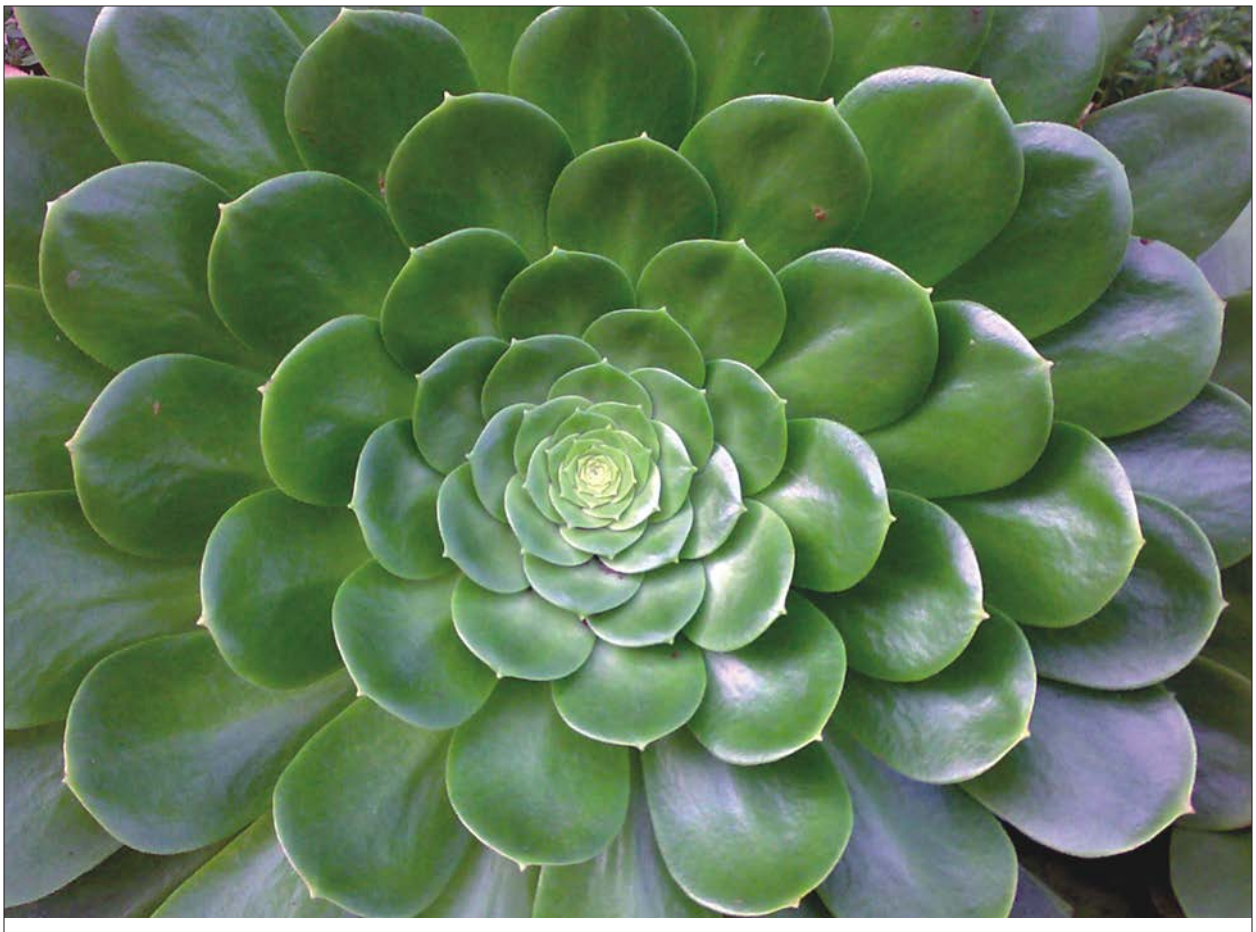

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Characterization of Genetic Similarity of a Hampton Road (Virginia) Live Oak (*Quercus virginiana* Mil. L.) to a Historic *Q. virginiana* Using Rapd Markers

Camellia Moses Okpodu, George F. Khan and Malikah Abdullah-Israel
Department of Biology, Norfolk State University, 700 Park Avenue, Norfolk, Virginia 23504-3993, United States of America

