Assessing genetic diversity of perennial ryegrass (*Lolium perenne* L.) from four continents by inter-simple sequence repeat (ISSR) markers

Tao Hu¹, Huiying Li¹, Deying Li², Jianming Sun¹ and Jinmin Fu¹*

¹Key Laboratory of Plant Germplasm Enhancement and Specialty Agriculture, Wuhan Botanical Garden, The Chinese Academy of Science, Wuhan City, Hubei, 430074, P.R. China.
²Department of Plant Sciences, North Dakota State University, Fargo, ND 58108-6050, USA.

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In this study, inter-simple sequence repeat (ISSR) markers were used to compare genetic diversity between commercial cultivars and natural germplasm which were obtained from Europe, Africa, Asia, and North America. There was a relatively high genetic variation in the whole collection judged by the polymorphism rate (97.16%), Nei's gene diversity (0.28), and Shannon's information index (0.44). Results indicate lower genetic diversity in commercial cultivars than natural germplasm. The European group showed the highest genetic diversity. The genetic distance (GD) between cultivars 'Exacta' and 'ABT-99-4.560' was the closest (0.19), while largest GD occurred between 'PI 632472' and 'PI 547390' (0.85). Based on Jaccard's similarity coefficient, 12 groups were distinguished with a cut-off point at 0.44. Using the concept of core collection, we suggested 'Headstast 2', 'PI 598909', 'Catalina II', 'PI 538976', 'PI 598440', 'PI 610925', 'PI 598877', 'PI 516605', and 'PI 619554' be included in a core collection of germplasm to accommodate maximum genetic diversity.

Key words: Genetic distance, genetic erosion, unweighted pair group method with arithmetic mean (UPGMA), cluster analysis, germplasm.

INTRODUCTION

Perennial ryegrass (*Lolium perenne* L.), native to Eurasia, is one of the most important forage and turfgrasses used in temperate region due to its rapid establishment, adaptability, and nutrition values (Thorogood, 2003). Because of the high economic value, breeders throughout the world have made a great deal of effort to develop elite cultivars. However, most of the breeding programs in the world have been relying heavily on very narrow genetic resources (Thorogood, 2003). As perennial ryegrass in old pastures and grasslands is being replaced by new cultivars without increasing the genetic diversity at the same time, there is a threat of genetic erosion (Adebooye and Opabode, 2004) despite its cross-pollination nature (Golembiewski et al., 1997). Although, this trend is difficult to quantify, comparison of variability of traits over geological distance has provided useful information on genetic diversity of ecological systems (Monestiez et al., 1994). Also, understanding the genetic diversity before, during, and after the release of cultivars is of vital importance to maintain broad genetic background (Cresswell et al., 2001; Günther et al., 1996).

It is well known that genetic diversity in natural and culture populations are increasingly declining because of over-exploration, changing environments and habitat fragmentation (Tang, 2007; Yang et al, 2011). Faced with the problem of preserving species diversity of perennial ryegrass, some biologists are now paying their concerns on genetic diversity in natural and culture populations (Balfourier and Charmet, 1991; Kollik et al., 1999; Roldan et al., 2000; Kubik et al., 2001; Ghariani et al., 2003; Bolaric et al., 2005). Roldan et al. (2000) revealed the high degree of genetic diversity within commercial...
ryegrass using amplified fragment length polymorphism (AFLP). Ghariani et al. (2003) examined genetic diversity of 16 wild perennial ryegrass accessions from Tunisian using ISSR and found large genetic diversity. Bolaric et al. (2005) assessed the genetic diversity within and among perennial ryegrass ecotypes from Germany using RAPD and found that genetic variation within cultivars (67%) was much larger than between them (33%). Although, great efforts have been focusing on its cultivation and natural germplasm, there is hardly any information on genetic diversity in the commercial and natural populations over larger geographical regions including Asia.

Inter-simple sequence repeats (ISSR) marker works by amplifying DNA segment between two SSR sequences based on polymerase chain reaction (PCR) method (Zietkiewicz et al., 1994). Compared to morphological, allozyme markers, random amplified polymorphic DNA (RAPD) technique, ISSR technique is simple, economical and reliable to assess the phylogenetic relationships and identify cultivars of various plants, which has been tested in both dicotyledon and monocotyledon species (Bornet and Branchard, 1991; Girma et al., 2010; Godwin et al., 1997; Singh et al., 2007). The objective of this study was to confirm that ISSR could provide sufficient polymorphism in perennial ryegrass collected from Europe, Africa, Asia and America. A second objective was to compare genetic diversity between commercial cultivars with natural germplasm to understand the current status of genetic erosion. And finally, to establish a core collection list that could facilitate germplasm collection for breeding.

