

Genetic Diversity and Population Size Change of *Balanites aegyptiaca* (L.) Delile Natural Populations

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ABSTRACT

Balanites aegyptiaca is an underutilized wild fruit tree with significant ethnomedicinal value. However, the species' population is under threat from overexploitation, and if action is not taken, the species could become extinct in the near future. Understanding the molecular properties and population dynamics of the species is crucial for devising effective plans for its domestication and conservation. This study aimed to evaluate the diversity of *Balanites aegyptiaca* within its native Nigerian populations to identify suitable sources for its domestication and conservation. Leaf samples had their DNA extracted using a standard method. Molecular analysis was conducted utilizing chloroplast DNA sequences from the matK gene area. Software programs MEGA and DnaSP were employed to construct a phylogenetic tree as well as calculate pairwise genetic distance, nucleotide diversity (Pi), haplotype diversity (Hd), the number of variable sites (S), parsimony-informative sites (Ps), average nucleotide differences and changes in population size.

The genetic diversity was found to vary among the population based on the nucleotide diversity (Pi) index measured. The highest Pi value was 0.264 in the Mashi population. Parsimony-informative sites ranged from 0.0 at Dumsai to 2.0 at Mashi. Variable site number ranged from 3.0 at Dumsai to 302.0 at Mashi, while the average number of nucleotide differences ranged from 2.0 at Dumsai to 151.7 at Mashi. In Buratai, a unimodal distribution curve was found, suggesting population expansion, whereas the other sources displayed bimodal and multimodal distribution curves, indicating minimal population changes. The high genetic trait recorded by the Mashi population is indicative of high genetic diversity within the population, and it could serve as a potential source of superior genetic material required for the conservation of *Balanites aegyptiaca*.

Keywords: desert date; nucleotide diversity; parsimony-informative sites; matK gene; plant conservation

INTRODUCTION

Conserving germplasm and making breeding decisions hinge on identifying real or potential variations within it, which is a highly complex task (de Vicente et al. 2005) and a crucial factor in trait improvement and/or breeding decisions (Kloch et al. 2015). In the past, the discovery of genetic variation in plants for conservation or breeding purposes was primarily based on morphological characteristics. Currently, the choice of genetic material for breeding is determined by genetic traits, with the use of DNA markers for the specific plant population in question (Mahajan and Gupta 2012). Characterization involves the identification or differentiation of plant or animal specimens through

a specific process. Molecular characterization involves the process of identifying variations in an organism's genetic makeup or its unique DNA sequences and specific genes. Conservation enables the preservation of genotype (Šijačić-Nikolić and Milovanović 2012). This involves the collection and management of plant genetic resources. Gene identification and value addition, as stated by de Vicente et al. (2005), make this possible. Comprehensive conservation efforts for plants or animals rely on a thorough understanding of taxonomy, domestication, and evolutionary processes, a knowledge gap that is only bridged by molecular characterization (Nwakanma et al. 2003). Molecular and morphological characterization can provide accurate information for evaluating genetic diversity, rates of

genetic divergence among populations, and the distribution of diversity in populations from different locations (Maestri et al. 2002, Ferguson et al. 2004, Figliuolo and Perrino 2004).

Previous studies have documented the characterization and assessment of genetic diversity in multiple tree species using molecular markers, as reported in Rowden et al. (2004), Jamnadass et al. (2005), Narayanan et al. (2007), Rasmussen and Kollmann (2008), Huo et al. (2009), Dahlia et al. (2019), Ouédraogo et al. (2019), and Andrade et al. (2022). The genetic characteristics of several tree species remain to be determined, particularly in the Sahel or arid regions of the world. The *B. aegyptiaca* tree is an important wild fruit tree valued for both its edible and medicinal uses. The population of the species is rapidly dwindling as a result of habitat destruction and excessive exploitation. This species is typically found in its natural habitat and is sometimes kept on farms by farmers. Currently, there is no documented proof that any conservation program exists for this vital species, which may be due to insufficient data on the genetic diversity within natural populations, particularly in arid and semi-arid regions.

