Evaluation of Genetic Diversity of Spring Onions (Allium fistulosum) Based on DNA Markers

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Article Information

ABSTRACT

The Alliaecae family is a widely cultivated vegetable family in Nigeria, with nutritional, culinary and medicinal usefulness. The objective of this study was to assess the genetic diversity using SSR and ISSR markers among ten (10) cultivars of spring onions. This study was carried out using one (1) SSR marker and three (3) ISSR markers. In this study, the genetic diversity among the cultivars were revealed. The four markers produced a total of 26 polymorphic alleles with PIC values of 0.7481, 0.6402, 0.675 and 0.7569 for ISSR 879, ISSR 866, ISSR 881 and SSR 62. The UPGMA dendrogram revealed that the ten spring onion cultivars could be divided into two main groups. The subgroup SPO06 and SPO04 showed no genetic distance among them revealing that there is a very close relationship and in total agreement with the factorial analysis. This study has revealed the efficiency of SSR and ISSR markers to estimate the extent of genetic variation of spring onion cultivars and will be a contribution for the conservation and management of Allium species.

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1. INTRODUCTION

Spring onions (Allium fistulosum), also called Japanese bunching onion or Welsh onion, is one of the most economically important and widely grown plants in the Alliaceae family. Spring onions is commonly cultivated in China, Korea, Indonesia and Japan, where both leaves and pseudostems are consumed either cooked or as a fresh vegetable [1]. The name ‘Welsh onion’ is derived from the old German word ‘welshe’ meaning foreign. Genus Allium comprises more than 800 species [2], making it one of the largest monocotyledonous genera. It is mostly grown as a perennial plant in home gardens to use as cut foliage in early spring [3].

According to USDA (2015), they are classified below:

- Kingdom: Plantae
- Division: Magnoliophyta
- Class: Liliopsida
- Subclass: Liliales
- Order: Liliales
- Family: Alliaceae
- Genus: Allium
- Species: fistulosum

Allium is a large plant taxon comprising of about 850 species; many of them have high economic value as spices, vegetables, medicinal plants and ornamentals [4] and they grow widely throughout the temperate to tropical and arid regions of the northern hemisphere. Other most common crops of the genus Allium are Allium cepa (onions), Allium sativum (garlic), Allium schoenoprasum (chives), Allium ampeloprasum var. porrum (leek), Alliumtuberosum (Chinese or garlic chives) and Allium chinense (rakkyo). Spring onions has several local names in different languages. It has several local/common names in different languages such as Welsh onion, bunching onion, Japanese bunching onion and stone leek in English; Ciboulette and ciboule in French; Zwiebel, schnittziebel and winterzwiebel in German, Cipolleta and cipolla dinverno in Italian; Negi and nebuka in Japanese; Chung in Taiwan; Pa in Korean; Cebolleta and Cebolla in Spanish; Alubosa elewe in Yoruba; Albasa in Hausa and Mmiri Yabasi in Igbo.

Spring onions originated in Asia and most common in Northeast India, temperate to tropical Asia and African countries. Spring onions is grown throughout the world in the wide range of climates -temperate to tropical conditions. Globally, it is cultivated in Siberia and Ciscaucasia (Russia), China, Japan, Korea, Taiwan, Thailand, Indonesia, Malaysia, Philippines, Northeast India, Norway, Germany, Sudan, Kenya, Cameroon, Congo, Zaire, Sierra Leone, Zambia and Zimbabwe; moreover China and Japan are the leading countries for its commercial cultivation. The whole plants; including green leaves, pseudo-stems as well as roots; are being consumed to flavor soups, steamed- boils, salads, vegetables and other culinary [5]. Under cultivation, the plants thrive best under acidic soil (pH 5.5- 6.5), well drained heavy soil, irrigated, humid, long-day conditions; but tends to bolt profusely in short days of winter months which is ultimately detrimental to leaf growth, quality and aroma. Early nipping (removal of apical buds) promotes vigorous shoot growth and thereby increases yield by 10-20% [5].

 Morphologically, there is a strong resemblance between Allium fistulosum and Alliumcepa but fistulosum does not develop a bulb or has a small bulb. Its plants stop growing and their leaves start withering out and die off under short days during start of winter season. A. fistulosum has more erect foliage and longer pseudostem with stronger pungency. A. fistulosum and A. cepa cross fertilize readily, yielding inter specific F1 hybrids and hybrid derivatives [6]. Allium fistulosum has been considered one of NASA’s Advanced Life Support Candidate Crops because of its characteristic flavor, short cropping cycle, suitability in dense planting compared with bulb onion, and ease of hydroponic culturing [7,8].

