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Molecular, morphological and growth studies of three *Annona species*: a phylogenetic approach

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Abstract

Molecular and morphological data have greatly enhanced plant systematics. When integrated with phylogenetic analysis, molecular evidence has revolutionized our understanding of plant evolution and prompted major revisions in taxonomic classifications across all levels. This study assessed the morphological, growth indices and molecular diversity among *Annona muricata*, *Annona squamosa*, and *Annona senegalensis* to determine their genetic relationships and systematic classification. Morphological evaluation covered key vegetative and reproductive characters including leaf length (6.5–20 cm), leaf width (2.5–6.5 cm), petiole length (0.5–2.5 cm), fruit diameter (5–20 cm), and seed size (1.0–1.7 cm long). These traits revealed significant interspecific variability, especially in leaf shape, fruit structure, and seed morphology. For molecular analysis, six Random Amplified Polymorphic DNA (RAPD) and nine Simple Sequence Repeat (SSR) markers were employed to evaluate genome variation. Cross-species amplification of all the SSR markers used, successfully amplified in *A. muricata* and *A. squamosa* but did not in *A. senegalensis*. RAPD markers showed cross species-amplification. Polymorphic Information Content (PIC) value ranged from 0.3457 (OPT05) to 0.5926 (OPH04) for RAPD markers with an average of 0.5103 indicating existence of sufficient genetic diversity among the three species studied genotypes. The PIC for SSR markers were uniform (0.5547). Dendrograms constructed from RAPD and SSR data revealed closer genetic affinity between *A. muricata* and *A. squamosa*, while *A. senegalensis* appeared more divergent. The study emphasizes that combining morphological traits with DNA markers is crucial for understanding genetic diversity in *Annona species*, offering essential insights for their conservation and improvement as underutilized crops.

Keywords *Annona species*, Plant height, Leaf area, SSR markers, Genetic diversity, Phylogenetic tree

1 Introduction

Phylogenetics involves reconstructing the evolutionary history of species using morphological or molecular data, often represented through phylogenetic trees or cladograms [19]. It aids in understanding evolutionary relationships and classifying taxa like species



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or genera. With advancements in genomics, phylogenetics plays a key role in revealing biological processes across plant lineages [32].

Annona species, including *A. squamosa* and *A. muricata*, are notable for their economic and medicinal value. Their fruits are rich in nutrients and bioactive compounds with antioxidant, antidiabetic, and anticancer properties, making them valuable in food and pharmaceutical industries [13, 16] (Fig. 1).

Although *Annona muricata* and *A. squamosa* have been extensively studied due to their commercial value, *A. senegalensis* remains largely uncharacterized at the molecular level. Most existing work on this wild species has focused on phytochemistry rather than genetics. There is limited cross-species validation of molecular markers, as SSRs developed for cultivated species often fail in *A. senegalensis* due to genetic divergence. Additionally, few studies integrate morphological and molecular data for phylogenetic analysis, and genomic resources for wild *Annona* species are scarce. As a result, *A. senegalensis* is underrepresented in breeding and conservation programs despite its potential (Table 1 and 2).

The influence of molecular data on plant systematics is profound and transformative. When combined with rigorous phylogenetic analysis methods, molecular evidence has significantly altered our understanding of evolutionary relationships and the delineation of taxa across all levels of the taxonomic hierarchy [7]. Phylogenetic trees now serve as a foundation not only for classification but also for investigating character evolution [41], hybridization [18, 26], polyploidy [9, 10], biogeography domestication processes [29], and the mechanisms driving speciation and species diversification [3, 4]. Molecular data have significantly advanced the systematics of *Annona* and related genera in the Annonaceae family by providing clearer phylogenetic relationships than morphological traits alone. For instance, studies utilizing chloroplast DNA sequences (such as *matK*, *rbcL*, *trnL-F*) and nuclear markers (e.g., *ITS*) have helped resolve species complexes and redefine interspecific relationships within *Annona*. Chatrou [6], employed plastid markers to reconstruct the phylogeny of Annonaceae, leading to a better-supported tribal classification (Fig. 2).

Phytomorphology is the study of the physical form and external structure of plants [40]. This is usually considered distinct from plant anatomy [40], which is the study of the internal structure of plants, especially at the microscopic level [12]. Recent studies in molecular biology started to investigate the molecular processes involved in determining the conservation and diversification of plant morphologies. In these studies

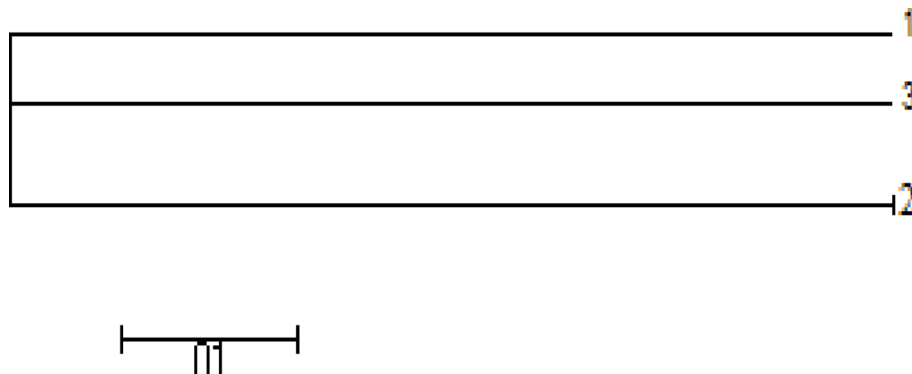


Fig. 1 Phylogenetic tree of the samples for SSR markers. 1- *A. muricata*, 2- *A. senegalensis*, 3- *A. squamosa*

Table 1 SSR primers**Specification of Oligonucleotides**

Name	Scale	Purification	5' Modification	Sequence	3' Modification	Bases
LMCH 4 F	0.01 (utnole)	Desalting	None	ATTAGAACAAGGACGAGAAT	None	20
LMCH 4 R	0.01 (utnole)	Desalting	None	CCTGTGTCTTTCATGGAC	None	18
LMCH 5 F	0.01 (utnole)	Desalting	None	CCCACTCTTCTACCTCAAC	None	20
LMCH 5 R	0.01 (utnole)	Desalting	None	CAAGTCCCTGTAAGAATCAGA	None	21
LMCH 29 P	0.01 (utnole)	Desalting	None	GTACCATCTTTTAGGAAATC	None	20
LMCH 29 R	0.01 (utnole)	Desalting	None	TGCAATCTATGTTAGTCAC	None	19
LMCH 33 P	0.01 (utnole)	Desalting	None	AAGAAATGGGAGTAAATAGTG	None	21
LMCH 33 R	0.01 (utnole)	Desalting	None	ACGGTTGTGAATAGTTGAGT	None	20
GM-S 3 F	0.01 (utnole)	Desalting	None	GCACTAGCACACGATTTGAT	None	20
GM-S 3 R	0.01 (utnole)	Desalting	None	CCCTCCTCTTCTTACTTC	None	20
GM-S 1 F	0.01 (utnole)	Desalting	None	CAGCCTCAGGACCTTCTACC	None	20
GM-S 1 R	0.01 (utnole)	Desalting	None	CTTCTCTCACCGAACAC	None	20
GM-S 2 F	0.01 (utnole)	Desalting	None	GGCAACTCTAGCGTTTTCTC	None	20
GM-S 2 R	0.01 (utnole)	Desalting	None	GTGTAATCACAGCAGGGAAGT	None	21
GM-S 4 F	0.01 (utnole)	Desalting	None	AGTGATTTAGGG CTG AGGTG	None	20
GM-S 4 R	0.01 (utnole)	Desalting	None	GCCAGTGGATGAAAGATG	None	18
GM-S 5 F	0.01 (utnole)	Desalting	None	CTATCACTGCACAGTGGCTT	None	20
GM-S 5 R	0.01 (utnole)	Desalting	None	ACACCCTTCTCCTTCAAATG	None	20

Table 2 RAPD Primers

OPT – 05	– 5 ¹ – GGG	TTT	GGC	A – 3 ¹
OPH – 01	– 5 ¹ – GGT	CGG	ACA	A – 3 ¹
OPH – 06	– 5 ¹ – ACG	CAT	CGC	A – 3 ¹
OPT – 06	– 5 ¹ – CAA	GGG	CAG	A – 3 ¹
OPT – 07	– 5 ¹ – GGC	AGG	CTG	T – 3 ¹
OPH – 04	– 5 ¹ – GGA	AGT	CGC	C – 3 ¹

transcriptome conservation patterns were found to mark crucial ontogenetic transitions during the plant life cycle which may result in evolutionary constraints limiting diversification [11]. Morphological characterization is a fundamental approach for analyzing plant diversity and grouping species [14, 37]. It has historically been the basis for identifying plant species and inferring their taxonomic positions [25, 40]. Furthermore, plant morphology is crucial for detecting desirable traits, identifying duplicate accessions, and organizing populations for conservation purposes [39, 42, 45]. However, this method is

