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Utilization of conserved genic SSR markers for phylogenetic analysis of Nigerian *Ipomoea* species

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Abstract

Ipomoea, with enormous economic importance, is a large genus with species that are highly evolutionarily diverged. There is dearth of information regarding the phylogenetic relationships of the Nigerian species of the genus *Ipomoea*. It is therefore imperative to use contemporary taxonomic evidences to understand the diversity of its distribution in a region and provide enhanced delimitation of the taxa. In this study, 6 genic simple sequence repeat (SSR) markers whose cross transferability have been established among the plant species were selected to appraise their level of polymorphism and used to study the phylogenetic relationships among 11 indigenous Nigerian *Ipomoea* species. The 6 SSR loci showed varying levels of polymorphism among the genotypes of all 11 species assessed and demonstrated 100% polymorphism when examined across the 11 species. A total of 55 alleles were produced. The PIC of the primers ranged between 0.2223 and 0.874 with an average value of 0.71885. Phylogenetic analysis clustered species into 3 major and 6 sub clusters wherein the species were clearly separated. This study has shown the effectiveness of developed genic SSR markers for establishing phylogenetic relationship in *Ipomoea* species.

Keywords

SSR markers, genic, phylogenetics, *Ipomoea*, conserved

Introduction

The genus *Ipomoea*, belonging to the family Convolvulaceae is widely distributed in the tropic and subtropic regions. The genus comprises of species which are annual and perennial herbs, vines, shrubs and even small trees (1-4). A striking morphological characteristic of the Convolvulaceae family, is twining or climbing woody or herbaceous plants often having heart-shaped leaves and funnel-shaped flowers (5). The genus is known anatomically with the existence of cells, which secrete resin glycosides in the foliar tissues and the roots. These glycoresins are a key chemotaxonomic marker of this family (6) and are implicated for the purgative characteristics of some species of the Convolvulaceae (7).

Several species of *Ipomoea* are used as ornamental plants, food or medicines. Interestingly, antimicrobial, analgesic, spasmolitic, spasmogenic, hypoglycemic, hypotensive, anticoagulant, anti-inflammatory, psychotomimetic and anticancer activities were reported in number of the species (8).

Examining the relationships among a group of closely related organisms to settle on their evolutionary history is core objectives in systematic

botany (9). It is imperative to consider characters that appear to be evolving fast enough to address the relationships of recently diverged taxa, especially among species belonging to large and taxonomically complex genera like *Ipomoea*. Problems exist in identification of *Ipomoea* especially among Nigerian species leading to a description of intraspecific species (10), thus the need to use modern taxonomic evidences for a better system of classification and delimitation of the species. Molecular markers have proven an important tool in plant genetic analyses. Microsatellites or simple sequence repeats (SSRs) are genetic markers that have recorded transferability at species level (11). In recent years, RAPD-PCR, SDS-PAGE of seed proteins and Inter-simple Sequence Repeat (ISSR) markers were used to assess phylogenetic analysis of *Ipomoea* species (12, 13). Among the various molecular makers, Simple Sequence Repeat (SSR)/Microsatellite makers are advantageous due to several attributes such as high abundance advantage, random distribution in the genome, cross transferability ability, co-dominance and high polymorphism information content (14). Furthermore, genic Simple Sequence Repeat (SSR) markers being derived from the conserved regions have been used for establishing phylogenetic relationships (15-17). They have been developed at great scale and lower costs with the aid of next generation sequencing. Considering various "Good markers" attributes, genic SSR makers have been used here for establishing phylogenetic analysis in *Ipomoea* species.

Genetic management of any given gene pool is consequent upon understanding the diversity of its distribution in a region. However, much is not known about the phylogenetic relationships of the Nigerian species of the genus *Ipomoea,* hence, the need for this study. This study was therefore aimed at examining the nature of genetic relationships among some *Ipomoea* species that occurs in Nigeria using genic SSR markers.

Materials and Methods

Species studied

Eleven (11) indigenous species of *Ipomoea* occurring in Nigeria, namely, *I. batatas*, *I. carnea*, *I. aquatic*, *I. involucrata*, *I. alba*, *I. nil*, *I. hederifolia*, *I. asarifolia*, *I. mauritiana*, *I. quamoclit*, and *I. cairica* were sampled and used in this study. Identification of the plant species was carried out at the herbarium of Forest Research Institute of Nigeria, Ibadan (FHI) Nigeria. Four different genotypes were collected per species. Details of the collected plants are shown in Table 1. Fresh leaves were collected and dried in silica gel.