Spring onions are used extensively to flavor up the soup, steamed, boils, fries, vegetables, salads and other cookeries especially in northern hemisphere of the world. In East Asia, it is used in diverse dishes including stir-fries and soups; in the west, it is used primarily used as a scallion, salad or green onion [2,5]. The Allium plants, including Allium fistulosum, are of great importance due to their nutritional composition, antioxidant properties, and used as flavoring agents, fragrance, therapeutics and medicinal food. The green leaf types contain higher levels of β carotene, vitamin B1, vitamin B2 and vitamin C.
The aim of study is to evaluate the level of genetic variation among different accessions of spring onion grown in Nigeria.

1.1 Specific Objective Includes

To assess the genetic relationship among ten (10) *Allium fistulosum* cultivars using simple sequence repeats and inter simple sequence repeat.

2. MATERIALS AND METHODS

2.1 Materials

Sample Collection for DNA Extraction: The materials that were used for the collection of samples for DNA extraction are; Scissors, Eppendorf tubes, 70% ethanol, tissue paper, thermo flask, ice packs, hand gloves, permanent marker and dispensing envelopes.

2.2 Methods

2.2.1 Plant materials.

The ten *Allium fistulosum* plants collected from six (6) states in Nigeria are listed in Table 1.

2.2.2 Plant collection

Following the collection of samples, each of the roots of the varieties were planted.

2.2.3 Planting procedure

The plants were kept in labeled plastics without covers in order to allow air into them and to avoid mould growth which can be encouraged by moisture. A mixture of loamy and sandy soil were collected and sieved to remove the coarse particles. Nursery bowls were gotten and labeled according to the code number given to each sample, and about 2 kg of the soil was added in each bowl. The soils in the bowls were moistened with water and the leaves of the spring onion were cut off with a sharp and sterile knife and was planted, by creating a shallow well on the soil surface. The bowls containing the plants were taken to the botanical garden of University of Lagos, Nigeria to allow for germination and growth. Watering was done very early every morning and weeding was done every three days.

2.3 DNA Extraction

The reagent used are; 200 mg (0.2 g) lyophilized leaf sample, 700 µl plant extraction buffer (PEB), 637.5 ml of double distilled water (ddH2O), 100 ml 1M Tris-HCl (pH 8.0), 100 ml of 0.5 M Ethylene Diamine Tetraacetic acid (pH 8.0), 100 ml of 5M NaCl, 62.5 ml 20% sodiumdodecylsulphate (SDS), 1% b-mercaptoethanol, 500 µl of 5 M of
Table 1. List of *Allium fistulosum* samples used in this study

<table>
<thead>
<tr>
<th>S/N</th>
<th>Code names</th>
<th>Location</th>
<th>Scientific name</th>
<th>Common name</th>
<th>Colour</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>SPO01</td>
<td>Saki, Oyo</td>
<td><em>Allium fistulosum</em></td>
<td>Spring onion</td>
<td>Green</td>
</tr>
<tr>
<td>2</td>
<td>SPO02</td>
<td>Oyo, Oyo</td>
<td><em>Allium fistulosum</em></td>
<td>Spring onion</td>
<td>Green</td>
</tr>
<tr>
<td>3</td>
<td>SPO03</td>
<td>Egba, Ogun</td>
<td><em>Allium fistulosum</em></td>
<td>Spring onion</td>
<td>Green</td>
</tr>
<tr>
<td>4</td>
<td>SPO04</td>
<td>Lagos</td>
<td><em>Allium fistulosum</em></td>
<td>Spring onion</td>
<td>Green</td>
</tr>
<tr>
<td>5</td>
<td>SPO05</td>
<td>Lagos</td>
<td><em>Allium fistulosum</em></td>
<td>Spring onion</td>
<td>Green</td>
</tr>
<tr>
<td>6</td>
<td>SPO06</td>
<td>Kwara</td>
<td><em>Allium fistulosum</em></td>
<td>Spring onion</td>
<td>Green</td>
</tr>
<tr>
<td>7</td>
<td>SPO07</td>
<td>Ekiti</td>
<td><em>Allium fistulosum</em></td>
<td>Spring onion</td>
<td>Light green</td>
</tr>
<tr>
<td>8</td>
<td>SPO08</td>
<td>Lagos</td>
<td><em>Allium fistulosum</em></td>
<td>Spring onion</td>
<td>Dark green</td>
</tr>
<tr>
<td>9</td>
<td>SPO09</td>
<td>Abuja</td>
<td><em>Allium fistulosum</em></td>
<td>Spring onion</td>
<td>Green</td>
</tr>
<tr>
<td>10</td>
<td>SPO10</td>
<td>Lagos</td>
<td><em>Allium fistulosum</em></td>
<td>Spring onion</td>
<td>Light green</td>
</tr>
</tbody>
</table>
Plate 2. Picture of samples growing in the screen house at the garden of University of Lagos, Akoka, Nigeria

