness is leading to rejection of registration for several agronomically valuable candidate varieties of alfalfa. In France, over the period 2001 to 2010, 11 out of 46 candidate varieties (23.9%) were rejected from registration because of insufficient VCU, whereas, 12 candidate varieties featuring sufficient VCU were rejected because of lack of the DUS requirement essentially as a result of insufficient distinctness (V. Gensollen, M.C. Gras, and B. Julier, personal communication, 2015). More recently (period 2012–2014), 21% of the alfalfa candidate varieties failed to exhibit sufficient DUS, compared with 3% lacking sufficient VCU (V. Gensollen, personal communication, 2015). Besides, over 70% of the accepted candidate varieties over the period 2001 to 2014 required one or two extra years beyond the ordinary 3-yr assessment for granting sufficient DUS (V. Gensollen, M.C. Gras, and B. Julier, personal communication, 2015). In the United Kingdom, over 12% of the candidate varieties of *Lolium* spp. and white clover (*Trifolium repens* L.) were rejected for insufficient distinctness over the period 2000 to 2008, reaching 19% in 2008 (Gilliland and Gensollen, 2010). Achieving sufficient distinctness can be particularly difficult for forage crop varieties issued by recurrent selection schemes, which are of special interest for genomic or phenotypic selection (Li and Brummer, 2012). Lack of morphophysiological trait-based distinctness may also hinder the registration of landrace cultivars in sui generis systems (Russi and Falcinelli, 1997; Annicchiarico, 2006).

The increasing difficulty of obtaining sufficient distinctness for agronomically valuable candidate varieties, and the high and rising costs of DUS testing, emphasize the interest of exploiting molecular marker diversity for assessing cultivar distinctness. This opportunity has been discussed within UPOV (2004) without reaching full consensus on its implementation. There is a concern that marker-based distinctness, as an alternative to morphophysiological distinctness, may imply greater risk of variety plagiarism because essentially derived candidate varieties could be obtained by selection within a successful variety aimed at altering the molecular identity for some specific allele frequencies without altering the phenotype (UPOV, 2004; Gilliland and Gensollen 2010). Actually, the concurrent VCU requirement (hence, the requirement for an essentially derived candidate variety to outperform its original variety) may keep this risk reasonably low. Besides, essentially derived candidate varieties could also be obtained based on morphophysiological distinctness (e.g., by selecting for higher or lower frequency of very dark blue-violet flowers in alfalfa). In any case, the risk of granting marker-based distinctness to essentially derived candidate varieties would decrease when requiring two (or more) allele differences (UPOV, 2004) while virtually disappearing for distinctness assessed on overall molecular diversity across a high number of markers.

UPOV (2004) showed consensus on the possible use of molecular diversity for excluding from DUS trials the commercial varieties that show high genetic dissimilarity to candidate varieties, assuming that high molecular dissimilarity would reflect high morphophysiological dissimilarity. Actually, this assumption requires verification at least for forage legume species such as alfalfa.

Genotyping bulked plants with independent bulks per cultivar acting as replicates, rather than many individual plants per cultivar, has been proposed for outbred forage species (Pupilli et al., 1996; Kölliker et al., 2001) as a means to access reasonably large plant population samples with a modest evaluation cost. High numbers of bulked plants (e.g., 100) offer the advantage of reducing the bias of the sampling effect that arises from rare marker alleles (Pupilli et al., 1996, 2000). For example, SSR (microsatellite) marker bands for 120 bulked plants per alfalfa cultivar were clearly visible only when their frequency in plants of the cultivar exceeded about 10% (Carelli et al., 2009). Restriction fragment-length polymorphism (RFLP) markers could distinguish various alfalfa cultivars by means of differences for allele frequency (Pupilli et al., 1996), and a few Italian cultivars according to the presence or absence of one or more marker bands (Pupilli et al., 2000). Amplified fragment-length polymorphism (AFLP) markers distinguished two varieties while failing to distinguish two Italian ecotypes of alfalfa (Zaccardelli et al., 2003). Several white clover (Kölliker et al., 2001) or perennial ryegrass (Roldán-Ruiz et al., 2001) varieties proved different in some

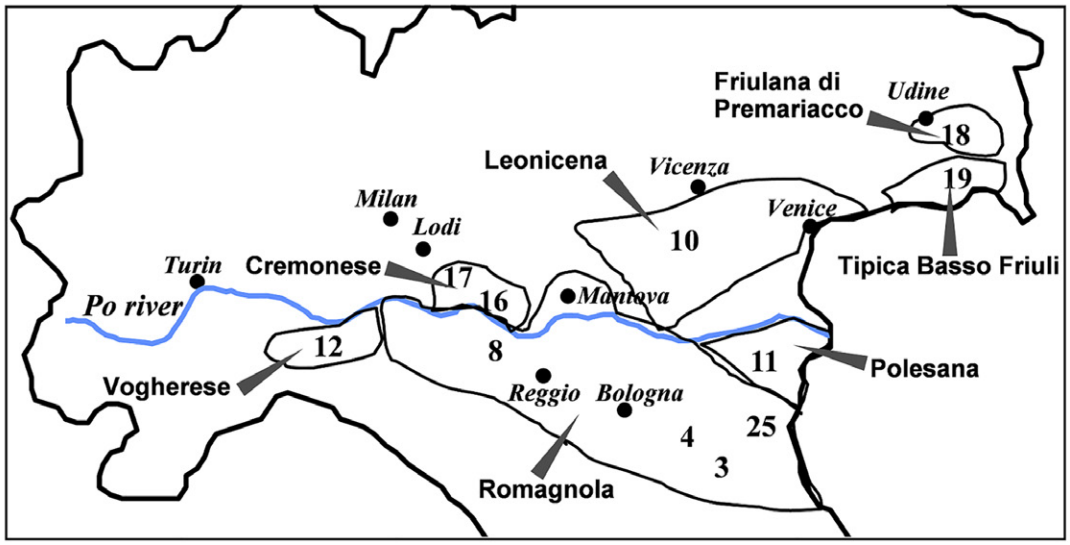

Fig. 1. Site of origin of 11 alfalfa landrace cultivars and production area of seven historical commercial ecotypes.

respect when assessing their AFLP marker diversity by ordination or classification methods. However, the high intrapopulation diversity that is a feature of forage crop varieties may hinder the reliable marker-based distinction of varieties through statistical tests (George et al., 2006). Simple-sequence repeat markers have higher interest than RFLP, AFLP, or random-amplified polymorphic DNA markers, owing to lower costs or higher quality and reliability of their information (Powell et al., 1996).