Corresponding Author: Camellia Moses Okpodu, Department of Biology, Norfolk State University, 700 Park Avenue, Norfolk, Virginia 23504-3993, United States of America Tel: (757) 823-8957 Fax: (757) 823-2000

ABSTRACT

Local Hampton Roads Virginia (USA) folklore has intimated for almost forty years that a *Quercus virginiana* called "Old Pointe" tree which had been targeted by some City planners to be removed, had a historical connection to the "Emancipation oak" (i.e., a tree that is historically significant in the US because it's the physical location where slavery ended in the US). As we come to the 150 years of the end of the US Civil war this paper shows how modern research can be used to answer a historical ethnological question. The objective of this research was to evaluate the genetic similarity of a Hampton Roads Virginia Live oak (*Quercus virginiana* Mil. L.) to a historic *Q. virginiana* called the "Emancipation oak" using random amplified polymorphic DNA. Cluster analysis was used to describe patterns of genetic similarity. Six primers produced 86 bands and had average polymorphisms of 54%. Three Zelkova trees [*Zelkova serrata* (Thunb.) Makino] were used as an out-group. Analysis using UPGMA dendrogram divided the Quercus and Zelkova species into two distinct groups. A similarity matrix was computed with Jaccard coefficient among the five trees. The cophenetic correlation for the goodness-of-fit of the cluster analysis to the similarity matrix was 0.99. This study successfully demonstrates that RAPD markers can be used in estimating the extent of genetic relatedness of live oaks.

Key words: RAPD, *Quercus* spp., UPGMA, genetic diversity, *Zelkova serrata*

INTRODUCTION

According to a Hampton Roads Virginia Historic Society (The Contraband Slave Historic Society which is an advocacy group for documenting the contribution of African Americans at Fort Monroe) (Willis, 2011), there are several trees in the Hampton Roads Area that were propagated from "the Emancipation oak". One tree in particular which we have named "Old Pointe oak" has been under consideration by the City of Hampton (ca. 1996) for demolition. "The Emancipation oak" and all its related off-spring (e.g., Old Pointe) possess both economical and psychological value. Therefore preserving these trees and the biodiversity they also bring through the use of biotechnological approaches has become an integral component in preserving them as part of the urban forest environment. Urban forests are now recognized as providing significant and increasing value to the urban environment (Dwyer *et al*., 1991). In addition, to providing improvement in air quality by reducing the carbon dioxide emission (Rowntree and Nowak, 1991; Anomohanran, 2011), urban

forests also provide cultural benefits that have been shown to lead to improvements in the overall quality of urban life. Urban trees provide "curb appeal" to a city neighborhood, provide privacy and influence the physical and biological environment of an area by offsetting and controlling rainfall runoff and flooding (Dwyer *et al*., 1992; Hull, 1992; Westphal, 2003; Ellis *et al*., 2006). After an ethnobotanical evaluation, that produced corroborative evidence which suggested that the Emancipation oak and Old Pointe oak trees were related (Okpodu, 2003) we decided to use Random Amplified Polymorphic DNA (RAPD) technique to calculate the percentage of relatedness of the "Old Pointe oak" to "The Emancipation oak." "The Emancipation oak" and its off-spring provide a biological legacy that is culturally important.

The objective of this study was to characterize the molecular diversity and relatedness of Old Pointe oak which had been under consideration to be removed by the City of Hampton, to the historic "Emancipation oak" by analyzing DNA amplifications products using RAPD molecular markers. This study reports the successful use of the RAPD method to examine the molecular polymorphisms as well as genetic relationship of the Old Pointe oak to "The Emancipation oak."

MATERIALS AND METHODS

DNA isolation: DNA samples were extracted from mature leaves (of all tree samples plant) using the combined DNAzol®-CTAB (hexadecyltrimethylammonium bromide) method as described by Okpodu and Abdullah-Israel (2011).