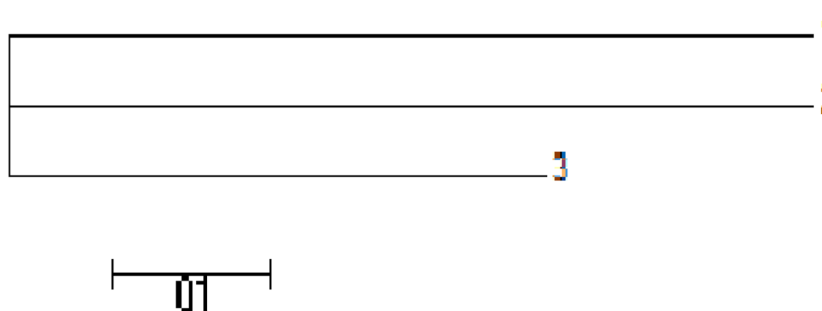


Image showing phylogenetic relationship among the samples

Fig. 2 Phylogenetic tree of the samples for RAPD markers. 1- *A. muricata*, 2- *A. senegalensis*, 3- *A. squamosa*

time-consuming, as it often requires the observation of a plant's full life cycle, including flowering and fruiting stages [14,]. In contrast, vegetative morphology—available year-round—offers a practical and significant source of key distinguishing characters for taxonomic studies (Table 3 and 4).

Traditionally, morphological traits have been employed to characterize plant germplasm for selecting high-yielding genotypes with superior fruit quality [13]. However, morphological markers are highly influenced by environmental factors such as soil and climate, limiting their reliability [22]. In contrast, molecular markers provide a more stable and precise approach to evaluating genetic diversity (Figs. 3 and 4).

Molecular systematics, which classifies organisms by analyzing differences in DNA and protein sequences, plays a crucial role in clarifying intricate taxonomic relationships that cannot be resolved through morphology alone [41, 43, 47]. Molecular systematics uses variations in protein and DNA molecules to determine how similar or dissimilar sets of organisms are. These molecular differences provide a much more accurate taxonomic picture. The real power of molecular systematics is that it allows the examination of how species have changed over evolutionary time, as well as of the relationships between species that have no common physical characteristics. Molecular changes can be used to explore phylogenetics. It has been suggested that the amount of change that takes place in DNA over time can act as a molecular clock, gauging how much evolutionary time has passed. The clock is set by first examining geological and historical records to determine how long two species have been physically separated (Figs. 5 and 6).

2 Materials and methods

2.1 Identification, collection, germination, and seedling establishment

The study involved *Annona species* collected from Uturu and Umuahia, both located in Abia State, Nigeria. Taxonomic identification of the specimens was carried out at the Department of Plant Science and Biotechnology, Michael Okpara University of Agriculture, Umudike. Seeds were extracted from freshly harvested fruits and sown without pre-treatment for dormancy, directly into polypots filled with sandy loam soil. Germination parameters were assessed by monitoring 20 seeds per species daily. Specific observations included the number of days to first leaf emergence [21], cotyledon abscission [20], and total germination percentage after 30 days (Fig. 7).

Following germination, seedlings were transplanted into new polypots (6 × 9 cm) filled with loamy soil from the same collection site. Watering was carried out at three-day

Table 3 Important folia morphological characters

<i>Annona species</i>	Plant habit	Leaf Shape	Leaf margin	Leaf base	Leaf surface	Leaf apex	Stipule	Peti- ole length	Leaf length	Leaf width	Leaf arrangement	Inter- node length	Venation	phyllotaxy	Vein number	Growth pattern
<i>Annona squamosa</i>	Tree	Lanceolate	Entire	Obtuse	Glabrose	Acuminate	Absent	0.5– 1.4 cm	5.5– 12.9 cm	3.0– 4.7 cm	Alternate	1.7 cm	Eucomptodro- mous	Distichous	12	Sympo- dial
<i>Annona muricata</i>	Tree	Obovate	Entire	Acute	Glabrose	Mucronate	Absent	0.3– 1.1 cm	4– 16.3 cm	2.3– 6.0 cm	Alternate	2.9 cm	Eucomptodro- mous	Distichous	11	Sympo- dial
<i>Annona senegalensis</i>	Tree	Elliptical	Entire	Round- ed	Glabrose	Obtuse	Absent	0.2– 1.4 cm	3.5– 17.5 cm	1.5– 9.3 cm	Alternate	6 cm	Eucomptodro- mous	Distichous	10	Mono- podial

intervals as recommended by standard nursery management protocols [1]. Growth performance were evaluated using a 3 × 6 Randomized Complete Block Design (RCBD), with data collection occurring biweekly [27]. Growth parameters included plant height (cm), stem girth (measured with a vernier caliper), number of leaves, and leaf area determined by tracing leaf outlines on graph paper [28, 33].

2.2 Morphological characterization

After one year in the nursery, leaf morphology was assessed across the three *Annona* species. Observations included leaf apex type (acuminate, mucronate, obtuse, etc.), base shape, overall leaf form (elliptical, lanceolate, or obovate), and venation pattern (notably eucamptodromous in all species). The number of visible veins were counted, and petiole length were recorded. Nodal length and diameter were measured, while abaxial and adaxial surfaces were examined for texture (glabrous or hairy). Leaf width was measured at the widest point using a standard metric ruler. These morphological features were used for species comparison and to identify diagnostic characters distinguishing one taxon from another, following standard botanical literature guidelines (Fig. 8).

2.3 Genomic DNA isolation and quality assessment

Genomic DNA was extracted from young, healthy foliage using the Cetyltrimethyl Ammonium Bromide (CTAB) method as described by Doyle and Doyle [38]. Approximately 2–3 leaves per sample were ground in extraction buffer, incubated at 65 °C for 45 min, and processed through chloroform and isopropanol precipitation. The resulting DNA pellets were washed with 70% ethanol, dried, and stored in TE buffer with RNase to eliminate RNA contamination. DNA concentration and purity were assessed using a spectrophotometer at absorbance values of 260 nm and 280 nm. Additional quality confirmation was done via 1.5% agarose gel electrophoresis stained with ethidium bromide and visualized under UV light using the Kodak EDAS 290 system.

2.4 Molecular marker analysis and data interpretation

PCR amplification was conducted using nine Simple Sequence Repeat (SSR) primers and six Random Amplified Polymorphic DNA (RAPD) primers. The 15 µl reaction mixture included 20 ng of template DNA, PCR buffer, dNTPs, MgCl₂, primers, Taq polymerase, and nuclease-free water. Amplification was executed on a thermal cycler under a modified protocol of initial denaturation at 92 °C, followed by 44 cycles of denaturation, annealing, and extension, and a final hold at 15 °C.

Amplified products were resolved on 1.5% agarose gels in TAE buffer and stained with ethidium bromide. Bands were scored manually as present (1) or absent (0) to develop a binary matrix. The Polymorphic Information Content (PIC) was calculated for each primer using the formula $PIC = 1 - \sum P_i^2$, where P_i is the frequency of each band. Cluster analysis and principal component analysis (PCA) were performed using NTSYS-pc version 2.01i and Darwin version 6, while genetic distance matrices and dendrograms were generated using UPGMA algorithms via PowerMarker software version 3.25 [26, 35].