Recent sequencing technologies such as genotyping-by-sequencing (GBS) (Elshire et al., 2011) and restriction-site-associated DNA tags (Baird et al., 2008) can generate large numbers of SNP data for a low cost in many crop species, including alfalfa (Li et al., 2014; Annicchiarico et al., 2015b). While having an obvious interest for marker-assisted selection, these techniques can also open new opportunities for germplasm diversity studies and variety fingerprinting. Work by Byrne et al. (2013), who have applied GBS for estimating allele frequencies of several thousand SNP markers in eight perennial ryegrass varieties, showed patterns of variety variation that were large and substantially consistent across replicates of independent bulked plants.

Alfalfa farm landraces represented the majority of the Italian seed market until recently (Sommovigo et al., 1999) before being banned from commercialization. They used to be marketed under the name of 14 commercial ecotypes depending on their area of production. Figure 1 shows the production area of the seven commercial ecotypes defined for northern Italy. Landraces represented the backbone of Italian breeding programs, owing to their outstanding agronomic value (Annicchiarico and Piano, 2005; Annicchiarico et al., 2012). Assessing their distinctness can be highly relevant not only for sui generis registration systems but also for comparing variety registration procedures, given the similar genetic base of Italian landrace and variety germplasm and the somewhat greater challenge of discriminating landraces that arises by virtue of their greater intrapopulation variation than most bred varieties (Annicchiarico, 2006). Evaluating distinctness between individual landrace cultivars rather than between ecotype groups is preferable because landrace phenotypic diversity proved to depend not only on ecotype groups but also on other collecting variables that differed within ecotype groups (e.g., the mowing frequency on the farm) (Annicchiarico, 2006).

The main objective of our study was to compare GBS-generated SNP marker variation with morphophysiological data and with SSR marker variation for their usefulness in assessing cultivar distinctness. Our study was based on 11 Italian landraces of alfalfa representative of the seven historical ecotypes from northern Italy (Annicchiarico, 2006). Molecular information obtained from DNA of replicated bulked plants for each marker type was evaluated for its value per se and when combined with morphophysiological information. The consistency between morphophysiological, SSR marker, and SNP marker information was concurrently investigated.

Materials and Methods

Plant Material

The study evaluated 11 landraces representative of the seven commercial ecotypes of northern Italy (Fig. 1). The cultivars were collected from farms that had multiplied their own seed for at least 20 yr, with further information reported previously (Annicchiarico, 2006).

Morphophysiological Data

These data were recorded in a rainfed field experiment established in Lodi (45°18′ N, 9°30′ E, 81 m elevation) in May 2002 by transplanting 2-mo-old seedlings previously grown in jiffy pots as described previously (Annicchiarico, 2006). Rainfall during the growing season (May–October 2002) amounted to 680 mm. The level of cold stress in winter 2003 was near the long-term site

average with 54 frost days and –7.8°C as the absolute minimum temperature.

The assessment of distinctness for synthetic varieties of open-pollinated species requires a minimal sample of 60 plants per cultivar subjected to individual measurements (UPOV, 1979, 2008; Gilliland and Gensollen, 2010). DUS trials in Italy are designed as randomized complete block experiments with four replicates per cultivar and 22 plants per replicate (M. Giolo, personal communication, 2015). For this experiment, plants of all cultivars were mixed in constant numbers into each of 128 units (grids) which, in turn, were arranged in four parallel columns of 32 adjacent units each. Plants within units were randomly arranged in a row–column layout as described in Annicchiarico (2004, 2006). For the purpose of this study, we always selected two plants' data per cultivar in each unit and considered each column of units as a complete block that included a sample of 64 plants per cultivar. This planting pattern, which allowed for larger plant sample size per replicate of each cultivar and for more similar growing conditions for sets of plants of different cultivars within each block, might have provided lower experiment error for quantitative traits relative to typical DUS trials. Error coefficients of variation in the analysis of variance (ANOVA) for single traits are reported in Table 1 as a reference.

The following 11 morphophysiological traits were recorded on individual plants as described in Annicchiarico (2006): (i) lateness of flowering (based on the visual score of first reproductive nodes proposed by Kalu and Fick [1981] modified as 1 = ripe seedpods, 2 = unripe seedpods, 3 = open flowers, 4 = floral buds, and 5 = vegetative); (ii) length of the main stem; (iii) number of stems per plant; (iv) length and (v) width of the central leaflet (assessed on the third leaf below the first reproductive node), and the proportions of (vi) variegated and (vii) very dark blue-violet flowers, all of them being assessed in early summer 2002 before the second forage harvest; (viii) plant height in autumn 2002 (trait strictly associated with winter dormancy); (ix) plant height at the onset of spring 2003; (x) summer plant mortality (as difference in plant number between spring 2002 and autumn 2002); and (xi) winter plant mortality (as difference in plant number between autumn 2002 and early spring 2003). We found no plants with cream, white, or yellow flowers.

The observed traits included those indicated as compulsory for DUS testing by UPOV (2005). We included all traits recommended by Rotili et al. (1999) for DUS testing in Italy with the exception of growth habit, leaf color and stem color (for which little variability was observed among cultivars), and shape of the central leaflet (reportedly useless for cultivar discrimination; Russi and Falcinelli, [1997]). The observed traits comprised, in addition, summer mortality and number of stems per plant (UPOV, 2005). The former displayed fairly large variation in our rainfed experiment, whereas the latter is a promising descriptor on the basis of its large cultivar variation that is highly consistent across testing environments (Annicchiarico, 2007; Annicchiarico et al., 2013). Additional information on experiment management and data recording is reported in Annicchiarico (2006).

Table 1. Experiment error coefficient of variation (CV) and P-level for cultivar variation in an analysis of variance, and range of cultivar mean values, for 11 morphophysiological traits assessed on 11 alfalfa cultivars.