Geographical location and plant material: A map from Horton *et al*. (2005) studies of the Chesapeake Bay Impact Structure were modified to show the geographical locations of the trees used in this study. *Zelkova serrata* (Thunb.) Makino was used as an outgroup. *Zelkova* like *Quercus* genus, are members of the sub-kingdom Tracheobionta (Judd and Olmstead, 2004). A non-native tree species, Zelkova genus is located in USDA hardiness zones 5 through 8. Ten species of Zelkova have been identified and three are native to China (Jin *et al*., 2009). Zelkova have been introduced in the U.S. as an answer to the American Elm that has been challenged by Dutch Elm disease. A total of five tree samples were used to compare genetic distance (three species of *Zelkova serrata* (Thunb.) Makino. and two *Quercus virginiana* L.).

RAPD analysis: Genomic DNA concentration was approximated using spectrophotometer readings at A_{260}. Quality of the DNA was examined on a 2% agarose gel in 1X TAE buffer, stained with 0.5 μg of ethidium bromide and visualized under UV light. RAPD PCR amplification was carried out using the Ready-to-go RAPD Analysis Kit with primers (Amersham Biosciences, Piscataway, NJ) per the manufacturer's instructions. Reaction conditions were as follows, 0.4 mM each of dNTP's, 2.5 μL of BSA, reaction buffer (3 mM $MgCl_2$, 30 mM KCl and 10 mM tris, pH 8.3), thermostable polymerases (AmpliTaq™ DNA polymerase and Stoffel fragment), 25 pmol of primer and 30-40 ng of genomic DNA, in a final volume of 25 μL. Samples were then placed in a Techne Progene Thermocycler and amplified using the following program: 1 cycle at 95°C for 5 min, 45 cycles of 95°C for 1 min, 36°C for 1 min and 72°C for 2 min. Amplification products were separated on a 2% agarose gel in 1X TAE buffer and stained with 0.5 μg of ethidium bromide. Gels were photographed under UV light with the ChemiDoc XRS Gel Documentation System and Quantity One software.

Screening primers: Genomic DNA was successfully extracted as described by the method of Okpodu and Abdullah-Israel (2011). This method allowed easily score bands. Initially, Operon Technologies (Alameda, CA) Operon Kit A was used for the RAPD analysis. The Operon Kit

contained 20 different primers (data not shown). Although, the Operon Kit has been shown to be effective for other tree samples (Hasan *et al*., 2009; Sesli and Yegenoglu, 2010), it was not consistent with our studies. Similar to the results of Pan *et al*. (1997) 19 of the 20 Operon primers produced artifact DNA bands in negative control reactions. In addition, the Operon Kit primers did not consistently generate amplification products with our test samples.

Gel scoring and data analysis: Only unambiguous and reproducible bands were used in scoring and analysis of the gels. DNA markers were scored as either present or absent in all samples used in the analysis. These data were used for the calculation of pairwise genetic distances between trees using the Jaccard coefficient similarity matrices. Similarity between clusters were calculated using Kendall tau Rank Correlation (v1.0.10) in Free Statistics Software (Wessa, 2008). The data sets were analyzed using DendroUPGMA, a dendrogram construction utility (DendroUPGMA, S. Garcia-Vallve, Biochemistry and Biotechnology Department, Universitat Rovira i Virgili, Tarragona, Spain (http://genomes.urv.es/UPGMA/), Garcia-Vallve *et al*., 1999). For comparison, angiosperm phylogeny relationship was constructed from Stevens (2001). Angiosperm Phylogeny Website. Version 8, June 2007. http://www.mobot.org/MOBOT/research/APweb/ which follows: Angiosperm Phylogeny Group [APG] classification for the orders and families of flowering plants (APG, 2003).

RESULTS AND DISCUSSION

Tree samples and RAPD analysis: The geographical location of tree samples used in this study are shown in Fig. 1. Both "The Emancipation oak" (*Quercus virginiana*) and Old Pointe oak