Table 4 Summary of the important floral and fruit morphological characters

Annona species	Pedicel surface	Pedicel colour	Pedicel length	Sepal surface	Sepal length	Petal colour	Petal length	Petal width	Num-ber of petal	Num-ber of sepal	Flower	Stamen	Fruit	Fruit length	Fruit breadth	Fruit shape	Fruit colour	Fruit stalk	Seed length	Seed breadth	Seed number	Ger-mination %
<i>Annona squamosa</i>	Pubescent	Greenish yellow	1.6 cm	Pubescent	0.2 cm	Greenish yellow	2.3 cm	0.7 cm	6	3	Chasmogamous	Many	Syncarpus	7.3	6.9	Oval	Yellowish Green	1.5	1.4	0.6	73	80
<i>Annona nutritica</i>	glabrous	Greenish brown	2.1 cm	Glabrous	0.3 cm	Deep yellow	2.7	2.9	6	3	Chasmogamous	Many	Syncarpus	15.4	10.3	Oval	Deep green	2.2	1.5	0.8	52	85
<i>Annona senegalensis</i>	Pubescent	Greenish brown	0.6 cm	Pubescent	0.3 cm	Deep yellow	1.1	0.9	6	5	Chasmogamous	Many	Syncarpus	4.1	4.5	Oblong	Yellow	2.2	1.0	0.7	39	70

3 Results

3.1 Morphological characters of the plants under study

Comparative foliar morphological characterization of three *Annona* species (*A. squamosa*, *A. muricata*, and *A. senegalensis*) revealed distinct and shared features that are taxonomically significant. All three species are trees, indicating a perennial woody growth form across the genus. Leaf shape varied among species, *A. squamosa* had lanceolate leaves, *A. muricata* displayed obovate leaves, and *A. senegalensis* showed elliptical leaves. These shape differences can aid in species differentiation. All species had entire (smooth) margins, suggesting uniformity in this trait. *A. squamosa* had an obtuse base and acuminate apex, *A. muricata* had an acute base and mucronate apex, *A. senegalensis* exhibited a rounded base and obtuse apex. These distinctions emphasize interspecific differences in leaf form. All species had a glabrous (smooth, non-hairy) surface. Absent in all three species, indicating a common trait within the genus. *A. senegalensis* showed the widest range (0.2–1.4 cm), *A. squamosa* ranged from 0.5–1.4 cm, *A. muricata* from 0.3–1.1 cm. These values reflect differences in leaf attachment flexibility. *A. senegalensis* had the longest leaves (3.5–17.5 cm) and widest leaves (1.5–9.3 cm), *A. muricata* had moderate size leaves (4–16.3 cm length, 2.3–6.0 cm width), *A. squamosa* had the shortest and narrowest leaves (5.5–12.9 cm length, 3.0–4.7 cm width). All three species exhibited an alternate leaf arrangement. *A. senegalensis* had the longest internode (6 cm), indicating wider leaf spacing, *A. muricata* had a moderate length (2.9 cm), *A. squamosa* had the shortest internode (1.7 cm). All species exhibited eucamptodromous venation and distichous phyllotaxy, which are consistent patterns across the genus. *A. squamosa* had 12 veins, *A. muricata* had 11, *A. senegalensis* had 10, indicating slight variations in vascular complexity. *A. squamosa* and *A. muricata* showed sympodial growth, *A. senegalensis* exhibited monopodial growth, marking a clear difference in branching architecture.

Significant variations were observed in the floral and fruit characteristics among the three *Annona* species studied. All three species produced chasmogamous flowers with six petals, but they differed in sepal number, petal size, pedicel length, and surface texture. *Annona muricata* exhibited cauliflorous or ramiferous inflorescences, with solitary or fascicled flowers. The pedicel was glabrous and measured approximately 2.1 cm in length. Each flower had three sepals (~0.3 cm, glabrous) and six deep yellow petals measuring 2.7 cm long and 2.9 cm wide. *Annona squamosa* bore flowers singly or in pairs in the axils of young shoots. The pedicel was pubescent and 1.6 cm long, with three sepals (~0.2 cm) and six petals that were greenish yellow, measuring 2.3 cm long and 0.7 cm wide. The petals fused in three pairs and were greenish-white internally. *Annona senegalensis* displayed paired flowers in the axils of young shoots. The pedicel was also pubescent but shorter at 0.6 cm. It had five sepals (~0.3 cm, pubescent) and six deep yellow petals, each about 1.1 cm long and 0.9 cm wide. These morphological features—including pedicel surface and length, sepal number, and petal color and dimensions—serve as reliable diagnostic characters for distinguishing species within the *Annona* genus.

The fruit characteristics also varied significantly across the species. *Annona muricata* produced large, syncarpous fruits that were deep green, measuring approximately 15.4 cm in length and 10.3 cm in breadth, with a short fruit stalk (~2.2 cm). The seeds were 1.5 cm long, 0.8 cm wide, with an average of 52 seeds per fruit, and a germination rate of 85%. *Annona squamosa* had oval, yellowish fruits, about 7.3 cm long and 6.9 cm



Fig. 3 Fruit and leaves [**A***Annona squamosa* (sugar apple), **B***Annona muricata* (soursop) and **C***Annona senegalensis*, (wildsop)]

wide, with a green fruit stalk and seed dimensions of 1.4 cm × 0.6 cm. The average seed number was 73, and germination percentage was 80%. *Annona senegalensis* bore oblong, yellow fruits, smaller in size at 4.1 cm length and 4.5 cm breadth, with 2.2 cm long fruit stalks. Seeds measured 1.0 cm × 0.7 cm, with a lower seed count (39 seeds) and 70% germination rate.

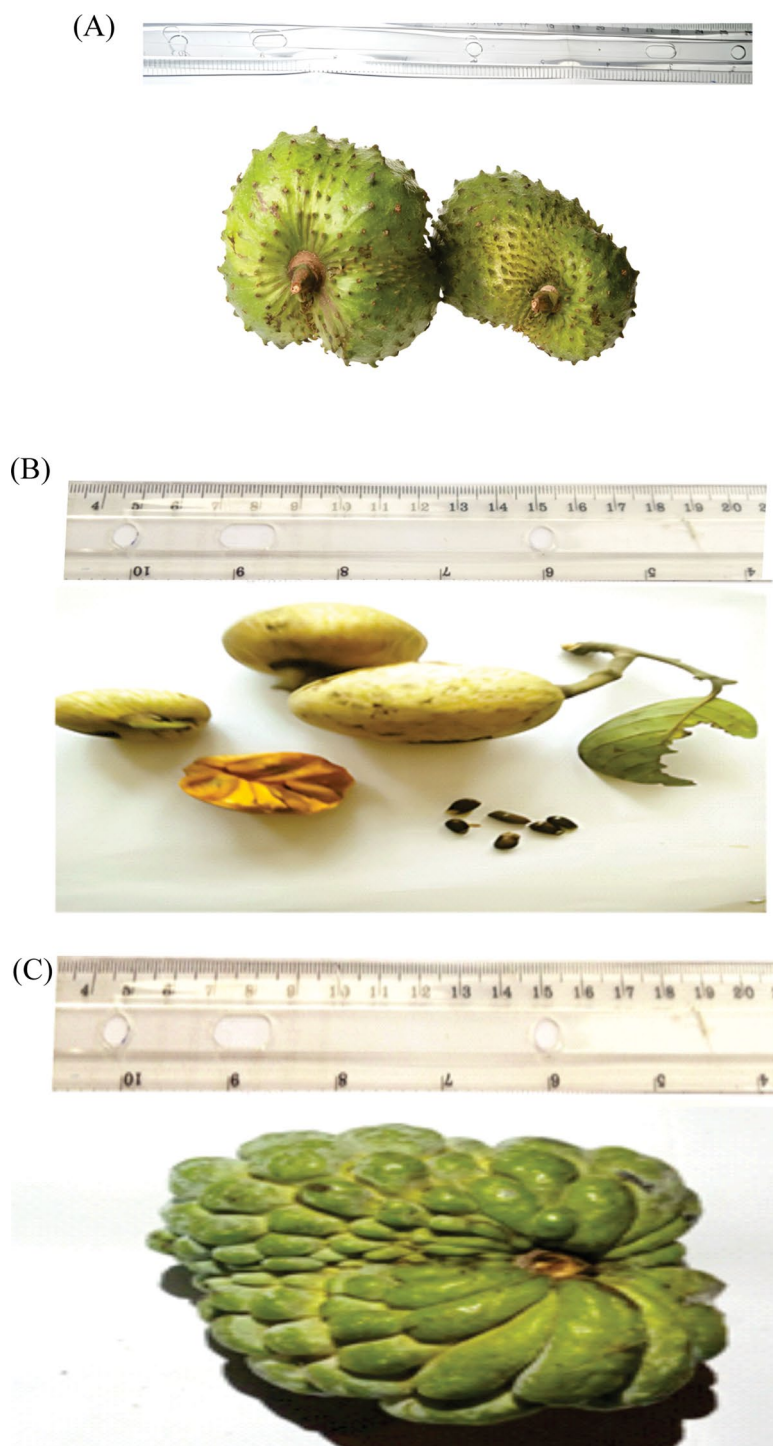


Fig. 4 Fruit [A *Annona muricata* (soursop), B *Annona senegalensis* (wildsop) and C *Annona squamosa*, (sugar apple)]

4 Growth parameters of the three species under study

Growth parameters of *Annona muricata*, *A. senegalensis*, and *A. squamosa* were evaluated from 2 to 48 weeks after planting (WAP). All traits examined differed significantly among species ($p < 0.05$).