MATERIALS AND METHODS

Plant materials

75 accessions of perennial ryegrass were obtained from 21 countries and four continents. These accessions included 47 commercial cultivars and 28 natural germplasm and were coded according to their origins (Table 1). The plant materials were established and maintained in a hydroponic system using half strength Hoagland solution (Hoagland and Arnon, 1950). Each accession was planted in a plastic tube 10 cm in diameter and 15 cm deep, which was filled with ceramsite to 12 cm depth and covered with a 0.5 cm layer of sand. A nylon screen was secured to the bottom of the tube to allow free passage of roots into the cultural solution. The tubes were inserted 8.5 cm below the surface of hydroponic solution through a supporting rack placed on the top of 45.2 L containers. The Hoagland solution was replenished weekly. Each accession had four replicates and a total of 12 containers were maintained in a greenhouse with temperature of 22/18°C (day/night). The plants were fully established 30 days after seeding and were cut to 6 cm height every other day.

DNA preparation and ISSR genotyping

At the 6-leaf stage, fresh newly developed leaves were cut with scissors and frozen immediately with liquid nitrogen before storing in a freezer under -80°C for further analysis. Total genomic DNA was extracted using a modified CTAB protocol described by Wang., (2009). Amplification reactions of ISSR analysis were carried out in a total volume of 25 μl per sample, which contained 1.0 U Taq DNA polymerase (BestBio, China), 1× polymerase buffer (BestBio, China), 1.5 mM MgCl2, 150 mM dNTP (Pharmacia, America), 0.2 mM primers, and 40 ng DNA template. PCRs were performed in a Biometra Uno II thermal cycler programmed for one cycle of 94°C for 5 min, followed by 38 cycles of 94°C for 45 s, 45 s annealing for different primer at 53 to 58°C, and 72°C for 90 s, with a final elongation at 72°C for 7 min. The amplified products were separated electrophoretically on a 1.6% agarose gel, stained in ethidium bromide (0.5 μg/ml) and digitally photographed under UV light using Gel Doc XR system (Bio-rad, America). The size of amplification products was estimated with a D2000 molecular marker (BestBio, China).

Sixty ISSR primers were initially synthesized based on the results from previous research (Fan et al., 2007; Haijun et al., 2007; Wei et al., 2007; Zeng et al., 2006), and twenty eight were chosen because of their stability, polymorphism, and reproducibility. The optimized annealing temperatures for the 28 primers were confirmed using Thermocycler T-gradient. All tests were repeated twice.

Data analysis

The distinct and reproducible bands of each ISSR were scored as either present (1) or absent (0) to represent the genetic identity of each individual sample. Genetic diversity parameters were calculated using the version 1.32 of PopGene software (Yeh et al., 2000), which included group size (GS), number of polymorphic loci (NPL), polymorphism rate (PR), observed number of alleles (Na), and the following.

Effective number of alleles (Ne) was estimated from:

\[
Ne = 1 + 4 \bar{N} \nu
\]

Where, \( \bar{N} \) is the effective size of a haploid population and \( \nu \) is the average mutation rate (Maruyama and Kimura, 1980).

Shannon’s information index (I) was estimated for each locus using the following equation:

\[
I = - \sum p_i \ln p_i (i = 1 - S)
\]

Where, \( p_i \) is the frequency of the \( i \)th allele and \( S \) is the sum total of alleles in the locus (Shannon and Weaver, 1949).

Average Nei’s (1973) gene diversity (He) was estimated from:

\[
He = 1 - \sum p_i^2
\]

Where, \( p_i \) is the frequency of the \( i \)th allele (Nei, 1987).

A pairwise genetic similarity matrix was analyzed using Jaccard’s coefficient between each and every accession (Jaccard, 1912). A dendrogram was constructed based on the Unweighted-Pair Group Method arithmetic Average (UPGMA) using the version 2.01 of numerical taxonomy multivariate analysis system (NTSYS) (Rohlf, 2000). Genetic distances (GD) and principal coordinate analysis (PCA) were also performed using NTSYS.

Polymorphism information content (PIC) values were calculated using the algorithm:

\[
PIC = 1 - \sum f_i^2 \quad i=1
\]

Where, \( f_i \) is the frequency of the \( i \)th allele (Smith et al., 1997). The partition, within- and among-group, of all parameters was analyzed using the version 6.1 of analysis of molecular variance (AMOVA) software in GenALEX (Peakall and Smouse, 2006).
Table 1. A list of 75 perennial ryegrass (*Lolium perenne* L.) accessions (germplasm and cultivars) used for genetic diversity analysis using inter-simple sequence repeats markers.