Trait	Error CV	Range	*P*-level
Lateness of flowering (score 1–5)	12.3	1.7–2.6	0.01
Length of the main stem (cm)	5.1	54.5–59.7	0.01
Number of stems per plant	16.2	4.5–8.0	0.01
Length of the central leaflet (mm)	3.8	28.1–31.7	0.01
Width of the central leaflet (mm)	3.9	15.1–16.2	0.01
Proportion of variegated flowers	33.0	2.7–14.2	0.05
Proportion of dark blue-violet flowers	27.1	2.7–15.3	0.01
Plant height in autumn (cm)	9.6	10.1–13.4	0.01
Plant height at onset of spring (cm)	6.7	13.0–14.8	0.01
Summer plant mortality (%)	34.7	2.1–22.7	0.01
Winter plant mortality (%)	25.1	2.3–19.9	0.01

Plant Sampling for DNA Analyses

Plant samples targeted for SSR marker or GBS analyses were obtained from plants grown in a greenhouse. Three bulks of 100 independent plants each (acting as replicates) were assembled for each of the 11 cultivars by pooling the central leaflet from the first trifoliate leaf of each relevant plant. Genomic DNA was extracted from each bulk using the Genelute plant genomic DNA kit (Sigma-Aldrich) checking DNA quality on a 1% agarose gel.

Simple-Sequence Repeat Marker Data

We selected 65 SSR markers derived from expressed sequence tag (EST) sequences or genomic DNA libraries on the basis of prior information by Diwan et al. (2000), Baquerizo-Audiot et al. (2001), Julier et al. (2003), Sledge et al. (2005), and S. Santoni (personal communication, 2003). The M13 tailing method described by Schuelke (2000) was used to label polymerase chain reaction (PCR) products. Each SSR marker was amplified by PCR independently in 10-μL reaction volume containing 20 ng of genomic DNA, 0.2 mM dNTP, 1.6 μM of each forward and labeled M13(−21) primer, 0.4 μM of reverse primer, 2.0 mM $MgCl_2$, and 0.2 U of Taq DNA polymerase in 1× PCR supplied buffer. Thermocycling conditions were as follows: 94°C for 5 min, 35 cycles of 94°C for 30 s, annealing at the appropriate temperature (from 55 to 65°C) for 1 min, 72°C for 1 min, 8 cycles of 94°C for 30 s, 53°C for 1 min, 72°C for 1 min, final extension at 72°C for 10 min. PCR products with different fluorescent labels and fragment size were pooled for detection. We used DreamTaq DNA Polymerase (Thermo Fisher Scientific) and an Applied Biosystems GeneAmp PCR System 9700 thermocycler. Typically, four PCR products (1 μL each) were combined with 15 μL of Hi-Di formamide and proper

volume of GeneScan-500 ROX internal size standard and analyzed on an ABI PRISM 310 Genetic Analyzer (Applied Biosystems). The PCR fragment size of the SSR loci was read using the GeneScan 3.7 software, scoring the different fragments as present (1) or absent (0).

Single-Nucleotide Polymorphism Marker Data

A GBS library was constructed according to the protocol by Elshire et al. (2011) with modifications. Briefly, 100 ng of each of the 33 bulked DNA samples (11 cultivars × 3 replicates) was digested with ApeKI (NEB, R0643L) and then ligated to a unique barcoded adaptor and a common adaptor that has "*W*" changed to "*A*" to reduce the number of target sites. Equal volume of the ligated product was pooled and cleaned up with QIAquick PCR purification kit (QIAGEN, 28104) for PCR amplification. In PCR, 50 ng template DNA was mixed with 25 pmol of each primer and KAPA library amplification readymix (Kapa Biosystems Cat # KK2611) in a final volume of 50 µL. Amplification was performed on a thermocycler for 10 cycles with 10 s of denaturation at 98°C, followed by 30 s of annealing at 65°C, and finally 30 s extension at 72°C. The resulting library was sequenced in one lane on an Illumina HiSeq 2000 at the Genomic Sequencing and Analysis Facility at the University of Texas at Austin, TX.

Raw reads (100 bp, single-end read) sequencing data were demultiplexed and quality filtered using the UNEAK pipeline (Lu et al., 2013). All reads beginning with the expected barcodes and cut site remnant were trimmed to 64 bp grouping identical reads into one tag. Tags with 10 or more reads across all 33 bulked DNA samples were retained for pairwise alignment, which aimed to find tag pairs that differed by 1 bp. Given our focus on allele frequencies within each cultivar, we estimated the fraction of each allele over the total number of reads separately for each bulked DNA sample. By a last filtering step, we kept for analyses only the SNP markers that showed at least 30 reads in all bulked samples. We empirically verified, by a discriminant analysis procedure described in the next section, that the 30-read threshold represented an optimal compromise between the contrasting desiderata of high number of markers (favored by a low threshold for number of reads) and high reliability of allele frequency estimates (favored by a high number of reads).

Statistical Analysis

Before ANOVA, quantitative morphophysiological data of each cultivar were averaged across plants of each replicate, whereas proportion data relative to cultivar mortality or flower color were submitted to the angular transformation. Lateness of flowering was analyzed as quantitative, since its distribution for the individual cultivars revealed just a slight trend toward a left-skewed and flatter distribution than the normal one. According to UPOV (1979) and to procedures adopted for DUS testing in Italy (Piano et al., 2001), pairs of cultivars were considered to differ for a trait when their difference exceeded Fisher's least significant difference (LSD) at $P < 0.05$.

We used a discriminant analysis to verify empirically the impact on cultivar distinctness of different minimum number of reads in all bulked samples for retention of individual SNP markers (10, 20, 30, 40, 50, or 60 reads). Distinctness ability was assessed as the proportion of paired cultivars out of the total number of pairs (i.e., 55) that showed distinctness. Discriminant analysis, although proposed occasionally even for morphophysiological diversity (e.g., Russi and Falcinelli, 1997), is not contemplated by UPOV's testing criteria for variety distinctness. We used a principal components analysis (PCA) performed on unstandardized SNP frequency data of the 33 bulked DNA samples (11 cultivars × 3 replicates) as a variable reduction tool (Afifi and Clark, 1984) applying the discriminant analysis to the first 30 PC axes (which always accounted for over 95% of total SNP marker variation). This procedure offered the additional advantage of using noncorrelated variables in the discriminant analysis (Jombart et al., 2010). We used a threefold stratified cross-validation procedure to assess the proportion of misclassified individual bulked DNA samples, granting distinctness to pairs of cultivars that featured 0% misclassification.