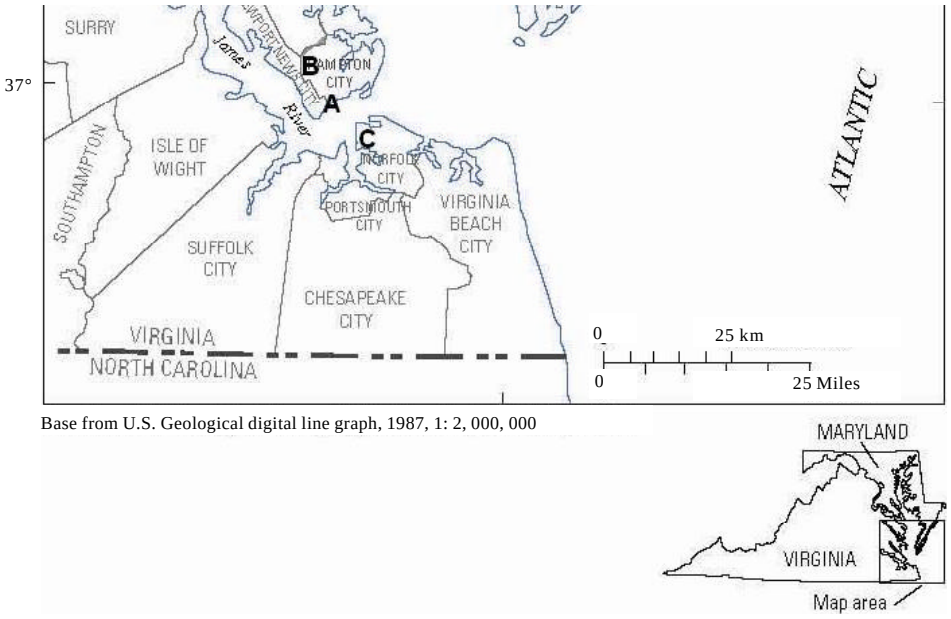

Fig. 1: Map of the geographical location of A: Emancipation oak, B: Old Pointe oak and C: Zelkova trees used in this study (Horton *et al*., 2005)

are members of the genus *Quercus* in the beech family Fagaceae (Dirr, 1990). *Q. virginiana* is also known as a live oak or a Virginia live oak. This species is native to the southeastern United States and northern parts of South America in USDA hardiness zones 8-11 (USDA, 1990). Its horizontal branches, leathery, oval and dark-green leaves can easily identify it. It is often classified separately from other oaks because it is an evergreen. The acorns are one-third covered by a deep cup. *Q. virginiana* are long-lived trees, with a life span of up to 2-3 centuries.

Amplification of the genomic DNA yielded 86 bands and only the polymorphic bands were scored. The amplification pattern results are summarized in Table 1. All the primers amplified bands with the number of amplified fragments ranging from 3-7 bands per lane. Of the 86 amplified bands, 54% were polymorphic. The percentage polymorphism ranged from 21% using primer 6 (RAPD-6) to a maximum of 81% using primer 3 (RAPD-3). The size of the bands varied 250-1034 bp. Profiles of the six RAPD primers for all tree samples are shown in Fig. 2.

Table 1: The decamer sequences of primers used in RAPD reactions, the resulting bands in the profiles are listed

Primer name	Primer sequence 5-3'	Total No. of bands	Total No. of polymorphic bands	% Polymorphism
RAPD-1	GGTGCGGGAA	16	12.0	75
RAPD-2	GTTTCGCTCC	22	15.0	68
RAPD-3	GTAGACCCGT	11	9.0	81
RAPD-4	AAGAGCCCGT	7	4.0	57
RAPD-5	AACGCGCAAC	16	4.0	25
RAPD-6	CCCGTCAGCA	14	3.0	21
Total		86	47.0	327
Average		14	7.8	54