<table>
<thead>
<tr>
<th>Accession code&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Accession name</th>
<th>Origin</th>
<th>Accession code</th>
<th>Accession name</th>
<th>Origin</th>
<th>Accession code</th>
<th>Accession name</th>
<th>Origin</th>
</tr>
</thead>
<tbody>
<tr>
<td>a1-1 PI 619033</td>
<td>Romania</td>
<td></td>
<td>c2 PI 502413</td>
<td>Uzbekistan</td>
<td></td>
<td>D2-23</td>
<td>BAR LP 4420</td>
<td>United States</td>
</tr>
<tr>
<td>a1-2 PI 610804</td>
<td>Romania</td>
<td></td>
<td>c3 PI 547390</td>
<td>Iran</td>
<td></td>
<td>D2-24</td>
<td>Panther GLS</td>
<td>United States</td>
</tr>
<tr>
<td>a1-3 PI 598453</td>
<td>Romania</td>
<td></td>
<td>d1 PI 403838</td>
<td>Canada</td>
<td></td>
<td>D2-25</td>
<td>Silver Dollar</td>
<td>United States</td>
</tr>
<tr>
<td>a2-1 PI 610795</td>
<td>France</td>
<td>D2-1</td>
<td>DP1</td>
<td>United States</td>
<td>D2-26</td>
<td>Pinnacle</td>
<td>United States</td>
<td></td>
</tr>
<tr>
<td>a2-2 PI 598439</td>
<td>France</td>
<td>D2-2</td>
<td>E-99</td>
<td>United States</td>
<td>D2-27</td>
<td>PST-217</td>
<td>United States</td>
<td></td>
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<tr>
<td>a2-3 PI 628693</td>
<td>France</td>
<td>D2-3</td>
<td>Linn</td>
<td>United States</td>
<td>D2-28</td>
<td>Premier</td>
<td>United States</td>
<td></td>
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<tr>
<td>a3-1 PI 632472</td>
<td>Italy</td>
<td>D2-4</td>
<td>Pizzazz</td>
<td>United States</td>
<td>D2-29</td>
<td>Friesta</td>
<td>United States</td>
<td></td>
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<tr>
<td>a3-2 PI 598928</td>
<td>Italy</td>
<td>D2-5</td>
<td>AF</td>
<td>United States</td>
<td>D2-30</td>
<td>Overdrive</td>
<td>United States</td>
<td></td>
</tr>
<tr>
<td>a4-1 PI 577254</td>
<td>Luxembourg</td>
<td>D2-6</td>
<td>Prosport</td>
<td>United States</td>
<td>D2-31</td>
<td>Nexus XD</td>
<td>United States</td>
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<tr>
<td>a4-2 PI 418722</td>
<td>Luxembourg</td>
<td>D2-7</td>
<td>PST-2L96</td>
<td>United States</td>
<td>D2-32</td>
<td>Sunshine 2</td>
<td>United States</td>
<td></td>
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<tr>
<td>a5 PI 619554</td>
<td>United Kingdom</td>
<td>D2-8</td>
<td>CAS LP84</td>
<td>United States</td>
<td>D2-33</td>
<td>Inspire</td>
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<tr>
<td>a6 PI 632510</td>
<td>Hungary</td>
<td>D2-9</td>
<td>APR 1232</td>
<td>United States</td>
<td>D2-34</td>
<td>Quickstart II</td>
<td>United States</td>
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<tr>
<td>a7 PI 628717</td>
<td>Bulgaria</td>
<td>D2-10</td>
<td>Phantom</td>
<td>United States</td>
<td>D2-35</td>
<td>Charger II</td>
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<td>Norway</td>
<td>D2-11</td>
<td>MP103</td>
<td>United States</td>
<td>D2-36</td>
<td>Citation Fore</td>
<td>United States</td>
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<td>a9 PI 577272</td>
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<td>D2-12</td>
<td>Koos R-71</td>
<td>United States</td>
<td>D2-37</td>
<td>Quick Trans</td>
<td>United States</td>
<td></td>
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<tr>
<td>a10 PI 598440</td>
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<td>D2-13</td>
<td>Yatsugeen</td>
<td>United States</td>
<td>D2-38</td>
<td>Salinas</td>
<td>United States</td>
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</tr>
<tr>
<td>a11 PI 422478</td>
<td>Germany</td>
<td>D2-14</td>
<td>Barlennium</td>
<td>United States</td>
<td>D2-39</td>
<td>Gray Star</td>
<td>United States</td>
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<tr>
<td>a12 PI 423136</td>
<td>Spain</td>
<td>D2-15</td>
<td>Exacta</td>
<td>United States</td>
<td>D2-40</td>
<td>Catalina II</td>
<td>United States</td>
<td></td>
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<tr>
<td>a13 PI 538976</td>
<td>Russian federation</td>
<td>D2-16</td>
<td>BAR LP 4317</td>
<td>United States</td>
<td>D2-41</td>
<td>Showtime</td>
<td>United States</td>
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<td>b1-1 PI 598877</td>
<td>Morocco</td>
<td>D2-17</td>
<td>ABT-99-4,560</td>
<td>United States</td>
<td>D2-42</td>
<td>Chaparral II</td>
<td>United States</td>
<td></td>
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<tr>
<td>b1-2 PI 516605</td>
<td>Morocco</td>
<td>D2-18</td>
<td>Headstast 2</td>
<td>United States</td>
<td>D2-43</td>
<td>Majesty II</td>
<td>United States</td>
<td></td>
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<tr>
<td>b2-1 PI 598909</td>
<td>Tunisia</td>
<td>D2-19</td>
<td>DCM</td>
<td>United States</td>
<td>D2-44</td>
<td>Transformer</td>
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<tr>
<td>b2-2 PI 610925</td>
<td>Tunisia</td>
<td>D2-20</td>
<td>PST-2LAN</td>
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<td>D2-45</td>
<td>Brightstar SLT</td>
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<tr>
<td>b3 PI 410155</td>
<td>South Africa</td>
<td>D2-21</td>
<td>Quicksilver</td>
<td>United States</td>
<td>D2-46</td>
<td>Uno(DO411T)</td>
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<tr>
<td>c1 PI 420124</td>
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<td>United States</td>
<td>D2-47</td>
<td>Fiestoc</td>
<td>United States</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup>Numbers immediately following letters represent populations of a country. Lower-case letter represents natural germplasm. Upper-case letter refers to commercial cultivars. The numbers after the hyphen represent population codes within the same country.