The consistency between morphophysiological-, SSR marker-, and SNP marker-based measures of cultivar diversity was assessed by computing Euclidean distances between cultivars for each information layer and then assessing their correlation by Mantel's test. For estimation of Euclidean distances, cultivar values for morphophysiological traits or SNP allele frequencies were averaged across replicates, whereas those for SSR alleles were obtained by summing up 0 or 1 values for each band over cultivar replicates. Morphophysiological-trait-based distances were computed on cultivar data standardized to zero mean and unit standard deviation. Mantel's tests results were averaged over 10,000 permutations.

Cultivar distinctness based on SSR or SNP marker information was assessed according to two strategies. The first focused on paired differences between cultivars for individual marker alleles, such as the presence or absence of one SSR band or the frequency of one SNP allele. Cultivar differences for presence or absence of single SNP alleles were ruled out, since only one marker in one cultivar displayed 100% frequency of one allele. Two cultivars were considered to differ for one SSR band if the difference was consistent across all replicates of these cultivars (i.e., in the absence of polymorphic bands within cultivar). Likewise, two cultivars differed for frequency of one SNP marker only if all nine possible paired comparisons between individual replicates of the two cultivars differed at $P < 0.05$ according to Fisher's exact test.

The second strategy focused on cultivar differences for overall genetic diversity. A PCA including data for the 33 bulked DNA samples (11 cultivars × 3 replicates) was performed separately on 1/0 (presence/absence) data of SSR marker alleles and on frequency data of SNP marker alleles using unstandardized values. Each PCA reduced the large number of original variables into a smaller set of derived principal components that was used to assess

cultivar distinctness. Consistent with UPOV's testing procedure for single morphophysiological traits, a separate ANOVA was performed on the 33 score values of each PC axis, declaring as different those pairs of cultivars whose difference in mean PC score exceeded Fisher's LSD at $P < 0.05$. The ANOVA started with the first PC axis (PC 1) and continued for lower-rank PC axes until three consecutive PC axes failed to display overall cultivar variation at $P < 0.05$. Cultivar differences were assessed only for PC axes displaying overall cultivar variation ($P < 0.05$).

The usefulness of each criterion for variety distinction was assessed as the proportion of paired cultivar comparisons that exhibited distinctness. We granted robust distinctness to pairs of cultivars that displayed two or more significant differences for each distinctness criterion based on morphophysiological traits, individual SSR bands, allele frequencies for individual SNP markers, or PC axes in the analysis of SSR or SNP marker diversity (e.g., two out of 11 morphophysiological traits), and weak distinctness to those displaying only one significant difference for a given criterion.

We also assessed the usefulness of each marker-based criterion when used in combination with morphophysiological diversity by computing the proportion of paired cultivar comparisons that showed weak and robust distinctness after combining the differences for the morphophysiological and the marker-based criterion.

Limited to SNP data, we also verified cultivar distinctness using a discriminant analysis as described above. Pairs of cultivars that featured 0% misclassification were granted robust distinctness. We did not consider weak distinctness for this approach in the absence of possibly relevant misclassification values (e.g., $\leq$5%).

ANOVAs for cultivar comparisons based on morphophysiological traits or marker-derived PC scores were performed using SAS v.9.2 (SAS Institute, 2008) statistical software. Discriminant analysis was performed using the Weka data mining framework (Hall et al., 2009). The remaining analyses were performed using R software (R Development Core Team, 2014).

Results

All morphophysiological traits exhibited overall cultivar variation at $P < 0.01$ and several significant differences between cultivars (assessed at $P < 0.05$) except for proportion of variegated flowers (Table 1), which was able to distinguish only one pair of cultivars (data not shown).

Forty-one SSR markers out of 65 showed at least one polymorphic band across the 33 bulked DNA samples. However, 12 out of these 41 markers failed to provide useful alleles for cultivar distinction (Table 2), because of inconsistency among replicates of a given cultivar. The most discriminating SSR marker displayed three useful alleles, which altogether, provided at least one different banding pattern for 28 paired cultivar comparisons out of 55 (Table 2). Thirteen other markers proved very useful, as they exhibited at least one different band in at least 10 paired comparisons (Table 2).

Barcoded reads issued by GBS were, on average, 530,514 per bulked DNA sample. The initial set of polymorphic SNP markers issued by the UNEAK pipeline without filtering for minimum number of reads across all bulked samples included 72,502 markers with an average read depth per bulked DNA sample of 8.6. Results of discriminant analyses using minimum number of reads for SNP marker retention in the range of 10 to 60 indicated a peak of cultivar distinctness between 20 and 30 (Fig. 2). We preferred the 30-reads threshold for further analyses because it reduced the risk of imputing noisy data in analyses of overall molecular variation (such as PCA) while still providing a high number of SNP markers (2902) for paired comparisons based on allele frequency of individual markers. We verified, however, that lowering the threshold to 20 reads did not improve the distinctness of cultivars in following analyses based on allele frequencies or scores on significant PC axes (data not shown). The resulting average read depth per sample, after the 30-reads threshold filtering, was 80.3.

According to Mantel's test and relative correlations results, the morphophysiological diversity between cultivars (as estimated by the matrix of Euclidean distances) was completely unrelated to SSR marker-based cultivar diversity ($r = 0.04$, $P > 0.30$) and poorly related to SNP marker-based diversity ($r = 0.23$, $P < 0.15$). The SSR and SNP marker-based indications on cultivar diversity were moderately consistent ($r = 0.62$, $P < 0.05$).

Significant cultivar variation ($P < 0.05$) emerged for (i) four PC axes (PC 1, PC 2, PC 3, and PC 5, jointly accounting for 33.2% of overall genetic variation) for SSR marker data and (ii) six PC axes (PC 1, PC 2, PC 3, PC 4, PC 5, and PC 7, jointly accounting for 28.6% of overall genetic variation) for SNP marker data. Principal component 4 for SSR and PC 6 for SNP data, which failed to achieve significance, explained a nearly identical amount of variation as their immediate higher-rank, significant PC axis. The ordination of bulked DNA samples in the space of significant PC axes indicated the occurrence of some inconsistency between replicates of each cultivar for both SSR and SNP marker data (Fig. 3) as expected from the effect of plant sampling variation. The coded cultivars 18 and 25 were sharply distinct from any other cultivar along PC 1 and PC 2 according to both marker types. The sharp molecular distinctness of 25 from cultivars 3, 4, and 8, also belonging to the historical ecotype 'Romagnola' (Fig. 1), confirmed that cultivar diversity and distinctness were not necessarily lower for cultivars belonging to the same ecotype group. Differences in cultivar PC scores were manifest also along other PC axes. For example, the cultivar 17 tended toward low values of PC 3 and high values of PC 5 in the analysis of SSR marker data, whereas the cultivars 4, 10, and 19 tended to display extreme PC 3 or PC 4 scores in the analysis of SNP marker data (Fig. 3).

