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GENETIC DIVERSITY OF TWENTY *COLA* ACCESSIONS USING INTER-SIMPLE SEQUENCE REPEAT (ISSR) MARKERS

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Abstract
Kola (Cola spp) is one of the important tree crops which have high nutritional, medicinal and industrial value. Molecular marker techniques have been considered to be the most accurate and suitable means for estimating genetic diversity. The aim of this study was to assess the genetic diversity among 20 accessions of Kola (Cola spp) using inter-simple sequence repeat (ISSR) markers. The alleles were scored in binary codes and percentage of polymorphism was calculated. Polymorphic Information Content (PIC), a measure of variability for each locus was calculated across the assay of units. The obtained results demonstrate that ISSR markers were highly polymorphic and generated allele number ranging from 5 to 17. Polymorphism Information Content (PIC) for Cola varied from 0.470 (UBC888) to 0.931 (UBC811), with an average of 0.749. Moreover, gene diversity was high (0.767), and dendrogram constructed using the Unweighted Pair Group Method with Arithmetic mean (UPGMA) based on inter-simple sequence repeat (ISSR) markers, grouped the accessions in to five major clusters, while a similarity coefficient of 0.72 for the markers indicated high level of variability among the Cola accessions. The study shows that ISSR marker system is useful for genetic characterization as it provides information on the inter-species phylogenetic status of the accessions and also showed genetic variability that could be exploited for varietal delineation and Cola improvement in Nigeria.

Keywords: Accessions, Polymorphic, Dendrogram, Clusters

Introduction
Kola nuts (*Cola spp*) are known for their high caffeine content, between 1.84 and 2.56% (Nyamien *et al.,* 2014). As a result, they are widely used in the food industry for the manufacture of energy drinks (Burdock *et al.,* 2009). It has a lot of traditional, social and medicinal importance, such as treatment of asthma and whooping cough (Adedayo *et al*., 2019; Dorathy *et al*., 2014). In the pharmaceutical industry, they are used to develop drugs against cardiovascular diseases (Madingou *et al.,* 2012) and are highly valued in traditional pharmacopoeia as a fertility regulator and as a remedy against migraines and indigestion (Esimone *et al.,* 2007). Generally, kola nut contains protein, starch, phenol, niacin, and riboflavin which are good for one's health (Livehealthy, 2014). Proximate results reported by Dewole *et al.* (2017) showed that the moisture content of kola nut were in respective range of 9.73 to 9.81%, ash 2.72 to 2.21%, fat 3.02 to 2.20%, protein 19.14 to 15.24%, crude fiber 7.30 to 4.18% and carbohydrate 58.09 to 66.45%. Kola nut contains considerable quantity of glucose compared to other stimulants like cocoa and coffee, and are three times greater in starch than cocoa but with relatively little fat. Substantial quantity of starch and glucose of kola nut have been reported to boost physical energy and suppresses hunger (Decker, 2017).

Kola nut also contains higher quantities of phenolic constituents than many fruits which play an important role in determining colour and flavour and have an impact on metabolic processes. Other uses of kola nuts include; cosmetics and textiles (Jayeola and Akinwale, 2002). In addition, Kola nut plays an important role in African society for cultural and customary ceremonies such as births and weddings (Durand *et al.,* 2015).

In spite of the health and socio-economic importance of *Cola*, sterility, self and cross incompatibilities, unpleasantly tall trees, low nut yield and longer gestation period are among the problems faced by growers of this tree crop. For its improvement and advancing its research attention, accurate genetic diversity study among the existing *Cola* germplasm in Nigeria is a prerequisite to assist in selecting suitable parents for breeding programmes. A number of projects have been embarked upon, including the collection of *Cola* accessions from different farmer's field from different locations in Nigeria, although with no distinguishing features. These are maintained as field gene banks with the view to effectively incorporate them in breeding programmes. Molecular characterization, which highlights the amount of genetic diversity and relationship among various groups of different accessions, is required for a direct and more reliable selection and distinguishing of *Cola* accessions.