### RESULTS

**Genetic diversity**

A total of 176 bands were generated from the 28 primers, 171 (97.16%) of which were polymorphic ranging from 100 to 2000 bp in size (Table 2). Each primer produced 2 to 9 polymorphic bands with an average of 6.1. The PIC values ranged from 0.13 for UBC842 to 0.31 for P7, with a mean of 0.23 for the 28 primers (Table 2).

Statistics with AMOVA revealed 12.09 and 87.91% variance among and within geographical population, respectively. It also showed 11.23 and 88.77% variance explained among and within population of both groups (cultivars and natural accessions). Variance differentiation was significant ($P < 0.001$) for all components (Table 3). This result suggests that genetic variance was high within groups and low among groups.

The European materials showed highest diversity judged from the means of $Ne$, $He$, and $I$, while Asia accessions showed the lowest diversity.
Table 2. Characteristics of the 28 ISSR primers used for the detection of polymorphism in 75 perennial ryegrass (*Lolium perenne* L.) genotypes.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5′-3′)</th>
<th>Annealing temperature (°C)</th>
<th>Total loci</th>
<th>Polymorphic loci</th>
<th>Polymorphism rate (%)</th>
<th>Size range of fragments (bp)</th>
<th>PIC&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>UBC807</td>
<td>(AG)&lt;sub&gt;3&lt;/sub&gt;T</td>
<td>55</td>
<td>9</td>
<td>9</td>
<td>100</td>
<td>350-1700</td>
<td>0.19</td>
</tr>
<tr>
<td>UBC817</td>
<td>(CA)&lt;sub&gt;3&lt;/sub&gt;A</td>
<td>53</td>
<td>8</td>
<td>8</td>
<td>100</td>
<td>300-1900</td>
<td>0.23</td>
</tr>
<tr>
<td>UBC821</td>
<td>(GT)&lt;sub&gt;3&lt;/sub&gt;T</td>
<td>55</td>
<td>7</td>
<td>7</td>
<td>100</td>
<td>400-1000</td>
<td>0.21</td>
</tr>
<tr>
<td>UBC823</td>
<td>(TC)&lt;sub&gt;3&lt;/sub&gt;C</td>
<td>53</td>
<td>7</td>
<td>7</td>
<td>100</td>
<td>100-1800</td>
<td>0.20</td>
</tr>
<tr>
<td>UBC835</td>
<td>(AG)&lt;sub&gt;3&lt;/sub&gt;GCC</td>
<td>55</td>
<td>4</td>
<td>4</td>
<td>100</td>
<td>450-1700</td>
<td>0.20</td>
</tr>
<tr>
<td>UBC836</td>
<td>(AG)&lt;sub&gt;3&lt;/sub&gt;YA</td>
<td>55</td>
<td>9</td>
<td>9</td>
<td>100</td>
<td>270-1800</td>
<td>0.28</td>
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<tr>
<td>UBC840</td>
<td>(GA)&lt;sub&gt;3&lt;/sub&gt;YT</td>
<td>58</td>
<td>8</td>
<td>8</td>
<td>100</td>
<td>260-1800</td>
<td>0.19</td>
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<tr>
<td>UBC842</td>
<td>(GA)&lt;sub&gt;3&lt;/sub&gt;YG</td>
<td>55</td>
<td>8</td>
<td>8</td>
<td>100</td>
<td>250-1600</td>
<td>0.24</td>
</tr>
<tr>
<td>UBC849</td>
<td>(GT)&lt;sub&gt;3&lt;/sub&gt;YA</td>
<td>55</td>
<td>9</td>
<td>9</td>
<td>100</td>
<td>400-2000</td>
<td>0.27</td>
</tr>
<tr>
<td>UBC855</td>
<td>(AC)&lt;sub&gt;3&lt;/sub&gt;YT</td>
<td>55</td>
<td>7</td>
<td>7</td>
<td>100</td>
<td>300-1400</td>
<td>0.30</td>
</tr>
<tr>
<td>UBC856</td>
<td>(AC)&lt;sub&gt;3&lt;/sub&gt;YA</td>
<td>55</td>