Given the overwhelming influence of cultivars 18 and 25 on cultivar diversity along PC 1 and PC 2 for SSR or SNP markers, we performed additional PCA and ANOVA analyses to verify whether the absence of these cultivars

Table 2. Name, origin, linkage group, locus, observed size range, numbers of total polymorphic alleles, useful alleles for distinguishing 11 alfalfa cultivars and distinguished pairs of cultivars out of 55 paired comparisons, for 41 SSR markers.

Marker name†	Origin‡	Linkage group§	Locus	Observed size range	Total alleles	Useful alleles¶	Distinguished pairs of cultivars¶
				bp			
AFCA1[1]	*Ms* genomic seq.	4	–	139–161	5	0	0
AFCA11[1]	*Ms* genomic seq.	6	–	136–161	4	3	23
AFCA16[1]	*Ms* genomic seq.	8	–	84–93	1	1	8
AFCT11[1]	*Ms* genomic seq.	–	–	185–196	2	1	2
AFCT45[1]	*Ms* genomic seq.	7	–	129–137	3	2	7
AW01[4]	*Mt* EST	6	AW559402	178–203	3	1	5
AW170[4]	*Mt* EST	2	AW695035	290–301	1	1	10
AW290[4]	*Mt* EST	5	AW688825	183–197	1	0	0
B14B03[3]	*Mt* EST	5	B14B03	142–184	2	1	7
BF106[4]	*Mt* EST	5	BT645633	193–216	6	2	15
BI90[4]	*Mt* EST	4	BF642442	249–278	4	2	17
E318681[3]	*Mt* EST	5	–	110–113	1	1	5
FMT05[5]	*Mt* genomic seq.	–	TPC63A	164–184	5	2	13
FMT06[5]	*Mt* genomic seq.	–	TPC42F	125–143	6	3	11
FMT07[5]	*Mt* genomic seq.	–	TPC67	100–104	1	0	0
FMT16[5]	*Mt* genomic seq.	–	TP43B	68–78	1	0	0
FMT51[5]	*Mt* genomic seq.	–	TA34	150–160	1	1	15
MAA660252[2]	*Mt* genomic seq.	–	MAA660252	97–114	3	1	0
MAA660456[2]	*Mt* genomic seq.	4	MAA660456	109–135	1	0	0
MAL368332[5]	*Mt* genomic seq.	–	MtBA023G11F1	123–129	1	1	9
MAL372597[5]	*Mt* genomic seq.	–	MtBA052C01F1	136–170	1	1	12
MAW127262[5]	*Mt* genomic seq.	–	MAW127262	146–203	3	1	9
MSA132930[5]	*Mt* genomic seq.	–	MSA132930	134–151	1	0	0
MSAJ2486[5]	*Mt* genomic seq.	–	MSAJ2486	203–214	1	1	10
Mt1D06[4]	*Mt* BAC seq.	2	AC138449	184–205	4	1	4
Mt1G05(2) [4]	*Mt* BAC seq.	5	AC122727	259–274	1	1	7
MTIC14[3]	*Mt* EST	6	–	125–131	1	0	0
MTIC153[3]	*Mt* EST	6	–	143–191	14	2	13
MTIC189[3]	*Mt* EST	3	–	126–156	7	1	1
MTIC237[3]	*Mt* EST	3	–	117–120	1	1	10
MTIC272[3]	*Mt* EST	6	–	127–142	5	1	2
MTIC289[3]	*Mt* EST	7	–	177–181	1	1	6
MTIC331[3]	*Mt* EST	4	–	92–98	2	0	0
MTIC338[3]	*Mt* EST	3	–	177–189	1	0	0
MTIC343[3]	*Mt* EST	6	–	135–158	3	0	0
MTIC347[3]	*Mt* EST	4	–	109–121	1	1	7
MTIC365[3]	*Mt* EST	2	–	127–152	9	3	28
MTIC447[3]	*Mt* EST	1	–	108–112	1	0	0
MTIC451[3]	*Mt* EST	2	–	149–167	8	4	19
MTIC475[3]	*Mt* EST	3	–	116–133	4	1	10
MTIC95[3]	*Mt* EST	1	–	117–147	2	1	5

† According to [1] Diwan et al. (2000); [2] Baquerizo-Audiot et al. (2001); [3] Julier et al. (2003); [4] Sledge et al. (2005); [5]S. Santoni (personal communication, 2003).

‡ *Mt, Medicago truncatula*; *Ms, Medicago sativa.*

§ According to Julier et al. (2003) or Sledge et al. (2005).

¶ Distinction only when allele differences are consistent across three replicates of bulked plants per cultivar.

would modify the distinctness results for the other cultivars. Significant cultivar variation ($P < 0.05$) in these new analyses emerged for PC 1 and PC 2 for SSR markers and PC 1, PC 2, PC 3, and PC 5 for SNP markers. In all cases for SSR marker diversity and in 24 cases out of 27 for SNP markers, robust distinctness between cultivars that emerged in the comprehensive analysis was confirmed in the analysis excluding the cultivars 18 and 25. Particularly for SNP markers, cultivar ordination along the first two PC axes in the latter analysis reflected closely the ordination along the two main significant PC axes other than PC 1 and PC 2 in the comprehensive analysis (see PC 1 and PC 2 in Fig. 4 vs. PC 3 and PC 4 in Fig. 3).