Leaf surface expansion was species dependent and varied over time (Table 5). In the earliest measurements (2–4 WAP), *A. squamosa* exhibited the largest lamina surfaces,



Fig. 5 C Seeds [A is *Annona muricata* (soursop), B: *Annona senegalensis* (wildsop) and C: *Annona squamosa*, (sugar apple)]

while *A. senegalensis* and *A. muricata* produced relatively smaller leaves. This early advantage of *A. squamosa* did not persist. From 6 WAP onward, *A. senegalensis* accelerated leaf enlargement, surpassing its congeners and maintaining the highest values for the remainder of the trial. By contrast, *A. squamosa* displayed a plateau in expansion after 22 WAP, while *A. muricata* followed an intermediate trajectory, continuing gradual enlargement but never reaching the dimensions achieved by *A. senegalensis*.

Leaf production increased throughout the growth period but with distinct temporal patterns (Table 6). *A. squamosa* generated the highest leaf counts during the early vegetative phase (up to 14 WAP), reflecting its rapid juvenile vigor. Thereafter, production

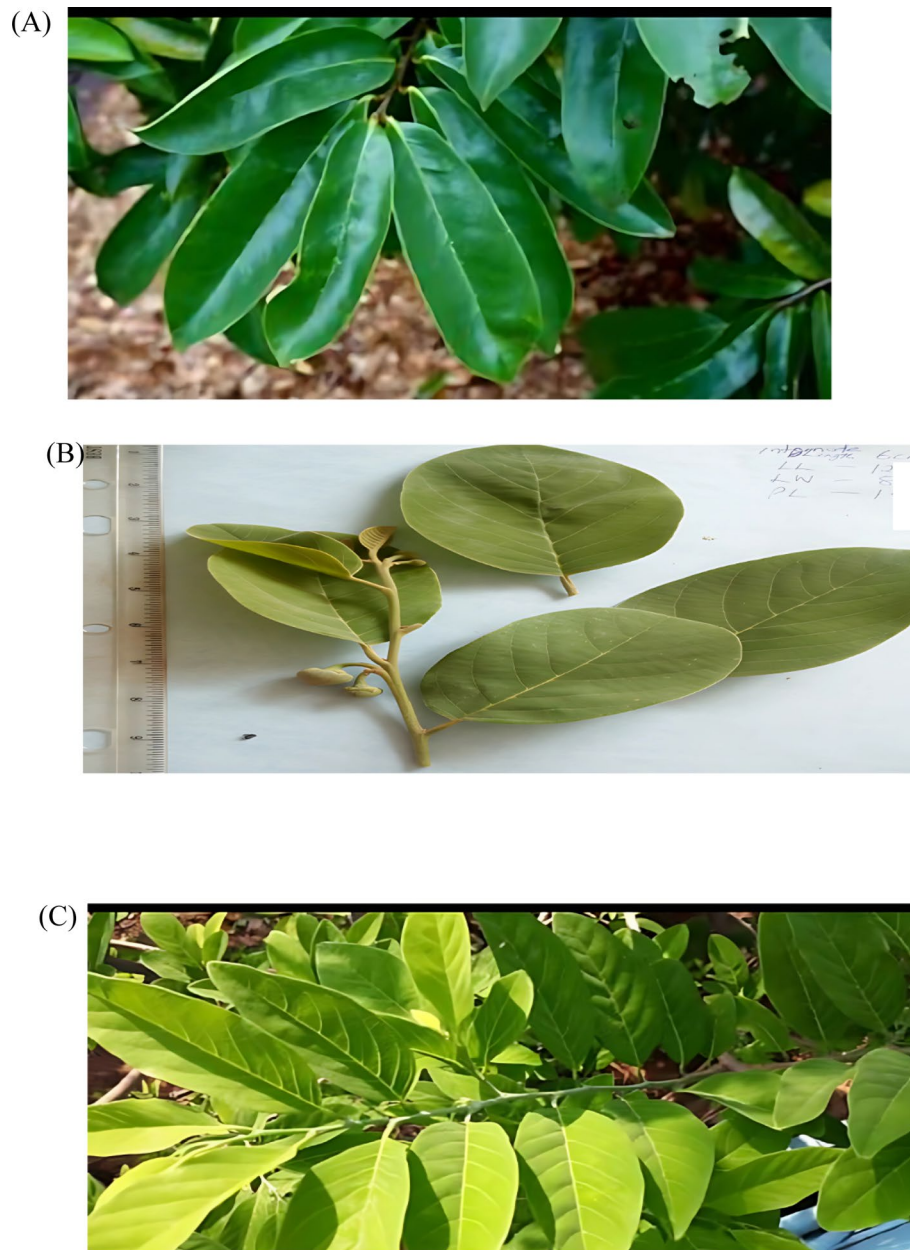


Fig. 6 Leaves [**A***Annona muricata* (soursop), **B***Annona senegalensis* (wildsop) and **C***Annona squamosa*, (sugar apple)]

slowed, and total leaf number stabilized from about 22 WAP. In contrast, both *A. muricata* and *A. senegalensis* continued producing new leaves throughout the experiment, ultimately exceeding *A. squamosa* by 48 WAP. Thus, *A. squamosa* adopted an early but short-lived leaf proliferation strategy, while the other species sustained leaf initiation over a longer duration.

Petiole elongation also revealed consistent differences (Table 7). *A. squamosa* had the longest petioles across nearly all measurement intervals, followed closely by *A. senegalensis*. *A. muricata* remained distinct with markedly shorter petioles at every stage, reaching a maximum that was approximately one-third lower than the other species. These results indicate contrasting strategies of lamina display: *A. squamosa* and *A.*

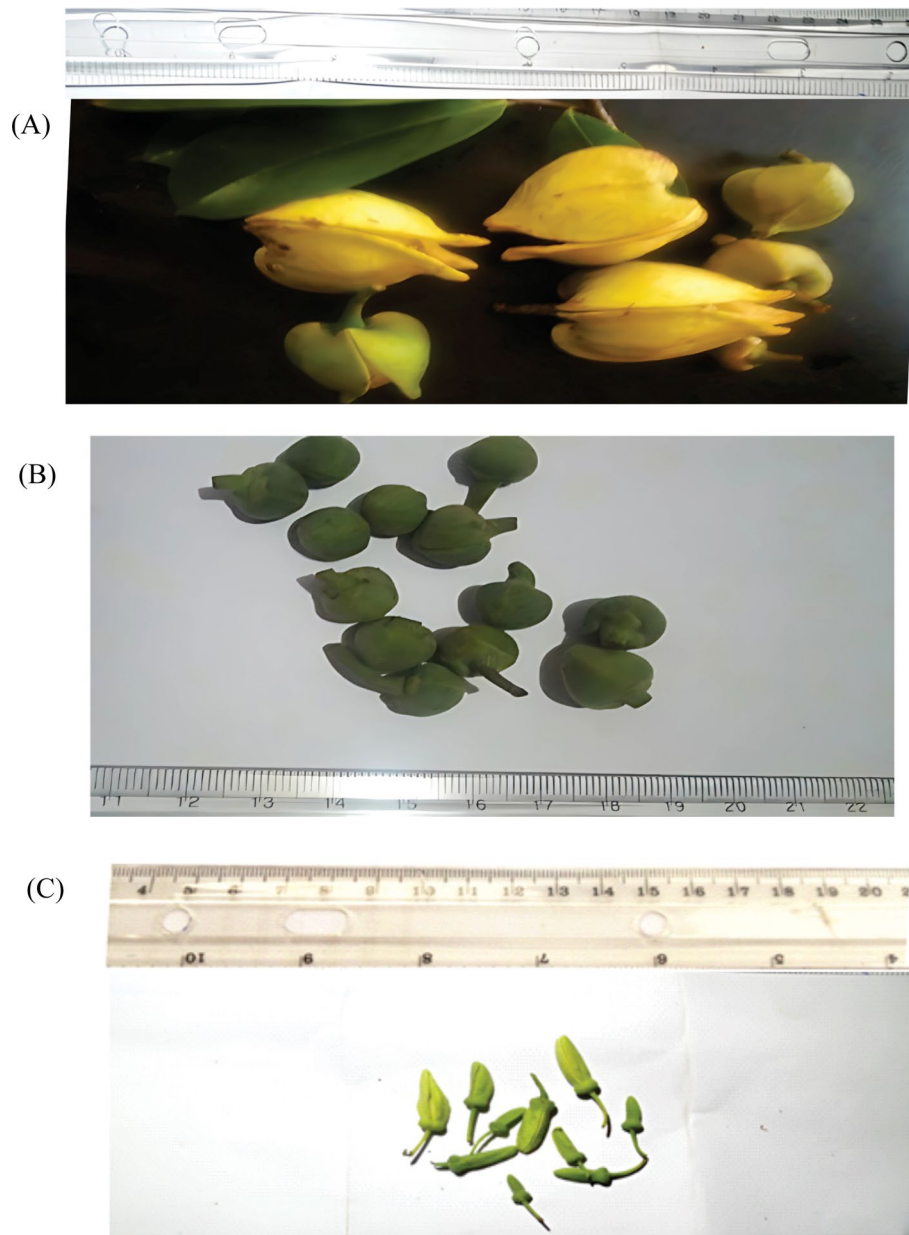
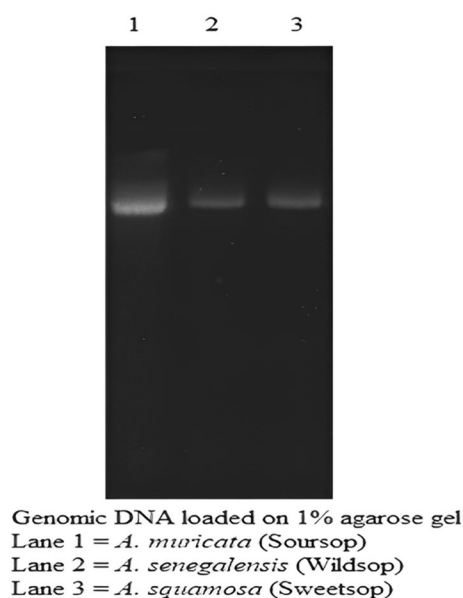