<td>7</td>
<td>7</td>
<td>100</td>
<td>370-1500</td>
<td>0.20</td>
</tr>
<tr>
<td>UBC857</td>
<td>(AC)&lt;sub&gt;3&lt;/sub&gt;YG</td>
<td>55</td>
<td>3</td>
<td>2</td>
<td>66.7</td>
<td>600-1800</td>
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<tr>
<td>UBC873</td>
<td>(GACA)&lt;sub&gt;3&lt;/sub&gt;</td>
<td>56</td>
<td>7</td>
<td>7</td>
<td>100</td>
<td>300-2000</td>
<td>0.30</td>
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<tr>
<td>UBC880</td>
<td>(GGAGA)&lt;sub&gt;3&lt;/sub&gt;</td>
<td>55</td>
<td>6</td>
<td>6</td>
<td>100</td>
<td>450-1500</td>
<td>0.17</td>
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<tr>
<td>P 1</td>
<td>(GA)&lt;sub&gt;3&lt;/sub&gt;YA</td>
<td>55</td>
<td>6</td>
<td>6</td>
<td>100</td>
<td>600-2000</td>
<td>0.22</td>
</tr>
<tr>
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<td>(GA)&lt;sub&gt;3&lt;/sub&gt;RC</td>
<td>55</td>
<td>5</td>
<td>5</td>
<td>100</td>
<td>250-1900</td>
<td>0.31</td>
</tr>
<tr>
<td>P 3</td>
<td>(GGGGA)&lt;sub&gt;3&lt;/sub&gt;</td>
<td>55</td>
<td>4</td>
<td>4</td>
<td>100</td>
<td>500-1000</td>
<td>0.21</td>
</tr>
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<td>P 4</td>
<td>(AC)&lt;sub&gt;3&lt;/sub&gt;GCT</td>
<td>55</td>
<td>8</td>
<td>8</td>
<td>100</td>
<td>270-1800</td>
<td>0.27</td>
</tr>
<tr>
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<td>(AC)&lt;sub&gt;3&lt;/sub&gt;TG</td>
<td>55</td>
<td>6</td>
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<td>0.27</td>
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<tr>
<td>P 6</td>
<td>(TC)&lt;sub&gt;3&lt;/sub&gt;TG</td>
<td>55</td>
<td>6</td>
<td>6</td>
<td>100</td>
<td>400-1400</td>
<td>0.28</td>
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<tr>
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<td>8</td>
<td>7</td>
<td>87.5</td>
<td>270-1300</td>
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<td>7</td>
<td>6</td>
<td>85.7</td>
<td>400-1600</td>
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<td>55</td>
<td>8</td>
<td>8</td>
<td>100</td>
<td>300-1600</td>
<td>0.31</td>
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<tr>
<td>P 10</td>
<td>ACT ACG ACT (TG)&lt;sub&gt;3&lt;/sub&gt;</td>
<td>55</td>
<td>6</td>
<td>5</td>
<td>83.3</td>
<td>500-2000</td>
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<tr>
<td>P 11</td>
<td>ACT CGT ACT (AG)&lt;sub&gt;3&lt;/sub&gt;</td>
<td>55</td>
<td>3</td>
<td>3</td>
<td>100</td>
<td>400-1700</td>
<td>0.23</td>
</tr>
<tr>
<td>P 12</td>
<td>CGT AGT CGT (CA)&lt;sub&gt;3&lt;/sub&gt;</td>
<td>55</td>
<td>3</td>
<td>2</td>
<td>66.7</td>
<td>500-1000</td>
<td>0.13</td>
</tr>
<tr>
<td>P 13</td>
<td>AGT CGT AGT (AC)&lt;sub&gt;3&lt;/sub&gt;</td>
<td>55</td>
<td>4</td>
<td>4</td>
<td>100</td>
<td>400-1500</td>
<td>0.22</td>
</tr>
<tr>
<td>P 14</td>
<td>(AC)&lt;sub&gt;3&lt;/sub&gt;CAG</td>
<td>55</td>
<td>3</td>
<td>3</td>
<td>100</td>
<td>350-750</td>
<td>0.20</td>
</tr>
</tbody>
</table>