Table 2. List of ISSR primers and their sequences used in this study

<table>
<thead>
<tr>
<th>S/N</th>
<th>Primer name</th>
<th>Sequences</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>ISSR 866</td>
<td>CTCCTCCTCCTCCTCCTC</td>
</tr>
<tr>
<td>2</td>
<td>ISSR 879</td>
<td>CTCTTTCACTTCA</td>
</tr>
<tr>
<td>3</td>
<td>ISSR 881</td>
<td>GGGTGGGTGGGTG</td>
</tr>
</tbody>
</table>

Table 3. List of SSR primer and its sequence used in this study

<table>
<thead>
<tr>
<th>S/N</th>
<th>Primer</th>
<th>Forward primer</th>
<th>Reverse primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>SSR 62</td>
<td>TTTCTCTCATTCCCCACAATTACCCTG</td>
<td>CGTTGAGACAAACAAAAACAAAGAGA</td>
</tr>
</tbody>
</table>

potassium acetate (CH3COOK), 700 µl chloroform isoamylalcohol, 700 µl ice-cold isopropanol, 100 µl cold 70% ethanol, 2 µl of 10 ng/ml RNase and 1% Agarose gel.

The extraction materials include; Centrifuge, Genogrinder-2000, two steel balls, 65°C water bath, -20°C freezer, fume-hood and incubator.

2.4 DNA Extraction Protocol

Total genomic DNA was extracted using the Dellaporta protocol [9]. Approximately 100 mg of lyophilized leaf sample was ground into powder form by using Genogrinder-2000. Two steel balls were used to enable grinding. 450µl of preheated plant extraction buffer was added to the tube and incubated at 65°C for 20 minutes mixing by occasionally inverting the tubes to homogenize the sample. The tubes were allowed to cool for 2 minutes, then 200µl of ice-cold 5M Potassium acetate was added and incubated on ice for 20 minutes to precipitate protein. The samples were centrifuged at 10000 rpm for 10 minutes and then transferred the supernatant into freshly labelled tubes. 450µl of Chloroform Isoamylalcohol (24:1) was added to further precipitate proteins and lipids. Then it was centrifuged at 10000 rpm for 10 minutes and the supernatant was transferred to labelled tubes.
2/3 volume of ice-cold isopropanol was added to the supernatant and mixed by gently inverting and were incubated at -80°C for 15 minutes to precipitate the DNA. The precipitated DNA was centrifuged again at 10000 rpm for 10 minutes for pellets to be formed at the bottom of the tubes. The supernatants were decanted and the tubes were placed face down on a paper towel to remove the last drops of isopropanol and air-dried at room temperature. 400µl of 70% ethanol was added to wash the DNA pellet and centrifuged at 10000 rpm for 10 minutes. The supernatant was decanted until the last drop and the pellet was air-dried (until ethanol smell disappears). 100µl of low salt TE was added to dissolve the pellets as well as 2µl of RNase. The samples were placed in the freezer and stored at -20°C until ready for use.

2.5 PCR Amplification

2.5.1 ISSR PCR amplification

The amplification reactions were made to for a final volume, containing 20 ng of DNA, a unit of Taq DNA polymerase (Invitrogen), 10mM of Tris-Hcl (pH 8.0), 2 mM of MgtCl2, 0.25 µM of each deoxyribonucleotide triphosphate (DNTPs) and 0.2 µM of oligonucleotide. The DNA amplifications were done using a MJ. Research, Inc. PTC100 Programmable Thermal Controller (Watertown USA) thermocycler, under the following conditions: 15 minutes at 95°C (initial denaturation) followed by 35 cycles of 30 seconds at 94°C (denaturation), 45 seconds at 50 or 55°C (ringing) and 2 minutes at 72°C (extension), with final extension for 7 minutes at 72°C. The products from the amplifications were separated in 2% agarose gel, crowned with Syber gold (Invitrogen), using the 25 bp Ladder 50µg (1.b µg/µL) (Invitrogen) marker and observed under an ultra-violet light and registered in a digital Vilber Lourmat photodocumentor.