Fig. 7 Flowers [A: *Annona muricata* (sour sop), B: *Annona senegalensis* (wild sop) and C: *Annona squamosa*, (sugar apple)]

senegalensis invested in petiole elongation, likely enhancing leaf positioning for light capture, whereas *A. muricata* maintained a compact architecture.

Plant height progressed steadily across species but with different intensities (Table 8). During the first ten weeks, *A. squamosa* plants were tallest, with *A. muricata* slightly shorter and *A. senegalensis* smallest. This pattern reversed later in development. From 30 WAP onward, *A. muricata* accelerated in height growth and became the tallest species at maturity, reaching nearly twice the final height of *A. squamosa*. *A. senegalensis* displayed moderate elongation, ending taller than *A. squamosa* but shorter than *A. muricata*. These observations suggest that *A. squamosa* emphasizes early vertical growth, while *A. muricata* invests in sustained stem elongation that ultimately produces greater stature.

**Fig. 8** Genomic DNA loaded on 1% agarose gel**Table 5** Leaf area (cm²) at weeks after planting WAP

Species	2 WAP	4 WAP	6 WAP	8 WAP	12 WAP	24 WAP	36 WAP	48 WAP
<i>A. muricata</i>	5.7±0.4	6.2±0.3	8.5±0.6	11.0±1.0	13.4±0.7	18.0±0.9	35.4±1.2	60.2±1.4
<i>A. senegalensis</i>	3.3±0.4	5.5±0.3	7.7±0.6	11.5±1.0	20.5±0.7	57.5±0.9	90.0±1.2	96.6±1.4
<i>A. squamosa</i>	11.6±0.4	12.2±0.3	14.1±0.6	16.4±1.0	19.1±0.7	29.3±0.9	33.7±1.2	36.6±1.4
Overall Mean	6.9±0.4	8.0±0.3	10.1±0.6	13.0±1.0	17.7±0.7	34.9±0.9	53.0±1.2	64.5±1.4

Table 6 Number of leaves at weeks after planting (WAP)

Species	2 WAP	4 WAP	6 WAP	8 WAP	14 WAP	20 WAP	30 WAP	40 WAP	48 WAP
<i>A. muricata</i>	3.0±0.0	4.0±0.0	7.0±0.3	9.3±0.8	14.7±0.2	17.7±1.0	19.7±0.6	28.3±0.8	33.7±1.6
<i>A. senegalensis</i>	4.0±0.0	6.0±0.0	8.3±0.3	10.3±0.8	16.3±0.2	18.7±1.0	22.3±0.6	29.7±0.8	32.3±1.6
<i>A. squamosa</i>	6.0±0.0	6.0±0.0	6.7±0.3	10.7±0.8	20.7±0.2	24.7±1.0	18.0±0.6	25.3±0.8	31.3±1.6
Mean	4.3±0.0	5.3±0.0	7.3±0.3	10.1±0.8	17.2±0.2	20.3±1.0	20.0±0.6	27.8±0.8	32.4±1.6

Table 7 Petiole length (cm) at selected weeks after planting (WAP)

Species	2 WAP	4 WAP	6 WAP	8 WAP	14 WAP	20 WAP	30 WAP	40 WAP	48 WAP
<i>A. muricata</i>	0.3±0.04	0.3±0.02	0.4±0.02	0.4±0.0	0.5±0.0	0.6±0.04	0.8±0.0	1.0±0.0	1.0±0.0
<i>A. senegalensis</i>	0.2±0.04	0.4±0.02	0.4±0.02	0.5±0.0	0.8±0.0	1.0±0.04	1.2±0.0	1.4±0.0	1.4±0.0
<i>A. squamosa</i>	0.6±0.04	0.8±0.02	0.8±0.02	0.8±0.0	0.8±0.0	1.0±0.04	1.2±0.0	1.2±0.0	1.4±0.0
Mean	0.4±0.04	0.5±0.02	0.5±0.02	0.6±0.0	0.7±0.0	0.9±0.04	1.1±0.0	1.2±0.0	1.3±0.0

Table 8 Plant height (cm) at weeks after planting (WAP)

Species	2 WAP	4 WAP	6 WAP	8 WAP	14 WAP	20 WAP	30 WAP	40 WAP	48 WAP
<i>A. muricata</i>	13.0±0.1	14.0±0.3	18.8±0.5	21.2±0.7	26.3±0.3	29.0±3.0	38.4±0.5	75.0±0.9	94.8±0.5
<i>A. senegalensis</i>	8.3±0.1	11.5±0.3	13.5±0.5	15.2±0.7	22.7±0.3	34.9±3.0	58.9±0.5	76.9±0.9	80.9±0.5
<i>A. squamosa</i>	14.2±0.1	15.2±0.3	17.5±0.5	20.2±0.7	28.5±0.3	34.1±3.0	47.4±0.5	49.7±0.9	53.2±0.5
Mean	11.8±0.1	13.6±0.3	16.6±0.5	18.8±0.7	25.8±0.3	32.7±3.0	48.2±0.5	67.2±0.9	76.3±0.5

Table 9 Stem girth (cm) at weeks after planting (WAP)

Species	2 WAP	4 WAP	6 WAP	8 WAP	14 WAP	20 WAP	30 WAP	40 WAP	48 WAP
<i>A. muricata</i>	1.2±0.1	1.5±0.0	1.6±0.1	1.3±0.0	1.4±0.0	1.6±0.1	2.0±0.0	2.9±0.0	3.3±0.1
<i>A. senegalensis</i>	0.2±0.1	0.3±0.0	0.3±0.1	0.4±0.0	0.7±0.0	2.3±0.1	2.6±0.0	2.9±0.0	3.0±0.1
<i>A. squamosa</i>	0.7±0.1	0.8±0.0	1.0±0.1	1.2±0.0	1.7±0.0	1.9±0.1	2.4±0.0	2.8±0.0	2.9±0.1
Mean	0.7±0.1	0.9±0.0	1.0±0.1	1.0±0.0	1.3±0.0	1.9±0.1	2.3±0.0	2.8±0.0	3.1±0.1

Table 10 Leaf length (cm) at weeks after planting (WAP)

Species	2 WAP	4 WAP	6 WAP	8 WAP	14 WAP	20 WAP	30 WAP	40 WAP	48 WAP
<i>A. muricata</i>	4.0±0.2	4.5±0.1	5.4±0.4	6.3±0.5	6.7±0.3	0.2±0.2	10.0±0.1	14.4±0.3	16.0±0.2
<i>A. senegalensis</i>	3.5±0.2	4.5±0.1	5.3±0.4	6.2±0.5	7.9±0.3	10.2±0.2	14.8±0.1	17.4±0.3	17.4±0.2
<i>A. squamosa</i>	6.1±0.2	6.2±0.1	6.9±0.4	8.0±0.5	9.8±0.3	11.8±0.2	11.9±0.1	12.4±0.3	13.0±0.2
Mean	4.6±0.2	5.1±0.1	5.9±0.4	6.8±0.5	8.1±0.3	9.7±0.2	12.2±0.1	14.7±0.3	15.5±0.2