<sup>a</sup>PIC, Polymorphism information content (Smith et al., 1997).

(Table 4). Although, the means were not necessarily statistically significant and comparable due to small sizes from Asia and Africa, the trend was consistent for all four parameters. Genetic erosion was shown from the comparison between commercial cultivars and natural germplasm, with the former had lower *Ne*, *He*, and *I* than the later (Table 5). The genetic diversity evaluated from NPL, PR, and Na supported the results from *Ne*, *He*, and *I* (Table 6). Again, European materials demonstrated higher variation than other regions. Cultivated varieties showed less genetic variation than wild germplasm.

**Genetic distance**

The genetic distance between accessions ranged
Table 3. Analysis of molecular variance (AMOVA) of profiles developed from inter-simple sequence repeats markers in 75 perennial ryegrass (*Lolium perenne* L.).

<table>
<thead>
<tr>
<th>Source of variation</th>
<th>df</th>
<th>Sum of squares</th>
<th>Variance component</th>
<th>Percentage of variation</th>
<th>P-value$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Analysis for four geographical groups</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Among groups</td>
<td>3</td>
<td>197.67</td>
<td>3.25</td>
<td>12.09</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Within groups</td>
<td>71</td>
<td>1678.15</td>
<td>23.64</td>
<td>87.91</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Total</td>
<td>74</td>
<td>1875.81</td>
<td>26.89</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Analysis for cultivars and natural accessions groups</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Among groups</td>
<td>1</td>
<td>130.09</td>
<td>3.03</td>
<td>11.23</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Within groups</td>
<td>73</td>
<td>1745.73</td>
<td>23.91</td>
<td>88.77</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Total</td>
<td>74</td>
<td>1875.81</td>
<td>26.94</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

$^a$Levels of significance were obtained through nonparametric procedures using 999 permutations.

Table 4. Variation of genetic parameters developed from inter-simple sequence repeats markers for different geographical groups of perennial ryegrass (*Lolium perenne* L.).

<table>
<thead>
<tr>
<th>Statistic</th>
<th>America</th>
<th>Europe</th>
<th>Africa</th>
<th>Asia</th>
</tr>
</thead>
<tbody>
<tr>
<td>Effective number of alleles, $N_e$</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean</td>
<td>1.39</td>
<td>1.49</td>
<td>1.48</td>
<td>1.35</td>
</tr>
<tr>
<td>Standard deviation</td>
<td>0.33</td>
<td>0.33</td>
<td>0.37</td>
<td>0.40</td>
</tr>
<tr>
<td>Minimum</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Maximum</td>
<td>2</td>
<td>2</td>
<td>1.92</td>
<td>1.80</td>
</tr>
<tr>
<td>Nei's gene diversity, $H_e$</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean</td>
<td>0.24</td>
<td>0.29</td>
<td>0.27</td>
<td>0.19</td>
</tr>
<tr>
<td>Standard deviation</td>
<td>0.17</td>
<td>0.16</td>
<td>0.19</td>
<td>0.22</td>
</tr>
<tr>
<td>Minimum</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Maximum</td>
<td>5</td>
<td>0.50</td>
<td>0.48</td>
<td>0.44</td>
</tr>
<tr>
<td>Shannon's information index, $I$</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean</td>
<td>0.38</td>
<td>0.44</td>
<td>0.40</td>
<td>0.27</td>
</tr>
<tr>
<td>Standard deviation</td>
<td>0.23</td>
<td>0.22</td>
<td>0.27</td>
<td>0.32</td>
</tr>
<tr>
<td>Minimum</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Maximum</td>
<td>0.69</td>
<td>0.69</td>
<td>0.67</td>
<td>0.64</td>
</tr>
</tbody>
</table>

Table 5. Comparison of genetic variation between commercial cultivars and natural germplasm using parameters developed from inter-simple sequence repeats markers.