DNA Isolation

Genomic DNA was extracted from silica gel dried leaf samples of *Ipomoea* species with the protocol outlined by (18), with minor modifications. About 2 - 3g of sample was crushed in liquid nitrogen by means of mortar and pestle. The crushed leaf tissues were then set in a pre-warmed (60 degrees) DNA isolation buffer solution (containing 1M tri-sHCl, 5M Nacl, 0.5M EDTA, 10% CTAB, β-mercapto-ethanol, Poly Vinyl Pyrolidone and distilled water) and incubated at 65 degrees for 1-2 hrs. 500 µl of chloroform:isoamyl alcohol (24:1) was added and mixed gently for 5 min and centrifuged at 2000 rpm for 15 min at room temperature. The aqueous solution was transferred into another tube and cold isopropanol added in the ratio of 2/3 with chloroform-isoamyl alcohol. The solutions were kept in the cold room for about 1 hr to facilitate precipitation and then centrifuged. The DNA which settled as a pellet was washed twice with 70% alcohol to remove ions, salts and proteins molecules. It was dried by inverting the tubes, re-suspended in 100 µl of TE buffer solution plus 10 µl RNAse (10 mg mL-1), incubated at 37 degrees for 2 - 3 hrs and after stored at -20 degrees. DNA concentrations were determined using spectrophotometer followed by quality check on ethidium bromide-stained agarose gels keeping known amounts of uncut λ DNA as standard.

Genic SSR primer designing and validation

Non-redundant (NR) transciptome dataset of *I. cairica* was used as query species for SSR markers mining. MISA (http://pgrc.ipkgatersleben.de/misa/), a microsatellite search tool was used to detect and catalog microsatellites in transcripts of *I. cairica*. Primer3 v2.23 was used to design primer pairs with default setting. PCR product size ranged from 100 to 350 bp. Six genic SSR primer pairs (Table 2) designed from the transcriptome of *I. cairica* were used for PCR amplifications and the reaction was conducted in a final volume of 10 µl containing 20ng DNA, 15 ng each of forward and reverse primers, 100 µM of dNTP's, 1× Taq buffer containing 1.5 mM MgCl2, 0.2 U of Taq polymerase and miliq water. The PCR reactions were performed using the following conditions: 4 min at 94 ^{0}C, 35 cycles of 1 min at 94 ^{0}C, 1 min s at the annealing temperature of each primer and 1 min at 72 ^{0}C with a final extension step of 5 min at 72 ^{0}C. The PCR products were subjected to electrophoresis on 1.2% non-denaturing polyacrylamide gels and stained by ethidium bromide. Amplification products were resolved on a 6% denaturing polyacrylamide gel (19:1 acraylamide: bisacrylamide) in 1× TBE buffer. The electropherograms were subsequently visualized by silver staining (Silver Sequence Staining reagents, Promega, Madison, USA), using a 50-bp DNA size standard (MBI Fermentas, Vilnius, Lithuania).

Table 1. Details of collections sites for 11 Nigerian *Ipomoea* species used in the study

Species names	Genotype	Location
	1	Opposite Fajuyi Hall, OAU University, Ile Ife, Osun State
Ipomoea carnea	2	Egbeada Road, Owerri, Imo State
	3	Ubakala Road, Abia State
	4	No 4 Old Road, Nekede, Imo State