To assess the genetic diversity of the germplasm established, inter simple Sequence Repeat (ISSR) marker system was employed. There has been no report of usage ISSR marker system for *Cola spp* diversity studies despite the several advantages of the marker system that has seen it utilized for tropical crops like *Cacao* and *Coffea*. Inter simple Sequence Repeat (ISSR) offers simple, fast and efficient techniques for selection of suitable parent(s) for a successful *Cola* breeding programme.

Materials and Methods

Plant Materials and Sample collection

Twenty accessions of *Cola spp* in a new germplasm collection at the Headquarters of Cocoa Research Institute of Nigeria, Ibadan, Nigeria, were used in this study (Table 1). Fresh young leaf samples of each of the selected twenty accessions of *Cola* were harvested into well labeled sample bags and tightly closed. The samples were placed on ice pack and immediately conveyed to biotechnology laboratory for DNA extraction and genetic profiling.

Table 1: List of the accessions used in the study

S/N	Accessions	Collection site
1	CN1	CRIN Ibadan
2	CN2	CRIN Ibadan
3	CN3	CRIN Ibadan
4	CN4	CRIN Ibadan
5	CN5	CRIN Ibadan
6	CN6	CRIN Ibadan
7	CN7	CRIN Ibadan
8	CN8	CRIN Ibadan
9	CN9	CRIN Ibadan
10	CN10	CRIN Ibadan
11	CN11	CRIN Ibadan
12	CN12	CRIN Ibadan
13	CN13	CRIN Ibadan
14	CN14	CRIN Ibadan
15	CN15	CRIN Ibadan
16	CN16	CRIN Ibadan
17	CN17	CRIN Ibadan
18	CN18	CRIN Ibadan
19	CN19	CRIN Ibadan
20	CN20	CRIN Ibadan

CRIN = Cocoa Research Institute of Nigeria

DNA Extraction

Collected leaf samples were prepared for DNA extraction by adding approximately 100 mg of silica gel dried tissues into an extraction tube. Two steel balls each were added into the tube to enable grinding. The dried tissue was ground into fine powder by vortexing at a high speed for 5 minutes. 750 µl of pre-heated plant extraction buffer was added. Tubes were incubated at 65^0 C for 20 mins, stirred occasionally by inverting the tubes to homogenize the sample. The tubes were removed from the incubator and

allowed to cool for 2 mins. 200 µl of ice-cold 5 M Potassium acetate were added. The tubes were again incubated for 20 minutes to precipitate protein**.** 500 µl of chloroform Isoamylalcohol (24:1) was added and mixed gently to further precipitate protein and lipids. The tubes were then centrifuged at 10000 rpm for 10 mins and the supernatant was transferred into freshly labelled tubes. 2/3 volume of ice-cold Isopropanol was added, mixed gently and incubated in -80ºc for 15 mins to precipitate the DNA and then Centrifuged at 10000 rpm for 10 min to pellet the DNA. The supernatant was gently decanted until the last drop. 400 µl of 70% ethanol was added to wash the DNA pellet and centrifuged at 10000 rpm for 10 min. **S**upernatant was gently decanted until the last drop and the pellet air dried (until ethanol smell was no longer perceived). 60 ul of TE was added to re-suspend the DNA. 2 ul of RNase was added and it was incubated at 37ºC for 30-40 minutes.

Confirmation of Extracted DNA using Agarose Gel Electrophoresis

Successful extraction of DNA from leaf tissue was determined through agarose gel electrophoresis. 1 g of agarose powder was measured into 100 mL 1xTAE in a microwavable flat bottom flask. It was placed in the microwave for 3-5 min until the agarose until agarose powder completely dissolved (but did not over boil the solution, to prevent evaporation of the buffer, which would alter the final percentage of agarose in the gel). The agarose solution was allowed to cool down to about 60°C (about when one could comfortably keep your hold the flask with the hand), about 5 mins. 10 µL EZ vision DNA stain was added. EZ vision binds to the DNA and allows one to visualize the DNA under ultraviolet (UV) light. The agarose was poured into a gel tray with the well comb in place. The gel was allowed to remain at room temperature for 20-30 mins, until it has completely solidified.