Genetic characteristics of *B. aegyptiaca* in Egypt were evaluated using random amplified polymorphic DNA (RAPD), as reported by Fathy and El-Kader in 2012, while amplified fragment length polymorphism (AFLP) was used to assess genetic variability of the species in Sudan, Egypt, Ethiopia, Saudi Arabia, and Yemen, as well as in Ghana (Khamis et al. 2017). Recently AL-Juhani et al. (2022) carried out a complete chloroplast genome sequence of the species in Saudi Arabia using multiple gene loci. The availability of information on the genetic diversity and population size change in *B. aegyptiaca* populations, as indicated by the maturase K (*matK*) chloroplast gene sequence, is limited, which hinders conservation and improvement, as well as breeding programme efforts.

This study was therefore carried out to assess the level and pattern of genetic variation as well as population size expansion or change in the population of *B. aegyptiaca* using *matK*. This information is crucial in designing conservation and improvement and/or breeding programmes for this important species. To the best of our knowledge, this is the first attempt made to assess the genetic diversity and population size change of *B. aegyptiaca* using *matK* gene loci in the Sahel.

MATERIALS AND METHODS

Sample Collection

Eight locations, namely Baure, Bauchi, Buratai, Dumsai, Gamawa, Gashua, Guri, and Kirikasama (Table 1), were purposively selected, based on the availability of *Balanites aegyptiaca* trees. Ten mature trees were randomly selected from each location. A minimum distance of 100 m between each selected species was maintained to avoid inbreeding (Abasse et al. 2011). Voucher specimens were accessioned and given the voucher identity number BUKHAN 0359 at the herbarium section of Bayero University Kano, Nigeria. The samples were inserted into small zip-lock bags, zipped, and labeled properly. The samples were stored in a vaccine storage cooler filled with ice blocks and labeled accordingly before being transported to the laboratory. The samples were still fresh when they got to the laboratory. The samples were transferred into a fridge in the laboratory before DNA extraction. One gram of leaf samples from each tree was utilised to determine the genetic characteristics of the selected trees, with chloroplast gene sequences from the *matK* region as outlined below.

Buffer Preparation

The buffer solution was prepared by diluting 2.5% cetyltrimethylammonium bromide (CTAB), 2 ml of 0.5 M ethylenediamine tetraacetic acid (EDTA), 1% polyvinylpyrrolidone (PVP), 4 ml of 1 M trisma base, 0.2% mercaptoethanol and 12 ml of 5 M sodium chloride (NaCl) in 17 ml of sterile distilled water.

DNA Extraction

This study adopted a modified version of the CTAB protocol for DNA extraction, as previously described by Varshney and Anis (2014), with the steps outlined below.

To each leaf sample, 2 ml of buffer was added and pulverized with a mortar and pestle. One millimeter (1 ml) of powdered sample was poured into 2 ml extraction tubes (labeled). The samples were incubated for one hour in a water bath heated to 650°C. Samples were shaken every 20 minutes to ensure an even distribution of chemical components. Each sample received 900 µl of chloroform isoamyl alcohol (CIA) and was thoroughly shaken to mix. This was done to prevent protein contamination, debris, and

Table 1. Geographic characteristic of *B. aegyptiaca* locations.