Table 11 Leaf width (cm) at weeks after planting (WAP)

Species	2 WAP	4 WAP	6 WAP	8 WAP	14 WAP	20 WAP	30 WAP	40 WAP	48 WAP
<i>A. muricata</i>	2.3±0.0	2.3±0.1	2.6±0.1	2.9±0.0	3.5±0.2	3.5±0.2	4.4±0.2	5.5±0.1	6.2±0.1
<i>A. senegalensis</i>	1.5±0.0	2.0±0.1	2.4±0.1	3.1±0.0	5.7±0.2	6.7±0.2	8.3±0.2	9.2±0.1	9.2±0.1
<i>A. squamosa</i>	3.1±0.0	3.2±0.1	3.4±0.1	3.4±0.0	3.7±0.2	3.9±0.2	4.2±0.2	4.5±0.1	4.6±0.1
Mean	2.3±0.0	2.5±0.1	2.9±0.1	3.1±0.0	4.3±0.2	4.7±0.2	5.6±0.2	6.4±0.1	6.7±0.1

Stem girth showed progressive increases in all species, but the sequence of advantage shifted over time (Table 9). Initially, *A. muricata* possessed the thickest stems, suggesting early allocation to supportive tissues. However, beginning around 18 WAP, *A. senegalensis* displayed accelerated secondary growth and, by the final assessment, matched the girth of *A. muricata*. *A. squamosa* thickened more slowly and consistently, reaching smaller diameters than the other species at maturity. These findings point to differing strategies of structural reinforcement: early investment in *A. muricata*, delayed but vigorous investment in *A. senegalensis*, and conservative, moderate investment in *A. squamosa*.

Differences in lamina elongation (leaf length) were clear across species (Table 10). In early development, *A. squamosa* produced the longest leaves, but its elongation rate slowed after 20–24 WAP, resulting in relatively short final laminae. *A. senegalensis*, in contrast, maintained a steady elongation trend throughout the growing period, ultimately producing the longest leaves by 48 WAP. *A. muricata* displayed intermediate values, with steady elongation leading to final lengths shorter than *A. senegalensis* but longer than *A. squamosa*. This indicates contrasting developmental schedules: rapid but short-lived elongation in *A. squamosa*, continuous elongation in *A. senegalensis*, and balanced growth in *A. muricata*.

Patterns of lamina broadening (leaf width) mirrored those of length (Table 11). *A. squamosa* had the widest leaves during the first few weeks, but its lamina width stabilized early and showed little further increase. *A. senegalensis* demonstrated vigorous and sustained broadening, surpassing the other species from mid-growth stages and maintaining the largest widths through to 48 WAP. *A. muricata* increased moderately, producing leaves of intermediate breadth. Together with leaf length, these results indicate that *A. senegalensis* exhibits the greatest potential for lamina expansion, while *A. squamosa* restricts development after early establishment.

4.1 Taxonomic key for three *Annona* Species

- 1a. Leaf shape obovate; leaf apex mucronate or cuspidate; pedicel glabrous; fruit deep green, large (15.4 cm long); flower with 3 sepals; petals deep yellow, 2.7 cm long
→ *Annona muricata*
- 1b. Leaf shape not obovate (lanceolate or elliptical); different leaf apex and pedicel characteristics—go to 2.
- 2a. Leaf shape lanceolate; leaf apex acuminate; pedicel pubescent; petals greenish yellow, 2.3 cm long; fruit oval and yellowish; 3 sepals present
→ *Annona squamosa*
- 2b. Leaf shape elliptical; leaf apex obtuse to rounded; pedicel pubescent; petals deep yellow, 1.1 cm long; fruit oblong and yellow; 5 sepals present — → *Annona senegalensis*.

5 Genomic and molecular studies

The genetic diversity of the studied *Annona species* was evaluated using nine molecular markers (GMS1–GMS5 and LMCH4, LMCH5, LMCH29, LMCH33) (Table 12). Each marker exhibited consistent values across the measured parameters, indicating uniform marker performance and genetic diversity across the sample. The major allele frequency for all markers was 0.5000, suggesting equal representation of dominant and alternative alleles at each locus. The sample size and number of observations were both 4.0000, reflecting the analysis of four individuals across all loci. Each marker revealed 3 alleles (AlleleNo=3.0000), indicating a moderate level of allelic richness per locus. Marker availability was complete (1.0000) across all samples, confirming successful amplification and scoring. The gene diversity (expected heterozygosity) was 0.6250 for each marker, indicating a relatively high level of genetic variability within the population. The Polymorphic Information Content (PIC) values were also consistent at 0.5547, showing that all markers were moderately informative and suitable for distinguishing among genotypes. The mean values for all parameters matched those of individual markers, reinforcing the consistency and reliability of the dataset. All markers demonstrated high consistency in allele frequency and polymorphism. The observed gene diversity (0.6250) and PIC values (0.5547) confirm that these markers are effective for assessing moderate to high genetic variation. These results underscore the utility of the selected markers for genetic diversity studies in *Annona species*, supporting their use in population structure, genetic mapping, or breeding program applications.

The genetic diversity of *Annona species* was further assessed using six RAPD markers (OPT05, OPH01, OPH06, OPT06, OPT07, and OPH04). The analysis revealed varying

Table 12 Genetic diversity and polymorphic information content (PIC) of SSR markers

Marker	Major.Alele.Frequency	SampleSize	No. of obs	AlleleNo	Availability	GeneDiversity	PIC
GMS1	0.5000	4.0000	4.0000	3.0000	1.0000	0.6250	0.5547
GMS2	0.5000	4.0000	4.0000	3.0000	1.0000	0.6250	0.5547
GMS3	0.5000	4.0000	4.0000	3.0000	1.0000	0.6250	0.5547
GMS4	0.5000	4.0000	4.0000	3.0000	1.0000	0.6250	0.5547
GMS5	0.5000	4.0000	4.0000	3.0000	1.0000	0.6250	0.5547
LMCH4	0.5000	4.0000	4.0000	3.0000	1.0000	0.6250	0.5547
LMCH5	0.5000	4.0000	4.0000	3.0000	1.0000	0.6250	0.5547
LMCH29	0.5000	4.0000	4.0000	3.0000	1.0000	0.6250	0.5547
LMCH33	0.5000	4.0000	4.0000	3.0000	1.0000	0.6250	0.5547
Mean	0.5000	4.0000	4.0000	3.0000	1.0000	0.6250	0.5547

levels of polymorphism and diversity among the markers. Major allele frequency ranged from 0.3333 to 0.6667, with a mean value of 0.4444, indicating moderate allele distribution across the loci. Markers OPT05 and OPH01 showed a higher dominance of one allele (0.6667), while others showed more balanced allele frequencies (0.3333) (Table 13). The sample size and number of observations were uniformly 3.0000 for all markers, indicating consistent sampling and data completeness. The number of alleles per locus ranged from 2 to 3, with a mean of 2.67, reflecting a moderate level of allelic richness. All markers showed 100% availability (1.0000), confirming successful amplification and scoring across all individuals. Gene diversity values ranged from 0.4444 to 0.6667, with a mean of 0.5926, indicating considerable genetic variation among the sampled accessions. The Polymorphic Information Content (PIC) values ranged from 0.3457 to 0.5926, with a mean PIC of 0.5103, suggesting that the markers are moderately informative.

6 Discussion

This study observed clear morphological distinctions in leaf shape, apex, base, venation, and floral structures among the three *Annona* species. These findings are consistent with those of Folorunso and Olorode [14], Wagner [46] who also reported obovate leaves and mucronate apices in *A. muricata*, and lanceolate leaves with acuminate apices in *A. squamosa*.

However, newly documented traits such as internode length, petiole variation, and vein number, especially for *A. senegalensis*, extend the baseline data available in the literature. Most previous studies in Nigeria [13] did not consider these traits, suggesting that this study provides a more comprehensive morphological dataset for regional taxonomic keys.

All the species studied are all perennials and habit encounter in these species studied are small tree in *A. muricata* and *A. squamosa* and shrubby tree in *A. senegalensis*. The leaf arrangement in all the three species are alternate. The leaf number which is found taxonomically useful in this study, was not used by previous authors. It also served as a diagnostic character.

Floral differences such as sepal number (three in *A. muricata* and *A. squamosa*, five in *A. senegalensis*) align with the work of Chatrou [6], who used floral traits for tribal classification within the Annonaceae. The pedicel colour is greenish-brown in both *A. muricata* and *A. senegalensis*, while in *A. squamosa* it is greenish-yellow. Pedicel length also varies among the species: *A. squamosa* has a length of 1.6 cm, *A. muricata* 2.1 cm, and *A. senegalensis* 0.6 cm. Despite these differences, all three species share a common trait of having six petals. Floral characteristics have proven to be valuable in distinguishing species within the genus.