Loading buffer was added to each of the DNA samples. Once solidified, the agarose gel was placed into the gel box (electrophoresis unit). Gel box was filled with 1xTAE (or TBE) until the gel is submerged. The molecular weight ladder was loaded carefully into the first lane of the gel. The samples were carefully loaded into the additional wells of the gel. The gel was run at 80-150 V for about 1-1.5 hours. The power was turned off; the electrodes were disconnected from the power source, and the gel was carefully removed from the gel box. The gel was visualized under UV transilluminator. Presence of DNA band indicated presence of DNA.

Polymerase Chain Reaction PCR)

The PCR mix was made up of 12.5 µL of Taq 2X Master Mix from New England Biolabs (M0270); 1 µL each of 10 µM forward and reverse primer respectively; 2 µL of DNA template and then made up with 8.5µL Nuclease free water. PCR Protocol was set at an initial denaturation at 94˚ C for 5 mins, followed by 36 cycles of denaturation at 94˚ C for 30 seconds, annealing at 55˚ C for 30 seconds and elongation at 72˚ C for 45 seconds. Followed by a final elongation step at 72˚ C for 7 minutes and hold temperature at 10˚ C forever.

DNA scoring and analysis

PCR product was resolved using agarose gel electrophoresis as described above and visualized under UV light. Gel images were taken, and the alleles (DNA bands) were scored in binary codes; clearly visible bands were assigned 1 for presence, 0 for absence and m for missing data. The percentage of polymorphism for each marker was calculated as the number of polymorphic bands over the total number of bands scored for the marker and multiplied by 100 (Martos *et al.,* 2005). The Polymorphic Information Content (PIC), a measure of variability for each locus was calculated

across the assay of units by the formula of Roldan-Ruiz *et al.* (2000):

$$PIC_i = 2f_i(1 - f_i)$$

where f_i is the frequency of the amplified allele (band present), and $(1 - f_i)$ is the frequency of the null allele (band absent) of marker i.

To estimate the level of genetic diversity, genetic similarities were evaluated using Nei and Li/Dice similarity index (Nei and Li, 1979) with the aid of the NTSYSpc software, version 2.11 (Rolf, 1998). A dendrogram was generated from the similarity matrix using the UPGMA (Unweighted Pair Group Method of Analysis using arithmetic averages) in NTSYSpc program.

Results and Discussion

Number of alleles, gene diversity, and polymorphic information content of *Cola* ISSR markers are presented on Table 2. The 10 ISSR markers exhibited extensive polymorphism among the *Cola* accessions. The major allele frequency ranged from 0.100 to 0.700, with a mean of 0.360. Genetic diversity of the primers used ranged from 0.490 to 0.935 with a mean of 0.767, where marker UBC811 revealed the highest genetic diversity. The allele number per ISSR marker ranged from 5 to 17, with a mean of 10. Polymorphism Information Content (PIC) for *Cola* varied from 0.470 (UBC888) to 0.931 (UBC811) with a mean of 0.749 (Table 2).

Figure 1 is a dendrogram showing overall genetic dissimilarity (variation) among the 20 accessions of *Cola* as revealed based on ISSR markers. At the 100% level of dissimilarity, all the accessions were distinct from one another. All the accessions formed a single cluster at 0.64 level of dissimilarity, indicating some degree of similarities among them. Five distinct clusters were formed from the analysis of the pooled ISSR marker data at the similarity coefficient of 0.72. Cluster I had only one accession CN8, indicating that CN8 is the most distinct among all the accessions. Cluster II and III had five accessions each II (CN9, CN7, CN6, CN5, CN4) and III (CN14, CN12, CN16, CN15, CN10) while Cluster IV had the largest number of accessions 7 (CN20, CN19, CN18, CN17, CN13, CN11, CN3), Cluster V composed of two accessions CN2 and CN1.