Location	Longitude	Latitude	Elevation (m)
Baure	08°43.496'	12°50.407'	403.7
Buratai	12°02.535'	11°01.488'	537.8
Dumsai	10°33.325'	12°51.615'	344.0
Gamawa	10°35.661'	12°11.397'	355.5
Gashua	11°00.770'	12°52.496'	334.1
Guri	10°24.755'	12°45.313'	347.9
Kirikasama	10°20.296'	12°40.213'	387.2
Mashi	07°56.316'	12°57.681'	507.2

Source: Wakawa and Akinyele 2024.

interphase material. The samples were then centrifuged at 15,000 rpm at 40°C for 10 minutes, and the supernatant was transferred to new tubes. Each sample was treated with 170 µl of 5 M NaCl and 340 µl of cold isopropanol before being frozen overnight at -40°C. The next day, samples were removed from the freezer and centrifuged at 15,000 rpm for 10 minutes before the supernatant was decanted to extract the white pellet. The samples received 70 µl of TE buffer and were incubated at -40°C for 2 hours. After incubation, each sample received 25 µl and 600 µl of potassium acetate and ethanol, respectively, and was incubated for 2 hours at -40°C. The dissolved pellets were spun at 15,000 rpm for 10 minutes at 40°C. Following the removal of the supernatants, the pellets were air dried. Finally, 50 µl of sterile distilled water was added to dissolve the pellets.

Preparation of Gel (1.2%) for Electrophoresis

To create 1% gel for a 60 ml tray, 0.6 g of agarose powder was dissolved in 60 ml of TBE buffer. Then, 0.6 g of agarose powder was multiplied by 12 g to obtain 1.2% gel. Ethidium bromide (5 µl) was added and allowed to cool before being put into the tray.

Electrophoresis

Each DNA sample of 2 µl was mixed with 2 µl of gel-loading dye on a parafilm paper loaded into each well of the electrophoresis machine and allowed to run at a constant voltage of 80 V for 15 minutes twice (i.e., 30 minutes).

Integrity Test

Samples on a gel tray were loaded in a gel documenting system (E-BOX CXG.TS Made in France) and photographed to assess DNA quality. Illuminating bright light reflection of the sample indicates quality DNA.

Polymerase Chain Reaction (PCR), Optimisation, and DNA Amplification

A polymerase chain reaction (PCR) cocktail of 12.5 µl volume was used, the constituents include the following 1.25 µl Buffer, 0.8 µl MgCl₂, 0.25 µl dNTPs, 0.25 µl matK F, 0.25 µl matK R, 6.64 µl sterile distilled water, 0.06 µl Taq, and 3 µl DNA template. PCR was done using the Eppendorf master cycler (Eppendorf AG 22331 Hamburg). Initial denaturation took 1 min at 94°C, followed by 35 cycles

of DNA denaturation at 94°C for 4 seconds. Annealing of primers took 40 seconds at 51°C; the initial extension took 40 seconds at 72°C, while the final extension lasted for 5 minutes at 72°C.

A pair of matK primers were used for amplification. The sequence of the primers is shown below:

MATK-KIM3F (5'-CGTACAGTACTTTTGTGTTTACGAG-3')

MATK-KIM1R (5'-ACCCAGTCCATCTGGAATCTTGTTTC-3').

DNA Sequencing

DNA sequencing was performed using standard procedures by Inqaba Biotech West Africa.

Data Analysis

Base calling using the BioEdit sequence alignment editor was carried out on the sequence data manually (Hall 1999). The data were subjected to BLAST on the National Centre for Biotechnology Information (NCBI) website (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>) to match them with similar sequences. Sequences belonging to the same genus (*Balanites*) of 625 lengths (bp) and above that using matK gene loci only were retrieved from the NCBI gene bank on 15-09-21 and used for the authentication (identification) of sample species. Sequences were aligned (alignment) using Molecular Evolutionary Genetics Analysis software (MEGA 7) to draw a phylogenetic tree based on the neighbor-joining method. The bootstrap consensus tree was inferred from 150 replicates. The pairwise distance was computed using maximum composite likelihood (Kumar et al. 2004). DnaSP 6.12.03 was used to determine nucleotide diversity (Pi), haplotype diversity (Hd), the number of variable sites, parsimony-informative sites, the average number of nucleotide difference (k), Tajima's D test and population size change (Rozas et al. 2017).