2.5.2 SSR PCR amplification

Using the primer pairs of an SSR marker, the genomic DNA of the varieties of each of the three species of spring onion was amplified in 25 µL reaction mixtures each containing 100 ng genomic DNA, 1 x PCR buffer 15 mM MgCl2, 200 µM DNTP, 0.2 µM each of the forward and reverse primers and 1 U Taq polymerase (Bangalore Genei) using the following PCR profile in an Eppendorf Mastercycler. Initial denaturation at 94°C for 30 minutes, followed by 39 cycles of 94°C for 30 seconds, 54 to 62°C for 1 minute, 72°C for 2 minutes and a final extension at 72°C for 10 minutes. The amplified products were resolved on 10% polyacrylamide gels and visualized following silver staining.

2.6 SDS Page Protocol (SSR Marker Allele Separation)

Polyacrylamide gel also known as PAGE was prepared following standard procedure. Samples were loaded into the gel and the gel was ran at 25mA in 1×SDS Running Buffer. The gel was placed in a plastic container, covered with isopropanol fixing solution and agitated at room temperature. Fixing solution was poured off then gel was covered with ethidium bromide staining solution and agitated at room temperature for two (2) hours. Staining solution was poured off. Then the gel was washed with distilled water to de-stain, agitating it at room temperature.

2.7 Genetic Diversity Analysis

Polymorphic SSR amplified product was considered to be a unit character and the populations were manually scored using binary data with presence as “1” and absence as “0” (Appendix). The numbers of monomorphic and polymorphic bands were derived from the agarose gel electrophoresis diagram. Genetic distances were measured based on shared allele frequencies. A dendrogram was constructed from a UPGMA (Unweighted Pair Group with Arithmetic Mean). Data obtained from SSR allelic diversity among cucurbit landraces were used to compute the Jaccard’s dissimilarity matrix using Darwin 5.0 software. The dissimilarity matrix was also used to construct a factorial analysis to reveal their molecular diversity. Polymorphic Information Content (PIC) and cross-species transferability was calculated as the presence of target loci to the total number of amplified loci for each SSR marker.

3. RESULTS

3.1 Marker Polymorphism

Reproducible, well resolved fragments were scored using Gel analyzer. Each scoreable band was scored as presence (1) or absence (0). The profiles generated in the spring onion cultivars were analyzed to compute polymorphic information content (PIC). A total of 26 alleles were revealed among the 10 cultivars from...
different locations using three (3) ISSR markers and one (1) SSR marker. ISSR 879 had 7 alleles, ISSR 866 had 5 alleles, ISSR 881 had 8 alleles and SSR 62 had 6 alleles. All four markers showed the same percentage polymorphism of 100%, each with a PIC (Polymorphism Information Content) of 0.7481, 0.6402, 0.675 and 0.7569 with their observed heterozygosity at 0.7755, 0.679, 0.7049 and 0.79 respectively (Table 2).

Table 4. Number of alleles per locus and PIC (Polymorphic Information Content) of ISSR and SSR markers used in this study

<table>
<thead>
<tr>
<th></th>
<th>ISSR 879</th>
<th>ISSR 866</th>
<th>ISSR 881</th>
<th>SSR 62</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of bands</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Number of monomorphic bands</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Number of polymorphic bands</td>
<td>7</td>
<td>5</td>
<td>8</td>
<td>6</td>
</tr>
<tr>
<td>% polymorphism</td>
<td>100%</td>
<td>100%</td>
<td>100%</td>
<td>100%</td>
</tr>
<tr>
<td>Observed Heterozygosity (He)</td>
<td>0.7755</td>
<td>0.679</td>
<td>0.7049</td>
<td>0.79</td>
</tr>
<tr>
<td>PIC</td>
<td>0.7481</td>
<td>0.6402</td>
<td>0.675</td>
<td>0.7569</td>
</tr>
</tbody>
</table>

Fig. 1. ISSR 866 amplified in the 10 cultivars of spring onions
3.2 Genetic Diversity Assessment

The UPGMA dendrogram (Fig. 3) showed that the 10 varieties of spring onions can be grouped into two (2) main groups (I and II). Group 1 contained 2 subgroups; subgroup 1 contained SPO06, SpO04, SPO01 and SPO05 while subgroup 2 contained SpO07, SpO02 and SPO08. SpO06 and SpO04 shows no genetic distance among them meaning that there is a very close relationship between them. Group II contained SPO10, SPO09 and SPO03. SPO10 and SPO09 shows a close relationship while SPO03 is further apart and seems to be an outlier. This dendrogram was constructed using ISSR and SSR markers (Figs. 1 and 2).