Ipomoea aquatica	1	Nembe Community, Bayelsa State
	2	Aladimma Hospital Road (Opp Christ Chapel Intl), Imo State
	3	Aladimma Post Office, Owerri (Opp Primary Sch), Imo State
	4	Iwo Road, Ibadan, Oyo State
Ipomoea involucrata	1	OAU University Botanical Garden, Osun State
	2	Nembe Community, Bayelsa State
	3	Fence of Aladimma Recreation Centre (Admirials Bar & Grills)
	4	Umueze Obazu Mbieri, Imo State
Ipomoea alba	1	OAU University along Rectas Hall, Osun
	2	Iwo Road near Mechanic workshop, Ibadan
	3	World bank Primary School, Owerri, Imo State
	4	Umueze Obazu Mbieri, Imo State
Ipomoea nil	1	Umueze Obazu Mbieri, Imo State
	2	IMSU Teaching Hospital, Orlu, Imo State
	3	Ubakala Road, Abia State
	4	Iwo Road near Mechanic workshop, Ibadan
Ipomoea hederifolia	1	Iwo Road near Mechanic workshop, Ibadan
	2	Ubakala Road, Abia State
	3	World bank Housing, Owerri, Imo State
	4	Area L ring road, Owerri
Ipomoea asarifolia	1	Opposite Crown Plaza Hotel, behind Federal Secretariat, Owerri.
	2	MFM Bus Stop, Iwo Road, Ibadan.
	3	Chukwuma Nwoha Junction Road, Owerri, Imo State
	4	Wetheral Road, Owerri, Imo State
Ipomoea mauritiana	1	Amaukoro, Umueze Obazu Mbieri, Imo State
	2	Umuchimanwiri Obazu, Mbieri, Imo State
	3	IMSU Teaching Hospital Hostel, Orlu, Imo State
	4	IMSUTH Orlu, Imo State
Ipomoea quamoclit	1	Beside ASO Rock building, Imo State University, Owerri
	2	Aladimma Hospital Road, Owerri, Imo State
	3	Obazu Mbieri Road, Imo State
	4	Orile Area, Lagos State
Ipomoea cairica	1	Nembe Community, Bayelsa State
	2	Ikoyi Lagos
	3	Obalende, Lagos State
	4	Nembe Community, Bayelsa State
Ipomoea batatas	1	Item Street, Ikenegbu Owerri, Imo State
	2	Obazu Mbieri, Imo State
	3	Ubakala Road, Abia State
	4	Ikoyi Area, Lagos State

Table 2. Characteristics of 6 *Ipomoea* genic SSR markers used in genetic diversity analysis

Primer	Orientation	Sequence	Motif	SSR	SSR Lenght	SSR type	Size	LOCALIZA-TION	FUNCTION
IPTR_43	FORWARD	GAAATGTGTCTTTGTCTACGC	GAA	GAAGAAGAAGAAGAA	15	Tri	15	3' UTR	PREDICTED: peptidyl-prolyl cis-trans isomerase FKBP19, chloroplastic isoform X1
	REVERSE	TCCACGTCAGTAACATTGAA							

IPTR_39	FORWARD	TACCAAGAACAGAACAGAGGA	TC	TCTCTCTCTCTCTC	14	Tri	18	3' UTR	PREDICTED: blue-light photoreceptor PHR2
	REVERSE	GGAAGAAATCTTTGAAACCAC							
IPTR_34	FORWARD	CCTGGTGAAGCACAAGTAGTA	GA	GAGAGAGAGAGA	12	Di	12	CDS	PREDICTED: GDP-mannose 3,5-epimerase 1
	REVERSE	TCACCGCTTTTATGTTATACG							
IPTR_21	FORWARD	AATCTCCTCAACTGACAAGTG	GTG	GTGGTGGTGGTGGTG	15	Tetra	20	CDS	PREDICTED: protein ECERIFERUM 1-like
	REVERSE	AAGCAACTGAATCCTTACAAA							
IPTR_37	FORWARD	CTATACTCTCCTTCGCCACTC	GA	GAGAGAGAGAGAGAGAGAGA	20	Tri	18	3' UTR	PREDICTED: uncharacterized protein At5g22580
	REVERSE	ACCACAGAACTCGCAGTAATA							
IPTR_8	FORWARD	TCTCTCTCCTTCTCCTCTCAC	CT	CTCTCTCTCTCT	12	Di	12	CDS	PREDICTED: 30S ribosomal protein S9, chloroplastic
	REVERSE	CTGGACTTGACATACTTCTGG							

Genetic Data Analysis

PCR amplified alleles of every individual genotype were scored. They were recorded as present (1) or absent (0) of the defined allele. Genetic statistics which included genic variation statistics and heterozygosity for all microsatellite loci were calculated using POPGENE version 1.32 software. DARwin software version 6.0.021 was used for diversity and phylogenetic tree analysis on the basis of evolutionary dissimilarities.