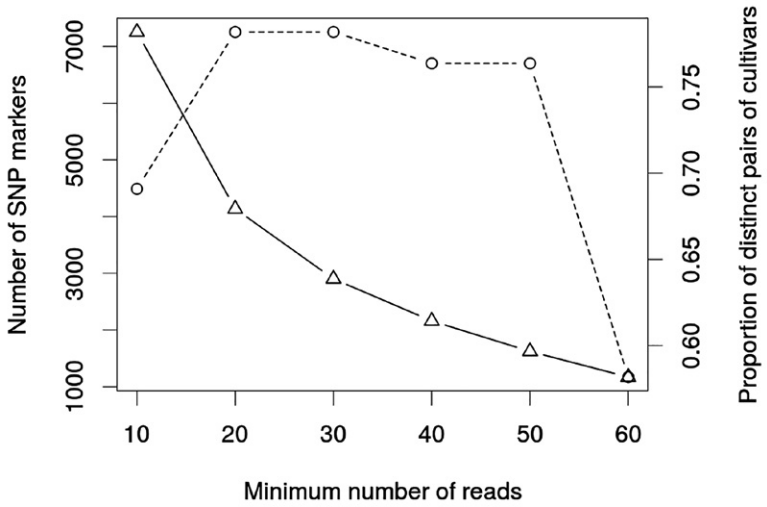

Fig. 2. Effect of different minimum number of reads for retention of individual single-nucleotide polymorphism (SNP) markers on (i) total number of markers (solid line) and (ii) the proportion of paired comparisons between 11 alfalfa cultivars that showed robust distinctness in a discriminant analysis (as 0% misclassification of individual bulked DNA samples) (dashed line).

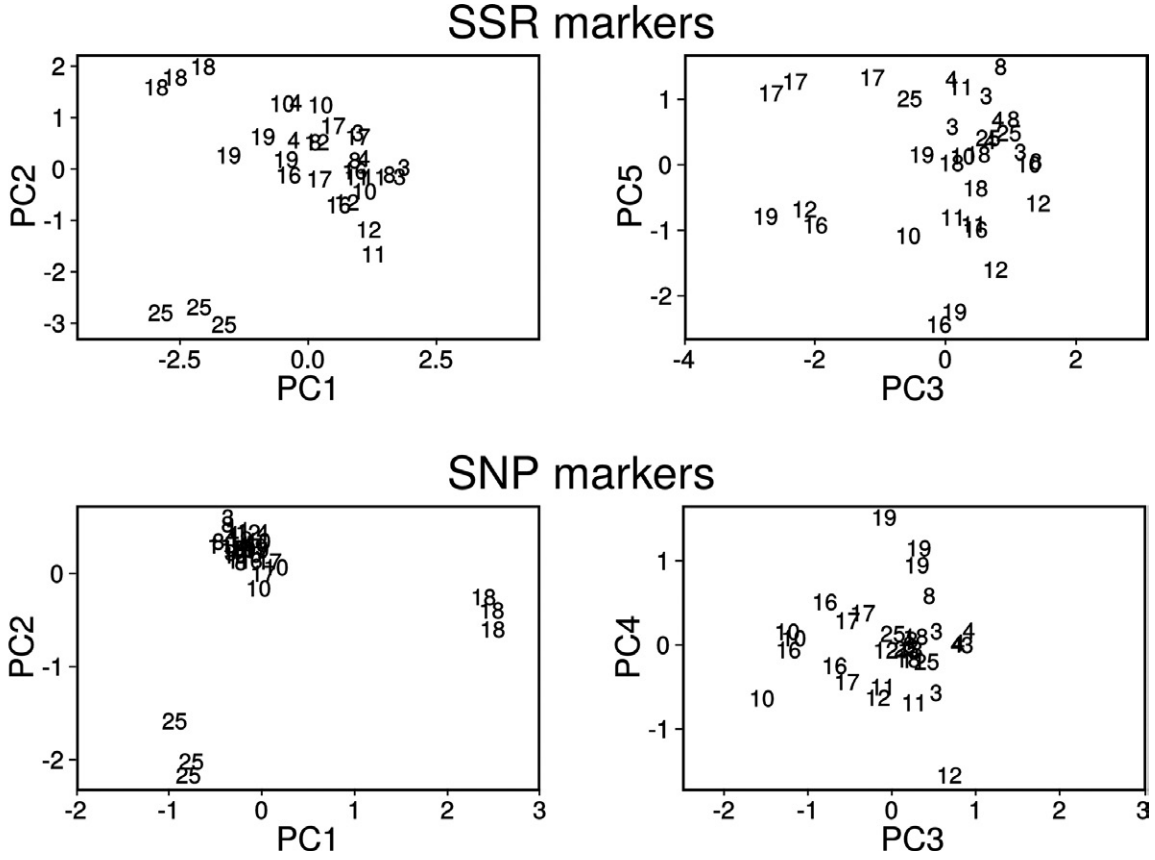

Fig. 3. Ordination of 11 coded alfalfa cultivars, each represented by three independent sets of 100 bulked plants, in the space of four principal component (PC) axes featuring significant variation for cultivar PC score separately for data of 41 simple-sequence repeat (SSR) markers and 2902 single-nucleotide polymorphism (SNP) markers. Overall variation explained by PC axes is 33.2 and 28.6% for SSR and SNP markers, respectively. See Annicchiarico (2006) for cultivar codes.

Based on morphophysiological trait-based distinctness, 12 cultivar pairs out of 55 were not different for any of the 11 traits, 14 showed a difference in only one trait, and 29 showed two or more differences (Table 3). Individual SSR bands allowed for distinguishing all cultivars from one another on the ground of weak distinctness (i.e., one difference) and 47 on the basis of robust distinctness (i.e., two or more different SSR bands; Table 3). Distinctness based on overall SSR marker variation via cultivar comparisons for individual significant PC axes displayed

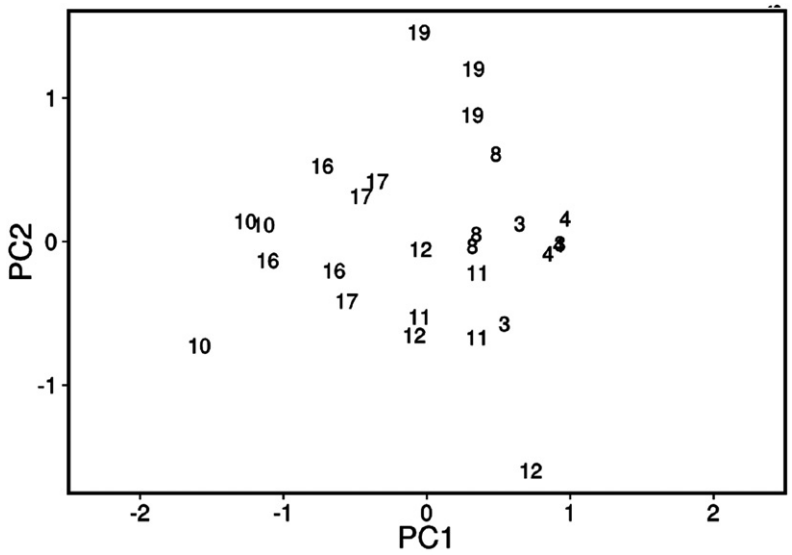

Fig. 4. Ordination of nine coded alfalfa cultivars (excluding the two most distinct cultivars of the set in Fig. 3), each represented by three independent sets of 100 bulked plants, in the space of two principal component (PC) axes featuring significant variation for cultivar PC score for data of 2902 SNP markers. Overall variation explained by PC axes is 20.2%. See Annicchiarico (2006) for cultivar codes.