Table 2: Number of alleles, gene diversity, and polymorphic information content PIC of *Cola* ISSR markers

Markers	Major Allele Frequency	No of Alleles	Gene Diversity	PIC	Sequence 5'- 3'
UBC888	0.700	6	0.490	0.470	ACACACACACACA
UBC881	0.150	15	0.915	0.909	GGGTGGGGTGGGGTG
UBC866	0.650	8	0.560	0.545	CTCCTCCTCCTCCTCCTC
UBC818	0.450	10	0.760	0.744	CACACACACACACACAG
UBC808	0.250	11	0.860	0.846	AGAGAGAGAGAGAGAGC
UBC840	0.150	12	0.900	0.892	GAGAGAGAGAGAGAGAT
UBC834	0.200	11	0.875	0.863	AGAGAGAGAGAGAGAGT
UBC811	0.100	17	0.935	0.931	GAGAGAGAGAGAGAGAC
UBC810	0.500	5	0.675	0.634	GAGAGAGAGAGAGAGA
UBC2	0.450	5	0.700	0.656	GAGAGAGAGAGAGAGAT
Mean	**0.360**	**10**	**0.767**	**0.749**	

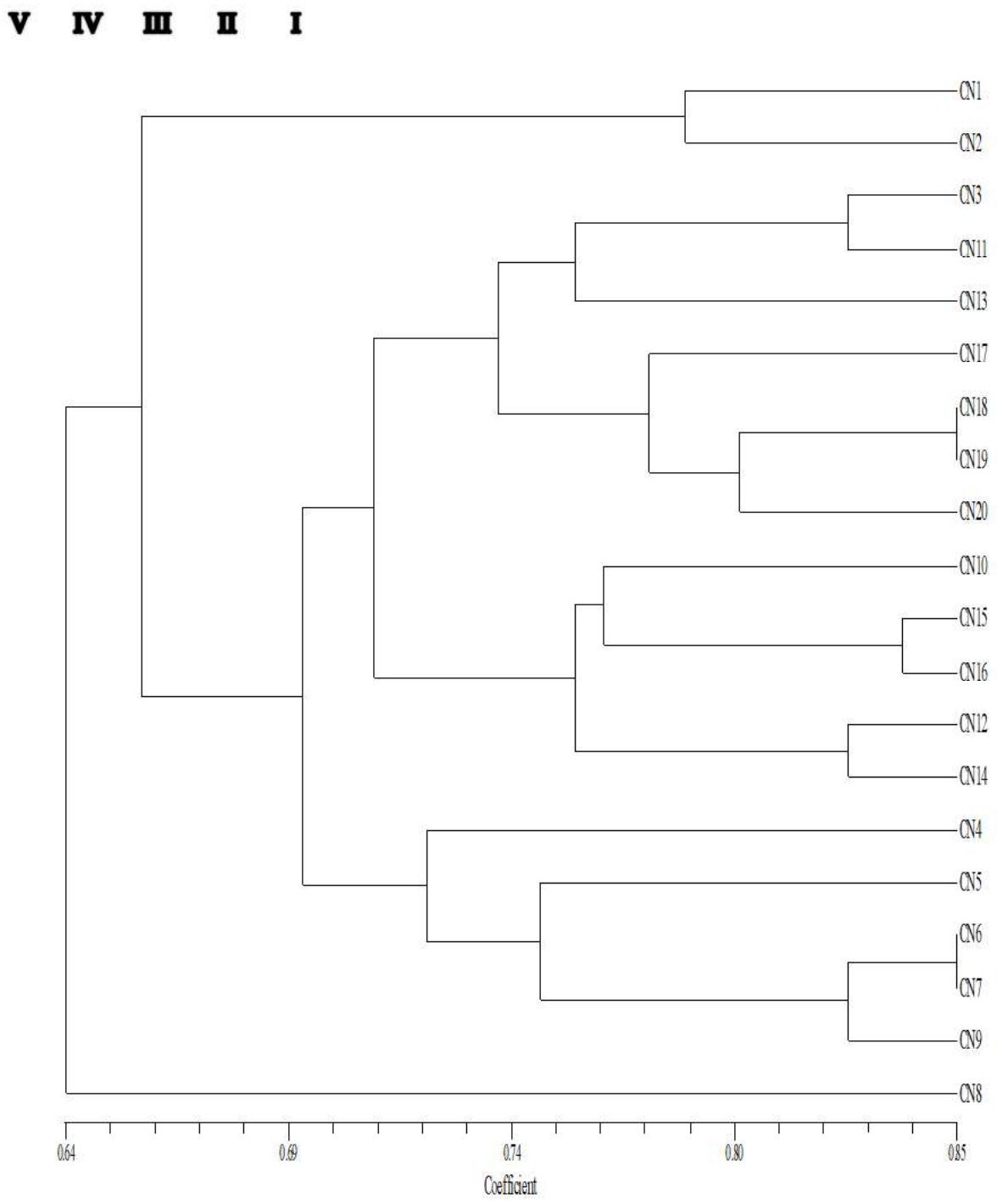

Figure 1: Molecular Dendrogram showing genetic dissimilarity among the twenty accessions of *Cola* based on ISSR markers.