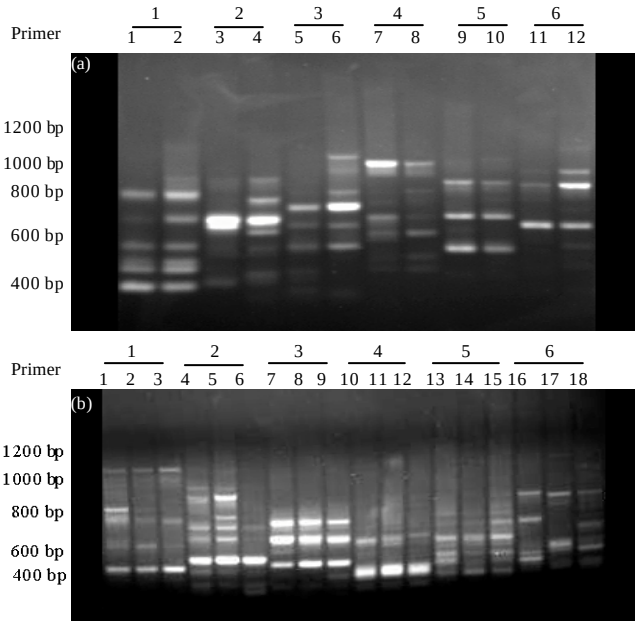

Fig. 2(a-b): RAPD bands detected in agarose gel electrophoresis profiles of DNA from (a) Emancipation oak (lane 1, 3, 5, 7, 9 and 11) and Old Pointe oak (lane 2, 4, 6, 8, 10 and 12) and (b) Zelkova samples using RAPD primers 1-6

Table 2: Similarity matrix computed with Jaccard coefficient among the five trees

Trees	Zelkova 1	Zelkova 2	Zelkova 3	Emancipation oak	Old Pointe oak
Zelkova 1	1				
Zelkova 2	0.78	1			
Zelkova 3	0.67	0.45	1		
Emancipation oak	0.00	0.00	0	1	
Old Pointe oak	0.00	0.00	0	0.79	1

The cophenetic correlation for the goodness-of-fit of the cluster analysis to the similarity matrix was 0.99

The Amersham RAPD primers generated reproducible results that were easier to score. No artifact DNA bands were observed in negative control reactions of the Amersham RAPD analysis beads and this assay provided strong evidence that there were no residual DNA molecules in polymerase enzyme solutions and that the polymorphic DNA bands shown in Fig. 2 were indeed amplified from the primers themselves. Using the RAPD analysis kit, a total of 86 bands were scored using the six different primers that were in the kit. A similarity matrix was computed with Jaccard coefficient among the five trees. The cophenetic correlation for the goodness-of-fit of the cluster analysis to the similarity matrix was 0.99 (Table 2).

Dendrogram analysis and statistical analysis: Random Amplified Polymorphic DNA (RAPD) techniques have been used to show genetic similarity in several species of plants (Lakshmi *et al*., 2008; Gorji *et al*., 2010; Kumar *et al*., 2010) and specifically trees for example, *Acer saccharum* and *Acer nigrum* (Skepner and Krane, 1998); *Betula maximowicziana* (Tsuda *et al*., 2004) and *Artemisia capillaris* (Hasan *et al*., 2009). RADP techniques are useful because the researcher does not have to know the complete genome and only small quantities of DNA are necessary. RAPD profiles provide valuable information that is comparable to isoenzyme results (Royo and Itoiz, 2004). In conversational plant biology RAPDs has become the technique of choice because it allows for low-cost and easy collection and storage of sample and reproducible results. Due to these advantages RAPDS have been used for genome mapping, cultivar identification and population genetic studies where researchers have looked at both inter and intra-species variation.

Six primers were applied to five individuals of trees for DNA amplification. The results showed that different primers generated different fragments numbers and length of products as shown in Fig. 3. The RAPD and DNA analysis mapped very similarly to the morphological phylogenetic relationship of major groups of Tricolplates (Eudicot) (Fig. 3a). Oaks and Zelkova belong to the subclass Hamamelididae and are paraphyletic; however, Zelkova belong to and the family Ulmaceae and oaks to the family Fagaceae (Judd and Olmstead, 2004). The overall evolutionary relationship for Fagales (Fagaceae) and Rosales (Ulmaceae) (Fig. 3b) demonstrated the same type of hierarchically nested clades as recognized by Chase *et al*. (1998) and APG (2003, 2009). The dendrogram produced from these samples produced two main clusters. The first cluster was between the three Zelkova trees. The second cluster was among the oak trees. The cophenetics correlation from the Jaccard analysis was 0.99. The strength of association within the clusters were calculated using Kendall's tau coefficients (Table 3). The value will be high (close to 1) in the case of a positive relationship, positive meaning that both values are increasing in the same direction (Rademaker *et al*., 2000). The closest relationships were observed between Zelkova trees 1 and 2 and "The Emancipation oak" and Old Pointe DNA. Both had values of 0.86. These high values support a positive association.