less cultivar differences than the criterion based on SSR single bands, achieving a performance roughly comparable with that based on morphophysiological traits (Table 3). The SNP-marker-based distinctness was best achieved by assessing cultivar differences for PC score on significant axes (44 cultivar pairs exhibiting robust distinctness) or through discriminant analysis (43 cultivar pairs displaying robust distinctness) (Table 3). In contrast, paired comparisons for individual SNP allele frequencies could not differentiate 26 of 55 cultivar pairs, and only 16 pairs exhibited two or more significant differences (Table 3).

The usefulness of best marker-based criteria for distinguishing morphophysiologically similar cultivars was manifest in some cases. For example, the coded cultivar 17 displayed robust distinctness from only two cultivars on the basis of morphophysiological differences, all ten cultivars on the basis of individual SSR bands, nine cultivars on the basis of PC scores for overall SNP marker variation, and eight cultivars by discriminant analysis based on overall SNP variation.

The combination of the morphophysiological trait-based criterion with one marker-based criterion always led to an increase in the proportion of distinct pairs of cultivars in comparison with results for each individual criterion (Table 3, 4). The number of pairs of cultivars that failed to achieve robust distinctness dropped below 10% when adding either of the two best-performing marker-based criteria, namely, cultivar differences for individual SSR bands or for significant PC axes summarizing SNP marker variation, to morphophysiological trait-based diversity (Table 4). Interestingly, robust distinctness from either of these procedures compared favorably even to weak distinctness based on morphophysiological traits (22% indistinct pairs of cultivars; Table 3, 4).

Table 3. Number of paired comparisons between 11 alfalfa cultivars that showed nil, weak, and robust distinctness out of 55 total comparisons, for cultivar distinction based on (i) 11 morphophysiological traits (Mor), (ii) 41 simple-sequence repeat (SSR) markers, and (iii) 2902 single-nucleotide polymorphism (SNP) markers.

Data type†	Distinctness criterion	No distinctness	Weak distinctness	Robust distinctness
Mor	LSD ($P < 0.05$) for single traits‡	12	14	29
SSR marker	Presence or absence of single bands§	0	8	47
SSR marker	LSD ($P < 0.05$) for single PC axes‡	8	19	28
SNP marker	Allele frequency difference ($P < 0.05$)¶	26	13	16
SNP marker	LSD ($P < 0.05$) for single PC axes‡	3	8	44
SNP marker	Discriminant analysis, 0% misclassification#	12	–	43

† Morphophysiological data, four replicates per cultivar; SSR or SNP marker data, three replicates per cultivar, each including 100 independent bulked plants.

‡ Weak and robust distinctness indicates one or more differences, respectively, between cultivar means, in analyses of variance for single morphophysiological traits or for scores of individual principal component (PC) axes summarizing SSR marker data (four significant PCs) or SNP marker data (six significant PCs).

§ Weak and robust distinctness indicates one or more differences, respectively, in individual SSR bands; differences consistent across the nine possible paired comparisons between individual replicates.

¶ Weak and robust distinctness indicates one or more differences, respectively, for allele frequency of individual SNP markers according to Fisher's exact test; differences consistent across the nine possible paired comparisons between individual replicates.

Using a threefold stratified cross-validation on data of individual replicates.

Discussion

Three molecular criteria, one based on individual SSR bands and two based on overall variation for SNP marker data analyzed either by cultivar differences on significant PC axes or discriminant analysis, exhibited distinctly better ability to distinguish alfalfa cultivars than the

Table 4. Number of paired comparisons between 11 alfalfa cultivars that showed nil, weak, and robust distinctness out of 55 total comparisons, for cultivar distinction based on 11 morphophysiological traits (Mor) alone or in combination with 41 simple-sequence repeat (SSR) markers or 2902 single-nucleotide polymorphism (SNP) markers.†

Data type	Distinctness criterion	No distinctness	Weak distinctness‡	Robust distinctness§
Mor	LSD ($P < 0.05$) for single traits	12	14	29
Mor + SSR marker	Presence/absence of single bands	0	3	52
Mor + SSR marker	LSD ($P < 0.05$) for single PC axes	2	6	47
Mor + SNP marker	Allele frequency difference ($P < 0.05$)	6	12	37
Mor + SNP marker	LSD ($P < 0.05$) for single PC axes	0	5	50
Mor + SNP marker	Discriminant analysis, 0% misclassification	5	2	48

† See footnotes of Table 3 for description of marker data and definition of weak and robust distinctness for Mor and individual marker-based distinctness criteria.

‡ Weak distinctness according to Mor and no distinctness according to the marker-based criterion or vice versa.

§ Either weak distinctness according to both Mor and the marker-based criterion; or robust distinctness according to Mor, the marker-based, or both criteria.

morphophysiological trait-based criterion. In contrast, the molecular criterion based on SNP allele frequency differences was less discriminating than morphophysiological variation, possibly because of the extreme intrapopulation variation for individual markers that is generally a feature of alfalfa cultivars (Brummer et al., 1991) including Italian landraces (Zaccardelli et al., 2003). Such variation is consistent with the large intrapopulation morphophysiological diversity featuring the cultivars of alfalfa and other perennial legume species (Annicchiarico, 2006; Annicchiarico et al., 2015a), which contributes to the difficulty of satisfying DUS requirements. Remarkably, all SNP alleles except one were present in all cultivars, confirming the difficulty of using information from individual SNP markers for assessing cultivar distinctness. As anticipated, distinctness based on individual markers is, anyway, less attractive than distinctness based on overall marker diversity because it implies higher risk of granting distinctness to essentially derived candidate varieties modified for some specific allele or allele frequency.