Table 13 Genetic diversity and polymorphic information content (PIC) of RAPD markers

Marker	Major.Allele.Frequency	SampleSize	No. of obs	AlleleNo	Availability	GeneDiversity	PIC
OPT05	0.6667	3.0000	3.0000	2.0000	1.0000	0.4444	0.3457
OPH01	0.6667	3.0000	3.0000	2.0000	1.0000	0.4444	0.3457
OPH06	0.3333	3.0000	3.0000	3.0000	1.0000	0.6667	0.5926
OPT06	0.3333	3.0000	3.0000	3.0000	1.0000	0.6667	0.5926
OPT07	0.3333	3.0000	3.0000	3.0000	1.0000	0.6667	0.5926
OPH04	0.3333	3.0000	3.0000	3.0000	1.0000	0.6667	0.5926
Mean	0.4444	3.0000	3.0000	2.6667	1.0000	0.5926	0.5103

The results showed *A. muricata* produced the largest, deep green fruits, *A. squamosa* had yellowish oval fruits, and *A. senegalensis* bore smaller, oblong fruits. These findings agree with the descriptions in Anuragi [2] and Kwapata [24], who also emphasized fruit size and shape as major discriminators among *Annona* species. Additionally, the relatively lower seed number and germination rate in *A. senegalensis* mirror patterns reported in wild relatives of cultivated species, reflecting lower domestication pressure.

For growth studies, *A. senegalensis* showed superior leaf area expansion and leaf width, particularly after 24 weeks, while *A. muricata* had greater plant height and stem girth. This contrasts with Kjellstrom [21], who found that under optimal cultivation, *A. squamosa* grew fastest in early stages due to higher leaf counts. However, the environmental conditions in this study likely more field-based favored *A. muricata* and *A. senegalensis*, both known to be better adapted to native soil and climate variability [17].

SSR markers are usually preferable for fingerprinting purposes and population structure analysis due to their high polymorphism, co-dominance nature, and reproducibility [23]. Development of SSRs is expensive, but once it has been developed, they are cost- and time-effective in comparison to RAPD. Combined study of the molecular composition of the three species, *A. senegalensis*, *A. muricata* and *A. squamosa* together have little research work. Cross-species amplification of all the SSR markers used, successfully amplified in *A. muricata* and *A. squamosa* but did not in *A. senegalensis* (Fig. 9). RAPD markers showed cross species-amplification. The failure of SSR amplification in *A. senegalensis* is most likely due to sequence divergence in primer-binding regions, reflecting its greater evolutionary distance from the other two species. RAPD markers, being non-locus specific, bypass this limitation and are thus better suited for cross-species comparisons in genetically diverse taxa like *Annona* [2].

PIC value ranged from 0.3457 (OPT05) to 0.5926 (OPH04) for RAPD markers with an average of 0.5103 indicating existence of sufficient genetic diversity and differing levels of

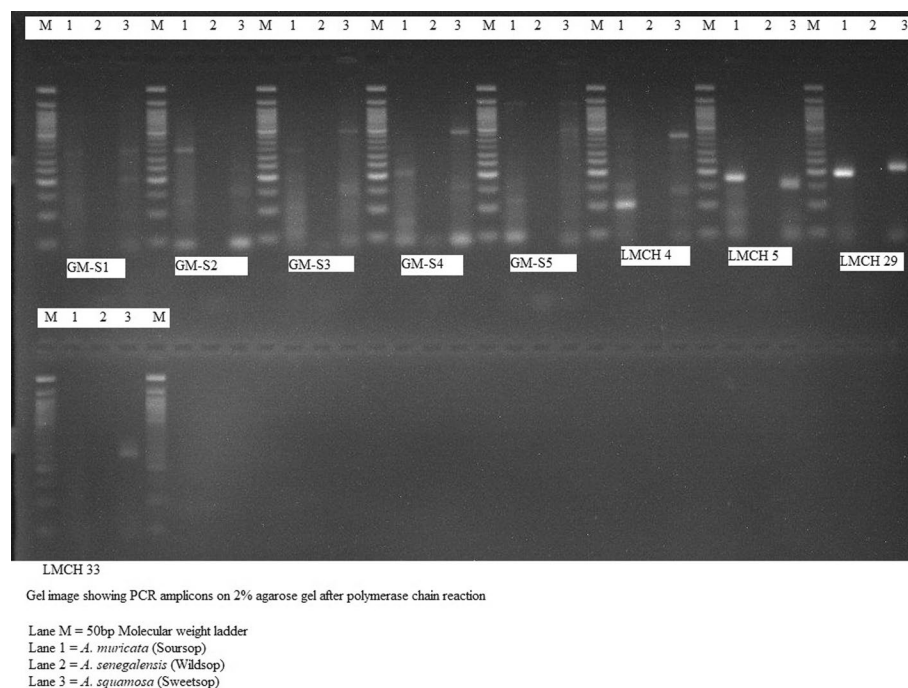


Fig. 9 Gel image of PCR amplicons on 2% agarose gel after polymerase chain reaction for SSR markers

polymorphism among the markers used in the three species studied genotypes. Online literature suggests markers with PIC values above 0.5 are highly informative, while those between 0.25 and 0.5 are moderately informative, which aligns with our report, as [5] stated. Significant genetic diversity have also been reported among the genotypes of *Annona* [2, 8, 24, 44]. The PIC for SSR markers were uniform (0.5547) this is in contrast to [2, 24] which ranged from 0.169 to 0.694 with average of 0.339. The high PIC suggests that the markers were derived from conserved, high-polymorphism loci [5]. Results of [24] demonstrated association between genetic and geographical distance in the *Annona species* indicating that large-scale geographical and ecotypic differentiation was reflected by the SSR markers. The high genetic diversity is attributed to biological characteristics of the tree species and habitat heterogeneity since they are of different origin [36, 46].

The uniform and relatively high (Polymorphic Information Content) PIC value in this study may be attributed to the deliberate selection of SSR markers with similar levels of polymorphism or the use of markers developed from conserved genomic region. Additionally, the genetic makeup and diversity of the specific accessions analysed can influence the allele frequency and consequently, PIC Values. Parida [34] in a study involving chickpea, SSR markers developed from various genomic regions, including intergenic regions and coding sequences, were evaluated for polymorphism. The markers located in coding sequences and exons showed higher polymorphism, whereas those in conserved exhibited lower variability (Figs. 10 and 11).

The high Polymorphic Information Content (PIC) values observed in this study—0.5547 for SSR markers and a mean of 0.5103 for RAPD markers indicate a substantial level of genetic diversity among the three *Annona species* studied. According to Botstein [5], PIC values above 0.50 are considered highly informative, making these markers valuable for distinguishing closely related genotypes and assessing allelic richness. Such high PIC values support the use of these markers in plant breeding programs, particularly for parent selection, genetic fingerprinting, and hybrid verification. They also provide a basis for marker-assisted selection (MAS) when linked with desirable phenotypic traits such as fruit size, seed number, or stress tolerance. From a conservation standpoint, the

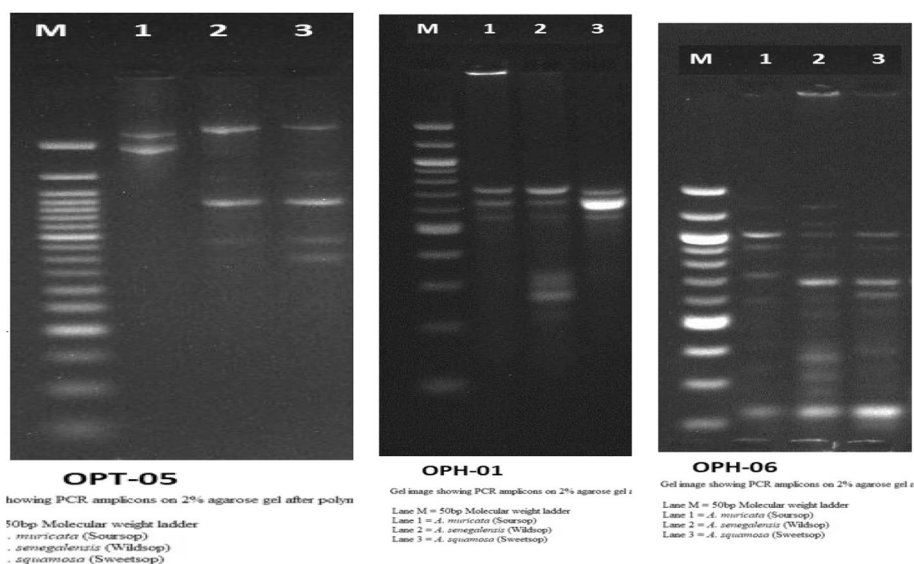


Fig. 10 Gel images of PCR amplicons on 2% agarose gel after polymerase chain reaction for RAPD markers