<table>
<thead>
<tr>
<th>Statistic</th>
<th>Cultivated varieties</th>
<th>Natural germplasm</th>
<th>Overall</th>
</tr>
</thead>
<tbody>
<tr>
<td>Effective number of alleles, $N_e$</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean</td>
<td>1.39</td>
<td>1.51</td>
<td>1.46</td>
</tr>
<tr>
<td>Standard deviation</td>
<td>0.33</td>
<td>0.32</td>
<td>0.32</td>
</tr>
<tr>
<td>Minimum</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Maximum</td>
<td>2</td>
<td>2</td>
<td>2</td>
</tr>
</tbody>
</table>

| Nei's gene diversity, $H_e$      |         |                  |         |
| Mean                             | 0.24    | 0.31             | 0.28    |
| Standard deviation                | 0.17    | 0.15             | 0.15    |
| Minimum                          | 0       | 0                | 0       |
from 0.18 to 0.94. The commercial cultivars ‘Panthers GLS’ and ‘Nexus XD’ from United States had the closest GD (0.18). The largest GD (0.94) occurred between ‘premier’ from United States and ‘PI 619554’ from United Kingdom. Natural perennial ryegrass generally had a greater GD than commercial cultivars. However, greater GD also observed between a few natural accessions and commercial accessions. For example, GD between ‘Headstast 2’ and ‘PI 516605’ reached 0.80. The genetic distance between ‘PI 628693’ and ‘PI 538976’ was 0.88. An average GD of 0.73 was observed between ‘PI 619554’ and the rest accessions.

**Phylogenetic analysis**

The Jaccard’s similarity coefficient ranged from 0.32 to 0.72 (Figure 1). Based on the polymorphic bands, 75 perennial ryegrass accessions were clustered into 12 groups (I–XII) with a cut-off point at 0.44. The accessions from same geological regions were likely to be clustered into the same group. Natural germplasm and commercial varieties were generally clustered into different groups. Group I included 56 accessions (74.7%), which consisted of 45 commercial cultivars and 11 natural accessions. Group I was further divided into 5 subgroups at Jaccard’s similarity coefficient of 0.48. Group I covered materials from United States (45) and Europe (11). The subgroup I-1 had 46 perennial ryegrass accessions, 43 of which were commercial cultivars from the United States, and the other three were natural accessions from different European countries. The subgroup I-2 was composed of 6 natural accessions from Europe. The subgroup I-3 included 2 natural accessions from two European countries. The subgroup I-4 and I-5 each had only one accession, ‘Quick Trans’ from United States and ‘PI 619033’ from Romania, respectively. Group III contained 3 natural accessions from Europe. Group VI included 7 natural accessions, 3 of which came from Europe, 3 others were from Asia and the last one from Africa. Group II, IV, V, VII, VIII, IX, X, XI and XII each had only one accession and collectively accounted for 83% of the total variation based on genetic diversity parameters (NPL, PR, Na, Ne, He, and l) (Table 6). They were ‘Headstast 2’, ‘PI 598909’, ‘Catalina II’, ‘PI 538976’, ‘PI 598440’, ‘PI 610925’, ‘PI 598877’, ‘PI 516605’ and ‘PI 619554’, respectively. This suggested that a core germplasm list can be potentially constructed, which enrich its diversity.

Two-dimensional plot based on PCA of ISSR data revealed a similar grouping result as from UPGMA. The first and second principle coordinates accounted for 11.28% of the total variation. ‘Headstast 2’, ‘PI 598909’, ‘PI

**Table 6. Differences of genetic diversity parameters among continents and between collections based on different classifications of perennial ryegrass (Lolium perenne L.) developed from inter-simple sequence repeats markers.**

<table>
<thead>
<tr>
<th>Comparison</th>
<th>Category</th>
<th>GS</th>
<th>NPL</th>
<th>PR</th>
<th>Na</th>
</tr>
</thead>
<tbody>
<tr>
<td>Continent vs continent</td>
<td>America</td>
<td>-</td>
<td>153</td>
<td>86.93</td>
<td>1.87</td>
</tr>
<tr>
<td></td>
<td>Europe</td>
<td>-</td>
<td>156</td>
<td>88.64</td>
<td>1.89</td>
</tr>
<tr>
<td></td>
<td>Africa</td>
<td>-</td>
<td>122</td>
<td>69.32</td>
<td>1.69</td>
</tr>
<tr>
<td></td>
<td>Asia</td>
<td>-</td>
<td>76</td>
<td>43.18</td>
<td>1.43</td>
</tr>
<tr>
<td>Cultivated vs natural</td>
<td>Cultivars</td>
<td>47</td>
<td>152</td>
<td>83.36</td>
<td>1.86</td>
</tr>
<tr>
<td></td>
<td>Natural germplasm</td>
<td>28</td>
<td>165</td>
<td>93.75</td>
<td>1.94</td>
</tr>
<tr>
<td>Recommended core vs whole</td>
<td>Recommended core</td>
<td>9</td>
<td>142</td>
<td>80.68</td>
<td>1.81</td>
</tr>
<tr>
<td></td>
<td>Whole collection</td>
<td>75</td>
<td>171</td>
<td>97.16</td>
<td>1.97</td>
</tr>
</tbody>
</table>

*GS, Group size; NPL, number of polymorphic loci; PR, polymorphism rate; Na, observed number of alleles.*
Figure 1. Jaccard’s similarity coefficient of 75 perennial ryegrass (Lolium perenne L.) based on inter-simple sequence repeats markers.