Our choice of marker-based criteria for cultivar distinctness was partly driven by preference for relatively simple approaches, which would favor their implementation in variety registration schemes. To facilitate further their possible acceptance by UPOV, two criteria based on overall SSR or SNP molecular diversity paralleled that used for morphophysiological traits, since they were based on statistically significant cultivar differences assessed through ANOVA on individual variables (represented in this case by derived PC variables summarizing uncorrelated main patterns of marker variation). Analyses excluding the two sharply distinct cultivars 18 and 25 suggested that distinctness results generated by this approach are affected only limitedly by the set of tested cultivars. We envisaged also discriminant analysis, although not contemplated by UPOV testing guidelines, owing to its possible interest for analyzing marker data expressed as continuous variables such as allele frequencies (Jombart et al., 2010). Cultivar classification by cluster analysis was considered less useful than cultivar ordination associated with ANOVA or cultivar discriminant analysis because its results can be affected sizably by the adopted grouping algorithm (Afifi and Clark, 1984).

The only moderate usefulness for cultivar distinction exhibited by morphophysiological diversity probably reflected what is encountered in ordinary DUS trials. Error coefficients of variation values observed here (Table 1), compared with those observed on average in DUS trials of Italy across 2011 and 2012 (M. Giolo, personal communication, 2015), were comparable or slightly smaller (e.g., 5.1 vs. 6.4% for length of the main stem; 6.7 vs. 6.9% for plant height in spring; 9.6 vs. 8.8% for plant height in autumn). The extent of morphophysiological distinctness might have been reduced if multiyear data were available, owing to cultivar × year interactions, which can be taken into account in the assessment of cultivar differences through different possible procedures (UPOV, 1996, 2008).

The ability of a simple criterion such as SSR banding pattern to distinguish all pairs of cultivars by at least one difference, and 85% of them according to at least two differences, is remarkable. These results would support its adoption for the assessment of alfalfa variety distinctness particularly on basis of two or more requested differences (which could increase its reliability and decrease the risks of variety plagiarism). Information reported in Table 2 can help define a set of SSR markers with highest potential for cultivar discrimination.

Our results, however, also showed the interest of methods for cultivar distinction based on overall SNP marker diversity. These methods, in comparison with that based on diversity for single SSR bands, would virtually eliminate any risk of essential derivation, given the high number of relevant SNP markers. They also imply somewhat greater complexity for preparation and analysis of DNA samples and bioinformatics analysis, but these disadvantages may decrease with time as a consequence of the increasing popularity of GBS data for marker-assisted and genomic selection.

The substantially independent information provided by morphophysiological and marker diversity can explain the excellent ability to distinguish cultivars that was achieved by combining the two types of information. This combination could be devised in different ways in DUS testing. For example, a preliminary assessment based on one molecular marker criterion could be considered sufficient when its results indicated robust distinctness, while requiring the morphophysiological assessment otherwise (UPOV, 2004). On the other hand, the little relationship

between morphophysiological and marker diversity discourages the exploitation of the latter to select control varieties for DUS trials on the basis of greater expected morphophysiological similarity with candidate varieties. Actually, this strategy of variety selection may show different potential depending on the species (UPOV, 2004). There is increasing evidence for substantial inconsistency between morphophysiological and marker diversity in forage legumes such as alfalfa (Crochemore et al., 1998), white clover (Kölliker et al., 2001; Annicchiarico and Carelli, 2014), red clover (*Trifolium pratense L.*) (Greene et al., 2004; Dias et al., 2008; Pagnotta et al., 2011), and forage grasses (Roldán-Ruiz et al., 2001). Interestingly, our study confirmed such inconsistency for both SSR and SNP markers.

The consistency between different marker types for indications on cultivar genetic distances has scarcely been investigated. The study by Roldán-Ruiz et al. (2001) reported modest correlation ($r = 0.42$) between distances among perennial ryegrass varieties estimated according to AFLP and STS markers, sampling cultivars by 18 to 54 individual plants for AFLP and 20 plants for STS markers. Moderately high consistency ($r = 0.62$) emerged here between SSR and SNP markers used on alfalfa cultivars represented by bulked plants.

Some degree of inconsistency across cultivar replicates represented by independent bulked plants is typical of phylogenetic analyses based on molecular markers in outbred species as reported by Kölliker et al. (2001) for AFLP markers and Byrne et al. (2013) for SNP markers. For SSR markers, alleles whose frequency in the cultivar is around 10% can easily show a present or absent band as a result of sampling effects (Carelli et al., 2009), which can be reduced by bulking large numbers of plants (Pupilli et al., 1996). Variation across cultivar replicates for SNP marker diversity may arise from inaccurate estimation of cultivar allele frequencies because of insufficient read depth of DNA fragments (here object of a separate assessment) or small number of bulked plants. The visual comparison of replication ordination along PC axes between this study and Byrne et al.'s (2013) suggests smaller variation across replicates in the latter study, which may arise from its larger number of bulked plants per replication (~200 vs. 100). We speculate that raising the number of bulked plants to 200 or more per replicates may ensure, for a modest additional cost, higher SNP or SSR marker-based cultivar distinction via lower inconsistency across cultivar replicates. However, specific work aimed to optimize the number of bulked plants for marker-based cultivar discrimination is required. Likewise, the impact on cultivar distinctness of higher GBS effort (i.e., higher number of total reads per bulked DNA sample) deserves investigation. Results from such studies could reinforce the potential interest of molecular criteria for assessing variety distinction in alfalfa or other forage crops.

The advantages of molecular distinctness over a morphophysiological one (i.e., faster and less expensive assessment and nearly nil interaction with testing conditions) are leading to its increasing consideration in lawsuits (where variety protection is practically into context) and do justify research work particularly for crops bred as synthetic varieties. Our results for alfalfa landraces, whose distinctness may be more challenging to achieve than that for varieties (as suggested by results in Pupilli et al. [2000] and Zaccardelli et al. [2003]), indicate that marker-based distinctness has high potential as a valid substitute (or complement) for morphophysiological distinctness in DUS testing of alfalfa varieties. Marker-based distinctness could be implemented also in sui generis registration systems aimed at marketing alfalfa landraces.

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