RESULTS AND DISCUSSION

Genetic Characteristics among *B. aegyptiaca* Populations

Nucleotide diversity (Pi) increased from 0.002 (Dumsai) to 0.264 (Mashi), while haplotype diversity ranged from 0.667 to 1.000 (Table 2). The average number of nucleotide differences (k) was the highest (151.667) in Mashi and the lowest (2.0) in Dumsai. The number of haplotypes (Nh) in this study ranged from 2 to 4. Variable or polymorphic sites (S) in this study increased from 3 in Dumsai to 302 in Mashi (Table 2).

Table 2. Genetic characteristics among *B. aegyptiaca* populations.

Location	Nh	S	Ps	Pi	Hd	k	Tajima's D test
Baure	4	55	0	0.095	1.000	27.500	-0.868ns
Buratai	3	10	0	0.008	1.000	6.667	-
Dumsai	2	3	0	0.002	0.667	2.000	-
Gamawa	3	7	0	0.006	1.000	5.333	-
Gashua	4	7	0	0.005	1.000	3.833	-1.202*
Guri	4	14	2	0.009	1.000	7.333	-0.403ns
Kirikasama	4	13	0	0.009	1.000	8.167	-1.217*
Mashi	4	302	2	0.264	1.000	151.667	-0.864ns

Note: Significant $p < 0.05$, "*" represents significance, ns indicates not significant, Nh = number of haplotypes, S = variable or polymorphic sites, Ps = parsimony-informative sites, k = average number of nucleotide difference, Pi = nucleotide diversity, and Hd = haplotype diversity.

The high values of genetic characteristics measured in this study, particularly in the Mashi population, suggest a high level of genetic diversity among the population studied in the given area. Khamis et al. (2017) findings concur with this outcome, which shows a high genetic diversity in *B. aegyptiaca* species from Sudan, Egypt, Ethiopia, Saudi Arabia, Yemen, and Ghana, as determined by amplified fragment length polymorphism (AFLP). According to research by Andrade et al. (2022), a relatively low degree of genetic variation was observed in wild mahogany populations following molecular analysis with microsatellite markers. In Burkina Faso, a limited genetic diversity among the *Zanthoxylum zanthoxyloides* (Lam.) population was noted after examining four DNA areas (GBSSI, ITS, psbA-trnH, and trnL-trnF) as part of the characterization process (Ouédraogo et al. 2019). This suggests varying degrees and types of genetic diversity among different species. Genetic characteristics including nucleotide diversity and haplotype diversity were effectively employed to evaluate genetic diversity in a number of organisms (Li et al. 2011, Ndoeye et al. 2013, Tamboli et al. 2016, Zhang et al. 2020).

The nucleotide and haplotype diversity levels observed in this study exceeded those found in baobab (*Adansonia digitata*), another important edible wild food tree of the Sahel region. The authors found a nucleotide diversity spanning 0.00060 to 0.00527 and a haplotype diversity of 0.363 to 0.648 (Ndoeye et al. 2013). Similarly, the average nucleotide diversity recorded in the population of Scottish pine according to Wachowiak et al (2011) was 0.0078. A genetic similarity index that ranged from 0.246 to 0.591

was reported among species of *B. aegyptiaca* sourced from Sudan, Egypt, Ethiopia, Saudi Arabia, and Yemen (Khamis et al. 2017). However, because they employed other indices to measure genetic diversity and a different molecular marker (AFLP), which is less discriminative than the sequencing utilized in this work, it is challenging to compare their findings with those of this investigation. MatK gene loci used in this study have been reported to have a success rate in the amplification of several species such as *Zygophyllum propinquum* Decne. of the family Zygophyllaceae, which shares the same family as *B. aegyptiaca* (Bafeel et al. 2011).

Phylogenetic Characteristics of *B. aegyptiaca* Tree Populations

Two main clades were identified by the phylogenetic tree that was produced. The populations of Buratai, Guri, Gamawa, and Kirikasama formed a clade with a bootstrap value of 4, whereas the populations of Mashi, Baure, Gashua, and Dumsai formed another clade with a bootstrap value of 0 (Figure 1).