538976’ and ‘PI 598877’ were all distinctly differentiated from the other accessions by the two principle coordinates, and were clustered into groups having one accession each (Figure 2).

**DISCUSSION**

Although, not necessarily comparable among geological regions, the present study showed polymorphism rates as high as 97.16% for the whole population. The interpretation was evidently affected by the population size, as only 43.18% was found in 3 Asian accessions. Nevertheless,
the polymorphism was comparable or higher than previ-ously reported in perennial ryegrass with comparable population size by several PCR-based molecular markers techniques (AFLP, RAPD, and SSR). Jones et al. (2001) detected 67% polymorphism in diverse genotypes with 2 to 7 alleles per locus based on SSR. Guthridge et al. (2001) reported a polymorphism of 89.6% in two perennial ryegrass populations based on AFLP. The present study also corroborated the ISSR results by Ghariani et al. (2003) and suggested that ISSR markers technique is one of the best in detecting genetic diversity in perennial ryegrass.

The present study indicated that the whole population of 75 perennial ryegrass accessions had a relatively high level of genetic diversity (PR=97.16%, $H_e=0.28$ and $I=0.44$). The present study indicated that GD ranged from 0.18 to 0.94 with an average of 0.48, which was in line with Ghariani et al. (2003), who found that natural perennial ryegrass population had a GD of 0.28 to 0.78. These results further suggested that there was a greater level of genetic diversity among the 75 perennial ryegrass accessions. The genetic diversity was contributed to the growing environment in different regions and the far geographical distance engendered gene isolation. The similar results were reported in some previous research (Galván et al., 2003; Hou et al., 2006; Song et al., 2006). This high level of genetic diversity in perennial ryegrass might imply complicated and independent evolutionary processes of this species.

In this study, a high degree of divergence was found between cultivated varieties and natural germplasm or among these accessions from different geographical regions by the analysis of phylogenetic relationships. This was in part due to the fact that an independent evolutionary history for these accessions themselves with little or no gene flow for a long time (Yang et al., 2011). Results from the present study indicated lower genetic diversity in commercial group than natural perennial ryegrass despite the open-pollination which enhances genetic hybridization and introgression. The results support the findings by Warpeha et al. (1998) and differ from that by Casler (1995). For example, the GD between two commercial cultivars ‘Exacta’ and ‘ABT-99-4.560’ was the closest (0.19). The greater GD occurred between ‘PI 632472’ and ‘PI 547390’ (0.85). Although reduction of genetic diversity may seem unavoidable due to the requirement of uniformity

**Figure 2.** Two-dimensional plot based on principle component analysis using inter-simple sequence repeats markers for 75 perennial ryegrass (*Lolium perenne* L.) accessions, where Dim-1 is the first component and Dim-2 is the second component.

![Figure 2](image-url)
in new cultivars, maintenance of diversity to a certain extent is desirable for adaptability to both biotic and abiotic stresses.

The present study reveals larger genetic variation within geographical groups (87.91%) than among geographical groups (12.09%). The European group showed the higher genetic diversity than the American group which supported the suggestion by Thorogood (2003) that the American breeding program has been based on a narrow germplasm mostly from Europe. The results also reflected the degree of genetic erosion in different continents and may be used as a benchmark to monitor the change of genetic diversity as new cultivars are released, which requires sampling and analyzing of plant materials in those regions over time.

We found that a random sample consisted of 9 accessions that accounted 12% of the initial collection maintains 83% of the diversity for perennial ryegrass species. Based on the concept of core collection, a minimum representative samples of the initial collection with maximum genetic diversity of a plant species and its relatives (Frankel, 1984), we suggested 9 germplasm (‘Headstart 2’, ‘PI 598909’, ‘Catalina II’, ‘PI 538976’, ‘PI 598440’, ‘PI 610925’, ‘PI 598877’, ‘PI 516605’ and ‘PI 619554’) be included in a core collection of germplasm. Further collections should be made to enrich the collection especially in those categories where only one accession was included.

ACKNOWLEDGEMENT

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REFERENCES