Results

Six genic SSR markers found to be transferable among the plant species were selected to appraise their level of polymorphism and phylogenetic analysis among the various *Ipomoea* species. These primers represented transcribed genes and had putative functions (Table 2). All the 6 genic SSR loci tested on the species of *Ipomoea* were found to be polymorphic. The primers demonstrated different levels of polymorphism among the genotypes of all eleven species assessed. Amplicon bands were detected for all 6 genic SSRs, with a total of 55 alleles. The number of alleles ranged from 6 to 12 with an average of 9.1667. The highest number of alleles was detected at loci IP_43 and IP_39 with both loci producing 12 alleles each. The lowest number of alleles was detected at loci IP_8 producing only 3 alleles. Two SSR loci, IP_34 and IP_ 37 each showed 63.64% polymorphism among genotypes within species whereas, the loci represented by IP_8 could not show polymorphism within species. IP_39 and IP_21 showed 36.36% polymorphism within species. However, all the 6 SSR loci showed 100% polymorphism when assessed across the eleven species. The PIC of the primers ranged between 0.2223 and 0.874 with an average value of 0.71885. The highest PIC value was found at locus IP_43 whereas, the lowest PIC value was found at locus IP_8 (Table 3).The genetic relationship among the *Ipomoea* plant species was analyzed by constructing dendrogram with allelic data acquired from the 6 genic SSR primer amplifications. The phylogenetic tree of the species/accessions is shown in Figure 1. The resulting dendrogram clearly separated each species and grouped all genotypes of each species together. The dendrogram showed that the species were divided into 3 major and 6 sub clusters, 2 in each group. Group 1 consisted of *I. quamoclit*, *I. carnea*, *I. asarifolia* and *I. involucrata*. Within this group, *I. involucrata*, was separated from the rest 3 as a subgroup. Group 2 included *I. hederifolia*, *I. alba* and *I. nil*. Group 3 comprised of the *I. aquatic*, *I. mauritiana*, *I. cairica* and *I. batatas* species. *I. aquatic* and *I. mauritiana* were separated as a subgroup from *I. cairica* and *I. batatas* within the group 3.

Table 3. Marker Information for all SSR loci

Locus	Sample Size	Number of alleles	Polymorphism within species	Monomorphism within species	% polymorphism within species	Polymorphism across species	PIC
IP_43	88	12.0000	5	6	45.45	100	0.8565
IP_39	88	12.0000	4	7	36.36	100	0.8253
IP_34	88	11.0000	7	4	63.64	100	0.8341
IP_21	88	6.0000	4	7	36.36	100	0.7009
IP_ 37	88	11.0000	7	4	63.64	100	0.874
IP_8	88	3.0000	0	11	0.00	100	0.2223
Mean	**88**	**9.1667**				**100**	**0.71885**