Inter simple sequence repeat (ISSR) technique is a PCR based method, and ISSR markers are highly polymorphic and are useful in studying genetic diversity, phylogeny, gene tagging, genome mapping and evolutionary biology. ISSRs have been successfully used to estimate the extent of genetic diversity level in a wide range of tree crop species including *Camellia sinensis* (Yogurtcu and Aygun, 2021), *Jatropha*

curcas (Arolu *et al.,* 2012), *and Juglans regia* (Potter *et al., 2002*). The technique provided useful information for the exploitation of available genetic variability. The number of alleles and the high gene diversity (0.767) observed in this study proved that significant genetic variability existed among the kola accessions. Botstein *et al*., (1980) reported that a marker with PIC value of more than 0.5 is considered as highly informative. The average PIC value of 0.749 obtained in the current study is highly informative suggesting that the ISSR marker employed in the study was very useful for diversity study in *Cola.* All the ten polymorphic ISSRs primers used in this study were found useful for the delineation of accessions showing high allelic variation of the kola accessions. These observations are similar to those reported by Arolu *et al.,* (2012) where ten primers were used to distinguish 48 accessions of *Jatropha curcas,* likewise a report by Potter *et al.,* (2002) where eight primers were used to distinguish 48 cultivars of *Juglans regia*, and also a report by Alansi *et al.,* (2016) showed that eleven primers, were used to distinguish 34 accessions of *Ziziphusspina-christ* Lin *et al.,* (2019) also reported that nineteen primers were used to distinguish 159 accessions of coffee. The grouping of *Cola* accessions based on the Unweighted Pair Group Method with Arithmetic Mean (UPGMA) in this study provides an opportunity for selection of parents for improvement programmes Based on the information from the dendrogram, accessions that are far from each other by virtue of diversity index are strongly recommended to be used as parent for crossing. This will bring about greater genetic diversity, thus resulting into increase in selection gain.

Conclusion

The result obtained showed that a valuable genetic diversity is present within the *Cola* accessions under study based on the ISSR technique. Cluster analysis grouped the twenty *Cola* accessions into distinct groups and revealed variation within the clusters.

References

Adedayo, L. D., Ojo, A. O., Awobajo, F. O., Adeboye, B. A., Adebisi, J. A., Bankole, T. J., Ayilara, G. O., Bamidele, O., Aitokhuehi, N. G. and Onasanwo, S. A. (2019). Methanol extract of *Cola nitida* ameliorates infiammation and nociception in experimental animals. *Neurobiology of Pain*, *5*, 100027. https://doi. org/10.1016/j.ynpai.100027

Alansi, S; Tarroum, M; Al-Qurainy, F; Khan, S; Nadeem, M. (2016). Use of ISSR markers to assess the genetic diversity in wild medicinal *Ziziphusspina-christi* (L.) Willd. collected from different regions of *Saudi Arabia, Biotechnology &Biotechnological Equipment*, 30(5): 942-947

Arolu, I. W., Rafii, M. Y., Hanafil, M. M., Mahmud, T.M.M., Latif M. A. (2012). Molecular characterizations of *Jatropha curcas* germplasm using inter simple sequence repeat (ISSR) markers in Peninsular Malaysia. *Australian Journal of Crop Science,* 6(12):1666-1673.

Botstein, D., White, R. L., Skolnick, M., Davis, R.W. (1980). Construction of a genetic linkage map in man using restriction fragment length polymorphisms. *Am. J. Hum. Genet.,* 32: 314-331

Burdock, G. A., Carabin, I. G. and Crincoli, C. M. (2009). Safety assessment of kola nut extract as a food ingredient. *Food and Chemical Toxicology,* 47: 1725–1732.