3.3 Factorial Analysis

Based on the factorial analysis, the ten (10) varieties were categorized into four (4) quadrants respectively (Fig. 4). The first quadrant had cultivars SpO02, SpO05, SpO06 and SpO04. SpO06 and SPO04 cluster the most and were overlapping. This showed close relationship. The second quadrant, SpO010 and SpO09 did not
really show a close cluster together with SpO07 as they were all far apart even though they were in the same cluster. SPO07 was close to SPO02 even though they are not in the same cluster. The third quadrant SpO03 seems to be an outlier as it was further apart from others. The fourth quadrant comprising of SpO01 and SpO08 seems to be apart as well which is also similar to the dendrogram as they are showing low genetic relatedness.

4. DISCUSSION AND CONCLUSION

DNA based molecular markers can demonstrate similarities and differences between cultivars. In this study, three (3) ISSR markers and one (1) SSR marker were designed to determine genetic relationship between ten (10) cultivars of spring onions (SPO01, SPO02, SPO03, SPO04, SPO05, SPO06, SPO07, SPO08, SPO09 and SPO10). A total of 26 alleles were generated with four primers (ISSR 879, ISSR 866, ISSR 881 and SSR 62) out of which 26 (100%) were polymorphic across all cultivars of spring onions. ISSR 879 generated 7 alleles with PIC value of 0.7481. ISSR 866 generated 5 alleles with a PIC value of 0.6402. ISR 881 generated 8 alleles with a PIC value of 0.675 while SSR 62 generated 6 bands with a PIC value of 0.7569. The pattern of grouping of the spring onion cultivars based on 3
ISSR markers and 1 SSR marker by dendrogram cluster analysis and factorial analysis were in close agreement.

The dendrogram grouped the 10 spring onion cultivars into two (2) main groups with subgroups for the cluster analysis at a distance of 1.00. Group I contained two subgroups; subgroup 1 contained SPO06 and SPO04. Subgroup 2 contained SPO05 and SPO02. Group II contained subgroups; subgroup 1 which contained SPO07. Subgroup 2 contained SPO09 and SPO10. Group III contained subgroup 1 which has SPO01 and subgroup 2 which has SPO08. Group IV only contain SPO03. This corroborated the research done by Hirashima et al. [10] where a dendrogram obtained by genetic distance showed two major clusters of Kaga and Senju, while two of the Kujo were closely related with Senju.

The dendrogram showed the ten (10) cultivars of spring onions arranged in two main groups with different subgroups. The analysis showed that there was zero distance between SPO06 and SPO04, i.e. they are the closest individuals among all evaluated cultivars and they are genetically related. Group I contained subgroups 1 which has SPO06, SPO04, SPO01 and SPO05 and are from Kwara, Lagos, Oyo and Lagos respectively. Hence geographical location is a major factor and they may have been sourced from similar location. Subgroup 2 contained SPO07, SPO02 and SPO06 even though SPO08 is a singleton. Group II contained SPO10, SPO09 and SPO03 even though SPO03 was further apart. SPO03 which is from Ogun state is clearly different from others in the dendrogram. The difference may be as a result of weather, topography or location. The factorial analysis showed four (4) clusters (Fig. 3) across the quadrants. The ten (10) spring onion cultivars form the four clusters. SPO02, SPO05, SPO06 and SPO04 formed cluster I in one quadrant, SPO10, SPO09 and SPO07 formed cluster II. Cluster III had SPO03 while cluster IV had SPO08 and SPO01. Even though SPO01 is in another cluster, it partially agrees with the dendrogram as it is also closely related to SPO06, SPO04 and SPO05. Hence, this clustering is in agreement with the dendrogram clustering because the two groups showed moderate genetic diversity among the cultivars. This further suggests that molecular markers such as ISSRs and SSRs are useful tools for assessment of genetic diversity in spring onion [11,12].

This study has shown that ISSR and SSR markers were able to distinguish genetic diversity among the set of spring onion studied. This establishes SSR and ISSR markers are reliable and effective markers for genetic diversity assessment and as such can help to establish genetic relationships, germplasm characterization and aid crop improvement through breeding to achieve desirable traits or characters. I suggest that more work should be done using more SSR markers to help in the plant conservation in the future which will give a broader view of the diversity of spring onions.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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