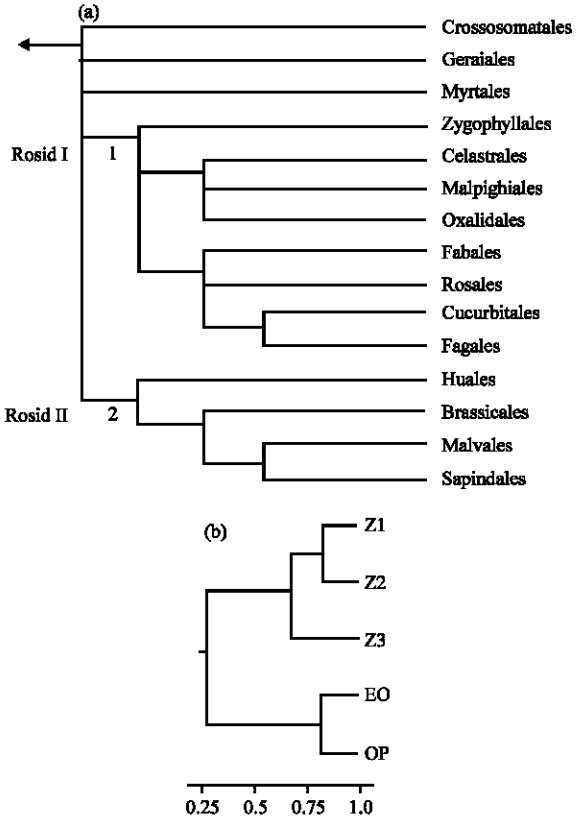

Fig. 3(a-b): Phylogenetic dendrograms, (a) The Angiosperm Phylogeny Group classification for the orders and families of flowering plants and (b) UPGMA dendrogram of trees based on DNA and RAPD analysis among Quercus and Zelkova trees, (APG, 2003; Stevens, 2001; Garcia-Vallve *et al*., 1999), Z1-3: Zelkova trees, EO: Emancipation oak and OP: Old Pointe tree

Table 3: Non-parametric Kendall's tau correlation coefficient. The significant correlation values (p>0.0001 level, two-tailed) are indicated by **.010201

Trees	Zelkova 1	Zelkova 2	Zelkova 3	Emancipation oak	Old Pointe oak
Zelkova 1	1.00				
Zelkova 2	0.86**	1.00			
Zelkova 3	0.79**	0.56**	1.00		
Emancipation oak	-0.11	-0.09	-0.09	1.00	
Old Pointe oak	-0.13	-0.11	-0.10	0.86**	1.00

CONCLUSION

The genus *Quercus* comprises more than 300 species spread over Asia, North America and Europe. In this study, we provide information on the genetic relatedness between two species of oak. Our finding can be used to make conservation decisions. The traditional method of propagating most species of oak (*Quercus* L. spp.) is by seed. According to local residences of Hampton VA (personal communication), the Old Pointe tree was generated from acorns. Sexual propagation of oaks is known to result in great phenotypic and genotypic variability (Flemer, 1962; Hartmann *et al*., 1990). Low-levels of differentiation between the two *Quercus* species suggest these

trees share ancestral polymorphism rather than recurrent gene flow. It is hard to envision gene flow being the answer for the shared polymorphism because these trees are at a physical distance (~5 miles apart) which should be sufficient to create a spatial barrier. Spatial isolation should prevent gene flow and therefore cannot be used as a probable account for this high level of genetic similarity among the oaks trees. Gurudeeban *et al.* (2011) demonstrated that RAPD analysis is sensitive enough to detect low levels of variation. From the data obtained in this study we can conclude that RAPD technology can be useful allowing the identification of different cultivars as well as the assessment of the genetic similarity among different genotypes of trees. In closing, RAPD analysis can be used in tree breeding programs and provide an important input into tree conservation biology.

Local Hampton Roads Virginia (USA) folklore has intimated for almost forty years that the *Quercus virginiana* called "Old Pointe" tree which had been targeted by some City planners to be removed, had a historical connection to the Emancipation oak (i.e., a tree that is historically significant in the US because it is the physical location where slavery ended in the US. As we come to the 150 years of the end of the US Civil war this paper shows how modern research can be used to answer this historical ethnological question. Present results provides definitive support to the folklore that the Old Pointe Tree has a close genetic association to the Emancipation oak.

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