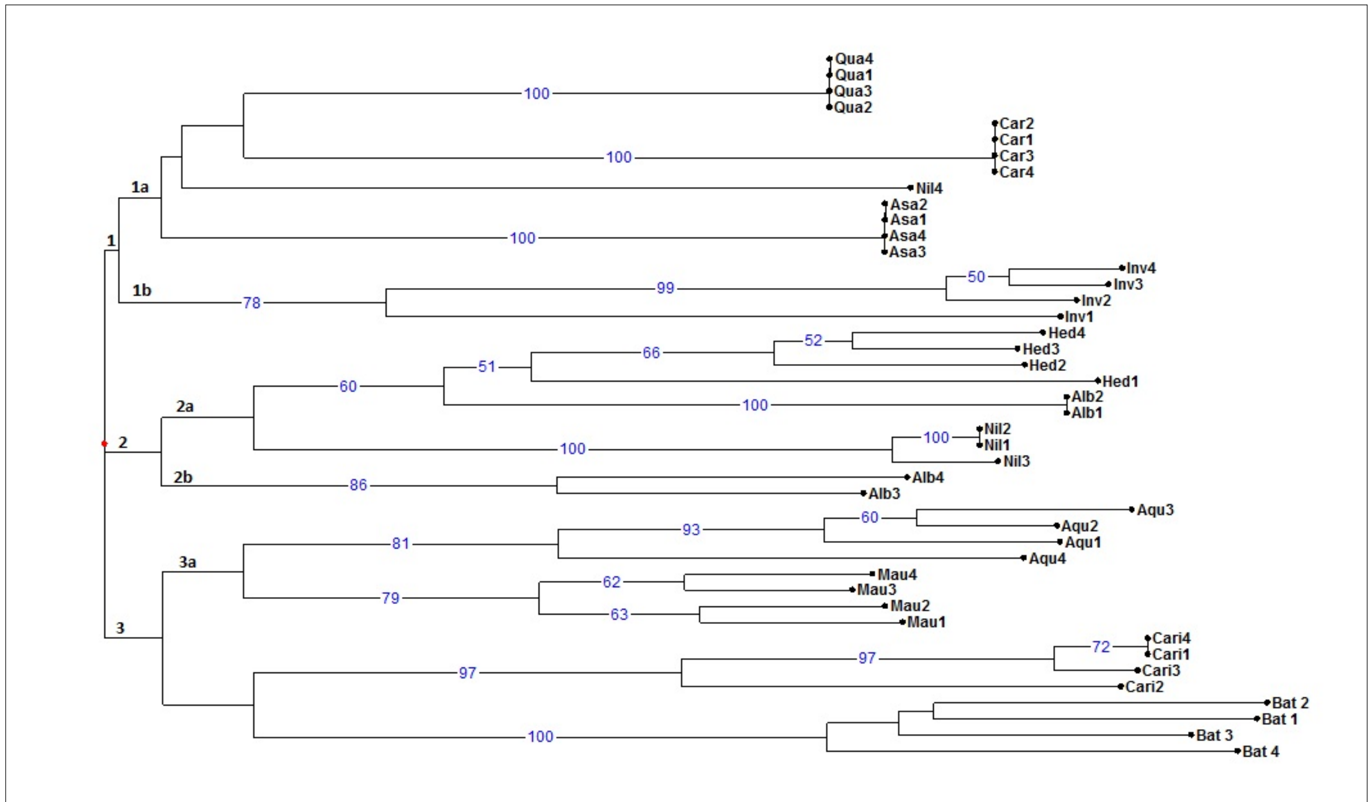

Fig. 1. Dendogram showing genetic diversity of eleven (11) Nigerian species of *Ipomoea*

Discussion

The genic SSR markers developed from *I. cairica* could amplify other species of *Ipomoea*. The amplification of these SSR markers is an indication of the high level of conservation of genic SSR primers to other related species and also lends credence to the validation of the markers. Microsatellites resulting from transcribed regions of the DNA, tend to be more conserved across species and thus show higher transferability rate (19). In addition, the coding regions are evidently more conserved across the genus than the non-coding parts of the genome (20). An average PIC value of 0.71885 was calculated for the primers. As pointed out by (21), PIC shows the discriminating ability of the marker which is dependent on the number and distribution alleles. As reported by (24), markers having a PIC value ≥0.5 are deemed to be highly informative markers whereas, markers having a PIC value range from 0.25 to 0.5 are deemed moderately informative. 5 out of 6 markers showed PIC values above 0.5 thus demonstrating the ability of the markers to ascertain polymorphism and can be useful in different *Ipomoea* species. Cluster analysis derived from the SSR data is an indication that the markers were helpful for evaluation of phylogenetic relationship among *Ipomoea* species with the formation of 3 groups and 2 sub groups within each group. Earlier phylogenetic study using DNA sequences recognized and supported *Ipomoea* as a paraphyletic group (9, 22). (23) proposed a tentative classification, wherein he recognized three subgenera and 13 sections within *Ipomoea* (subgenera *Ipomoea*, *Quamoclit*, *Eriospermum*). *I. quamoclit*, *I. alba* and *I. alba* were however grouped in the subgenus *Quamoclit* although the result of this study using SSR markers placed them in different groups as seen in the dendogram. The results of this study supports that *Ipomoea* is a highly evolutionarily diverged genus but however showed close relationship amongst Nigerian *Ipomoea* species.

Conclusion

This study has demonstrated the usefulness of developed genic markers in analyzing phylogenetic relationship among the taxonomically more related *Ipomoea* species. The developed markers can additional be utilized in other genetic studies regarding the genus as the markers developed from genic SSR are more likely to be conserved and transferable in other species as well.

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Authors contributions

CU and IA conceived the study and participated in its design and coordination. CU carried out the laboratory studies and drafted the manuscript. All authors read and approved the final manuscript.

Compliance with ethical standards

Conflict of interest: Authors do not have any conflict of interests to declare.

Ethical issues: None.

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