The genetic compositions of *B. aegyptiaca* populations in Buratai, Guri, Gamawa, and Kirikasama are found to be comparable, similarly as the trees in Mashi, Baure, Gashua, and Dumsai. Further confirmation came from the pairwise distance matrix (Table 3). Bootstrap values were extremely low for both clades. The level of confidence in the similarities between populations within each clade was extremely low, indicating that it cannot be definitively concluded that they are closely related, even though they possess comparable genetic characteristics. Studies by Hillis and Bull (1993)

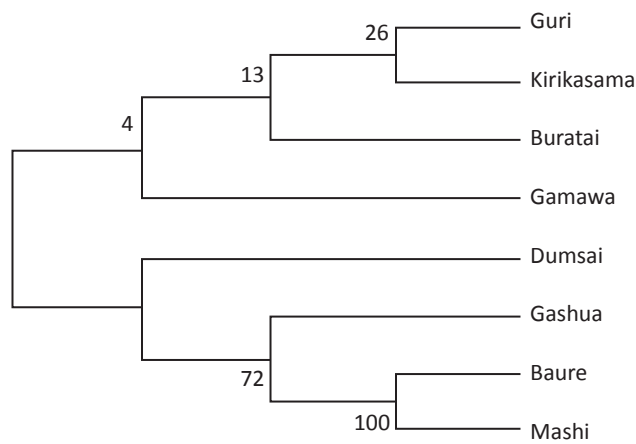


Figure 1. Phylogenetic trees among sources of *B. aegyptiaca* trees. Note: The numbers at the branches are confidence values based on Felsenstein's bootstrap method. *B* 150 bootstrap replications.

suggest that a bootstrap value below 73 indicates weak bootstrap support, implying that the associated phylogenetic confidence level is statistically insignificant, thus lowering the overall confidence in the phylogenies.

B. aegyptiaca tree populations in Baure and Mashi formed a monophyletic clade (sub-clade) with a very high bootstrap value (100), and similar was observed in Gashua, Baure, and Mashi, which had a bootstrap value of 73. This suggests that samples from each distinct monophyletic clade share a common ancestral parent. The pairwise distance matrix also provided evidence, revealing that these populations have a closer genetic distance as indicated in Table 3.

The high bootstrap values associated with both monophyletic groups suggest a strong level of confidence or assurance in the validity of their sibling relationship. The Baure and Mashi populations appear to represent distinct ecotypes. This was not unexpected, given that both populations exhibit comparable proximity and share analogous climatic and soil attributes. The proximity of the two populations may facilitate cross-pollination and seed dispersal between them. It is possible that one population has resulted from the transfer of seeds between the other population by either animals or humans. Phylogenetic trees of DNA sequences have been used to identify ecotypes in a population of *Adansonia digitata* (L.) in Senegal, as reported by Ndoye et al. (2013).

Population Size Changes among *B. aegyptiaca* Populations

The population of *B. aegyptiaca* in Buratai demonstrated a unimodal distribution of mismatch (Figure 2), indicating a possible shift in the population dynamics. Xu et al. (2009) found that a unimodal distribution curve represents a population's change. Changes to the genetic composition of *B. aegyptiaca* populations in Buratai may have occurred due to population decline, potentially affecting genetic diversity in the location. According to Harpending et al. (1998), changes in genetic diversity are commonly associated with changes in population size. This could be attributed to the high level of exploitation or disturbance faced by the population in the location. *B. aegyptiaca* tree species are

cherished for their edible fruit production and medicinal properties, therefore exposing them to over-exploitation.