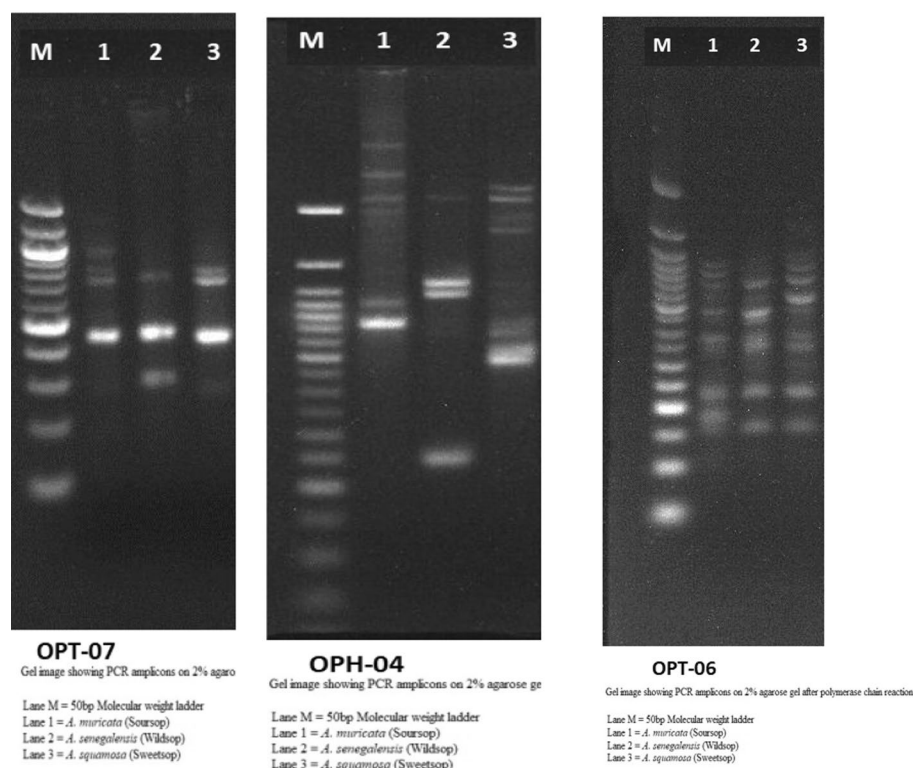


Fig. 11 Gel images of PCR amplicons on 2% agarose gel after polymerase chain reaction for RAPD markers

high level of polymorphism reflects the presence of rare alleles and broad genetic base, especially in wild species like *A. senegalensis*. This aligns with observations by Hamrick and Godt [17], who reported that long-lived, outcrossing, and undomesticated species tend to exhibit higher genetic diversity. Such diversity is essential for germplasm conservation, adaptive evolution, and core collection development. Comparable studies on *Annona squamosa* by Anuragi [2] and on guava by Kumar [22] also found high PIC values to be useful in cultivar identification and germplasm evaluation. These findings collectively affirm the suitability of the markers used in this study for future breeding, phylogenetic analysis, and systematic classification of *Annona species*.

Our report of a lower major allele frequency (MAF) of 0.3333 (GENOTYPE) to 0.6667 (OPB04) implies the presence of rare alleles, which play a critical role in genetic differentiation and adaptive evolution, as also reported by [30, 47].

Analysis of dendrograms derived from RAPD and SSR marker data indicated a closer genetic relationship between *Annona muricata* and *Annona squamosa*, whereas *Annona senegalensis* exhibited greater genetic divergence. The high level of genetic diversity is due to out breeding nature of *A. senegalensis* and this is a typical characteristic of wild tree species [24].

High genetic diversity in tree species is often attributed to their inherent biological characteristics—such as outcrossing breeding systems, long lifespan, and wide dispersal abilities as well as the heterogeneity of their habitats, which fosters population differentiation and local adaptation [24, 31].

Phylogenetic tree showed genetic relatedness of the three species studied. SSR markers indicated that *A. muricata* and *A. squamosa* are monophyletic and are more closely related than they are to *A. senegalensis*, while RAPD markers indicated that *A.*

senegalensis and *A. muricata* are more related than they are to *A. squamosa*. Folorunso [15] reported evidences of common evolutionary origin in the genus *Annona*. There are intrageneric relationships among the *Annonas*.

The genotypes characterized based on molecular marker supports the present day-classification of the three species into the same genus.

The intrageneric morphological features that unite the three species include the absence of stipules, entire leaf margins, six petals, alternate leaf arrangement, syncarpous fruits, chasmogamous flowers, yellow petals, eucamptodromous venation, a tree growth habit, and distichous phyllotaxy. The general morphological traits that differentiate the species include variations in petal length and width, pedicel length, leaf shape, leaf base, leaf apex, petiole length, leaf length, internode length, and fruit colour. Diagnostic morphological features—those that distinctly separate one taxon from its close relatives include the oblong fruit shape in *A. senegalensis*, the greenish-yellow petal colour in *A. squamosa*, and the glabrous surfaces of the pedicel and sepals in *A. muricata*.

The confirmation of the identities of the studied species has been better enhanced through the use of morphological and molecular characters. The variation among these characters has a great evolutionary significance. Characters of an organism are all the features and attributes that are possessed by the organisms, which may be compared, measured, counted, described or otherwise assessed. Diagnostic features are usually obtained from differential characters.

The observed high genetic diversity, especially in *A. senegalensis*, supports the hypothesis that wild *Annona species* maintain broader allelic variation due to their outcrossing reproductive systems, ecological heterogeneity, and limited artificial selection [17, 24]. Furthermore, the identification of stable diagnostic characters (e.g., pedicel texture, sepal number, petal dimensions) enhances species delimitation, and contributes valuable data for *Annona* improvement, conservation, and biogeographical classification.

7 Conclusion

Phylogenetic analysis provides valuable insights into the evolutionary relationships among organisms by comparing morphological and molecular characteristics. In this study, both morphological and molecular data were used to assess the relationships among three *Annona species*. The findings revealed a number of shared traits as well as distinguishing features, contributing to a better understanding of their taxonomic placement. The molecular data, particularly from SSR and RAPD markers, supported genetic differentiation among the species, while also indicating varying degrees of relatedness. These results are consistent with their current classification within the genus *Annona*, while also highlighting the importance of integrating multiple data sources for more accurate systematic studies. Incorporating Next-generation sequencing approaches such as genotyping-by-sequencing (GBS) and whole plastome sequencing could provide higher resolution in phylogenetic inference. Integration of cytogenetic analyses (e.g., karyotype and chromosome banding) with metabolomic profiling would further strengthen chemosystematic insights. Additionally, linking molecular diversity with ecological adaptations and ethnobotanical traits will deepen understanding of evolutionary processes and support conservation strategies for *Annona*.

Supplementary Information

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Supplementary Material 1

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Author contributions

All authors contributed to the study conception and design. Okechukwu, C. L.: Writing—original draft, review and editing, Conceptualization. Omosun, G.: Writing—review, editing and Conceptualization. Nwaru, C. E.: Writing—review and editing, Onyeabor, C. P. N.: Writing—review and editing. Ndukwe, N. N.: Writing—review and editing. Anosike, O.: Writing—review and editing.

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Data availability

The datasets generated and analysed during the current study are available in the [Repository.mouau.edu.ng] repository, [<https://repository.mouau.edu.ng/work/view/phylogeneticstudies-of-three-species-of-thegenus-annona-7-2>].

Declarations

Ethics approval and consent to participate

Plant collection and handling complied with institutional, national, and international guidelines, including the IUCN Policy Statement on Research Involving Species at Risk of Extinction and CITES regulations. Not applicable as this study did not involve human participants.

Consent for publication

Not applicable as this study does not include identifiable human data.

Plant reproducibility

Annona muricata, *Annona squamosa*, and *Annona senegalensis* were collected in Uturu and Umuahia, Abia State, Nigeria. The plants were identified by Dr. Chikodi Lilian Okechukwu, and voucher specimens are stored in the Herbarium of Abia State University under accession codes ABSU/FBS/45, ABSU/FBS/46, and ABSU/FBS/47.

Competing interests

The authors declare no competing interests.

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