Dewole, E. A., Dewumi, D. F. A., Alabi, J. Y. T. and Adegoke A. (2013). Proximate and Phytochemical of *Cola nitida* and *Cola acuminata*. *Pakistan Journal of Biological Sciences*, 16: 1593-1596.

Dorathy, I. U., Okere, S. O., Daniel, E. E., Mubarak, L. L. (2014). Phytochemical

Constituents and antidiabetic property of *cola nitida* seeds on alloxan-induced diabetes mellitus in rats. *British Journal of Pharmaceutical Research International*, 4(23): 2631–2641

Durand, N. D., Adoukonou-Sagbadja, H., Nafan, D., Adjanohoun, A., Baba-Moussa, F., Sina, H., Sezan, A. and Baba-Moussa, L. (2015). Indigenous knowledge and socioeconomic values of three kola species (*Cola nitida, Cola acuminata and Garcinia kola*) used in southern Benin. *European Scientific Journal,* 11(36): 206–227.

Esimone, C. O., Adikwu, M. U., Nworu, C. S., Okoye, F. B. C. and Odimegwu, D. C. (2007). Adaptogenic potentials of *Camellia sinensis* leaves, *Garcinia* kola and *Cola nitida* seeds. *Scientific Research Essay*, 2(7): 232–237.

Jayeola, C.O. and Akinwale, T.O. (2002). Utilization of kolanut and cocoa production beverages. *Nutrition and Food Science*, 32 (1):21–23.

Lin, Y., Collins, O., Lifang, H., Xiaoyang, W., Hua, Z., Yulan, L.,Yuzhou, L.,Yunping, D. and Yuepeng, H. (2019). Genetic Diversity and Population Structure of Coffee Germplasm Collections in China Revealed by ISSR Markers *Plant Molecular Biology Reporter*, (https://doi.org/10.1007/s11105-019-01148-3)

Madingou, N.O.K., Souza, A., Lamidi, M., Mengome, L.E., Mba, C.E.M., Bayissi, B., Mavoungou, J. and Traoré, A.S. (2012). Study of medicinal plants used in the management of cardiovascular diseases at Libreville (Gabon): an ethnopharmacological approach. *International Journal of Pharmaceutical Science and Research,* 3(1):111–119.

Martos, V., Royo, C., Rharrabti, Y., and Garcia del Moral, L. F. (2005). Using AFLPs to Determine Phylogenetic Relationships and Genetic Erosion in Durum Wheat Cultivars Released in Italy and Spain throughout the 20th Century. *Field Crop Res*., 91:107-116

Nei, M. and Li, W.H. (1979). Mathematical model for studying genetic variation in terms of restriction endonucleases. *Proceedings of the National Academic of Science*, USA 76: 3269-3273

Nyamien, Y., Adje, F., Niamke, F., Chatigre, O., Adima, A. and Biego, H. (2014). Caffeine and phenolic compounds in *Cola nitida* (vent) Schott and Endl and *Garcinia kola* Heckel grown in Côte d'Ivoire. *British J. Applied Sci. Technol,* 4(35)*:* 4846–4859.

Potter, D., Gao, F., Aiello, G., Leslie, C. and McGranahan, G. (2002). Intersimple Sequence Repeat Markers for fingerprinting and determining genetic relationship of walnut (*Juglans regia*) cultivars. *J. Amer. Soc. Hort. Sci*., 127(1):75-81

Rohlf, F.J. (1998). NTSYSpc Numerical Taxonomy and Multivariate Analysis System. Vs. 2.0. Exeter Software, Setauket, New York

Roldán-Ruiz, I., Dendauw, J., Van Bockstaele, E., Depicker, A. and De Loose, M. (2000). AFLP markers reveal high polymorphic rates in ryegrasses (*Lolium* spp.). *Molecular Breeding,* 6**:**125–134

Yogurtcu, B., and Aygun, A. (2021). Characterization of tea (*Camellia sinensis* L.) genotypes grown in turkey by ISSR markers. *Applied ecology and environmental research,* 19(5):4103-4114.