A bimodal/multimodal pattern of mismatch distribution curve was evident in all the additional sources examined, namely Baure, Dumsai, Gamawa, Gashua, Guri, Kirikasama, and Mashi. A bimodal or multimodal pattern implies that the population of *B. aegyptiaca* in these locations has not undergone any significant changes, but instead that it is in a stable state (Xue et al. 2014). Such a stable population size is typically characteristic of populations that are not influenced by human activity or other external factors. Equilibrium in population size is a characteristic associated with populations that are free from human disturbance or other factors. This is usually found in organisms that are under protection (Cruz-Salazar et al. 2021). However, *B. aegyptiaca* populations in these locations were not observed to be protected, therefore the lack of size change could be attributed to the lack of significant exploitation of the tree by humans and animals in the location.

To validate the mismatch distribution curve results, a Tajima D test was conducted on the populations with at least four (4) haplotypes, which are required for the neutrality test. Non-significant results were observed in Baure (-0.868), Guri (-0.403), and Mashi (-0.864) (Table 2). A non-significant Tajima D test result suggests no changes in a population, whereas a significant negative Tajima D test outcome indicates population change (Li et al. 2011, Tamboli et al. 2016). It can be stated with considerable confidence that these populations, namely Baure, Guri, and Mashi, have undergone little to no significant transformation. Significant negative Tajima D test results were found for the Gashua population at -1.202 and the Kirikasama population at -1.217, contradicting the mismatch distribution curve's indication of substantial change. Ndoye et al. (2013) also observed a similar contradiction between the results of the Tajima D test and mismatch analysis in baobab tree populations. This discrepancy may be attributed to the rapid population increase in Gashua and Kirikasama, which was not taken into account by the mismatch analysis. The Tajima D test is highly sensitive to recent population growth, which causes test results to shift towards more negative values as reported by Tajima (1989).

Table 3. Pairwise distance matrix among sources of *B. aegyptiaca*.

	Buratai	Gamawa	Guri	Kirikasama	Baure	Mashi	Dumsai	Gashua
Buratai	-							
Gamawa	0.000	-						
Guri	0.002	0.002	-					
Kirikasama	0.000	0.000	0.002	-				
Baure	0.117	0.117	0.120	0.117	-			
Mashi	0.111	0.111	0.114	0.111	0.004	-		
Dumsai	0.000	0.000	0.002	0.000	0.117	0.111	-	
Gashua	0.002	0.002	0.004	0.002	0.114	0.108	0.002	-

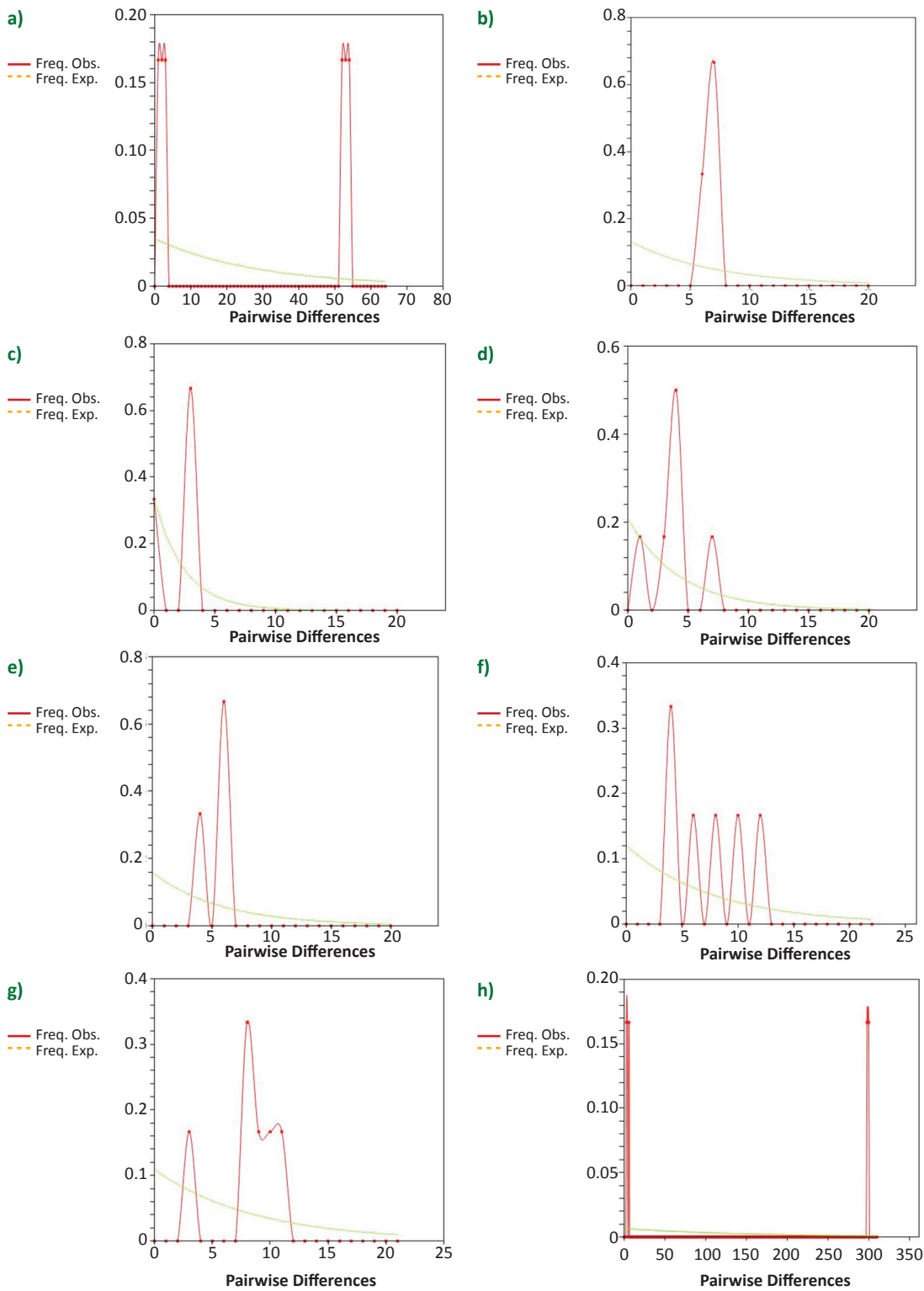


Figure 2. The mismatch distribution curve of *Balanites aegyptiaca* populations among sources: (a) Baure, (b) Buratai, (c) Dumsai, (d) Gashua, (e) Gamawa, (f) Guri, (g) Kirikasama, (h) Mashi.

CONCLUSIONS

This study assessed the genetic diversity and population size change among eight (8) natural populations of *B. aegyptiaca*, an important wild fruit tree with high medicinal and nutritional value. Genetic variation was found within the population of *B. aegyptiaca*. The Mashi population exhibited the highest genetic characteristics (S, Pi, Ps, k), signifying a considerable level of genetic diversity. In Baure, Gamawa, Dumsai, Kirikasama, and Mashi, a bimodal pattern of mismatch distribution curve was observed, in contrast to Gashua and Guri, which displayed a multimodal distribution pattern. These populations appear to have experienced minimal disruption, suggesting no substantial alteration in their genetic diversity. We noted a sign of change in the population size of *B. aegyptiaca* in Buratai population, as evidenced by a unimodal pattern in the mismatch distribution curve. Populations of Baure and Mashi form a monophyletic clade, supported by a very high (100) bootstrap value, suggest that they may be closely related and represent a distinct ecotype compared to others. The genetically diverse population in Mashi offers a superior source of germplasm required for the conservation and

improvement of *B. aegyptiaca* species, and could also play a significant role in germplasm collection.

Author Contributions

LDW conceived and designed the research, performed laboratory analysis, collected samples, secured the research funding, processed the data, performed the statistical analysis and wrote the final manuscript. AOA supervised the research and helped with the design of the experiment and also draft the manuscript.

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Conflicts of Interest

The authors declare no conflict of interest

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