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Genome-Wide Association Mapping of Oil Content and Seed-Related Traits in Shea Tree (*Vitellaria paradoxa* subsp. *nilotica*) Populations

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Abstract: Shea tree (*Vitellaria paradoxa*) is an important fruit tree crop because of its oil used for cooking and the industrial manufacture of cosmetics. Despite its essential benefits, quantitative trait loci linked to the economic traits have not yet been studied. In this study, we performed association mapping on a panel of 374 shea tree accessions using 7530 Single-Nucleotide Polymorphisms (SNPs) markers for oil yield and seed-related traits. Twenty-three SNP markers significantly ($-\log10\ (p) = 4.87$) associated with kernel oil content, kernel length, width, and weight were identified. The kernel oil content and kernel width had the most significant marker–trait associations (MTAs) on chromosomes 1 and 8, respectively. Sixteen candidate genes identified were linked to early induction of flower buds and somatic embryos, seed growth and development, substrate binding, transport, lipid biosynthesis, metabolic processes during seed germination, and disease resistance and abiotic stress adaptation. The presence of these genes suggests their role in promoting bioactive functions that condition high oil synthesis in shea seeds. This study provides insights into the important marker-linked seed traits and the genes controlling them, useful for molecular breeding for improving oil yield in the species.

Keywords: linked; marker association; annotation; genes; SNPs; shea

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1. Introduction

The shea tree (*Vitellaria paradoxa* C. F. Gaertn.) is an important economic tree crop known for its oil used to produce valuable products in the food and cosmetic industries [1]. The tree is endemic to Sudano-Sahelian Africa, covering 21 countries [2], where it adds to the sustainability of sociocultural and economic wellbeing of the communities. The shea tree: *Vitellaria paradoxa* C. F. Gaertn., has two described subspecies: *V. paradoxa* subsp. *paradoxa* and *V. paradoxa* subsp. *nilotica*. The two subspecies vary for their morphological characteristics [3]. The subspecies *nilotica* has larger flowers and a dense "woolly" appearance that remains on young leaves and persists on leaf veins and midribs. It is characterized

by dense ferruginous indumentum on pedicels and outer sepals, with the constituent hairs being longer, spreading and imparting a woolly appearance to the parts during the bud stage. The hermaphroditic and actinomorphic flowers are always in dense clusters on the twigs that have not formed leaves [4]. In the subspecies *paradoxa*, the flowers have longer styles measuring 12–15 cm [5].

The tree is a diploid (2n = 24), highly outcrossing that has undergone domestication in the savannah parklands of Africa for over 1000 years [6]. A molecular marker study by Allal et al. [7] placed the centre of origin of *V. paradoxa* in West Africa, where three genetic groups corresponding to West, Central, and East African types were found. The shea genome size is up to 658.7 Mbp, consisting of 38,505 coding genes [8]. There is an observable variation in stand densities within the shea parklands, due to the differences in land use, localities, soils, rainfall, temperature, daylight length, and ecological conditions [9], forming seven different morphological and structural forms [10].

The shea tree is recognized as the second-highest oil-producing plant, after the oil palm [8]. The global market for shea products was reported to be USD 30 billion in 2020 [11]. This high demand is owed to its use in the confectionary and cosmetic industries. The demand for these natural and organic cosmetics in the European market reached EUR 3.90 billion in 2019 [12]. The cosmetic sector alone exceeded USD 530 in 2020 and is expected to rise to USD 1025 million in 2027. Among this, the US market alone is projected to rise from USD 240 million in 2020 to USD 390 in 2027 and expected to grow at a compound annual growth rate (CAGR) of 7%, due to the increasing demand in the cosmetics industry [13]. The total export of oils from different plants to Europe in 2020 was estimated at 300,000 tonnes, with the Netherlands and France being the leading importers. However, both processed and unprocessed products are sold in national and international markets, contributing to national income through foreign exchange in the shea-producing countries. The leading producers of shea in Africa are Nigeria (361,017 tons/year), Mali (49,640 tons/year), Burkina Faso (45,183 tons/year), and Ghana (33,878 tons/year) [11]. There is a huge and untenable supply deficit due to heightened international demand, necessitating breeding interventions to boost production across its range.

The first recognizable shea tree improvement efforts were through a participatory selection trial of plus trees from three countries in West Africa [14]. A larger collection (from Burkina Faso, Benin, Nigeria, Ghana, Cameroun, Niger, and Mali) of carefully selected plus shea trees raised from clonal materials was also established in Mali by the World Agroforestry (CIFOR-ICRAF) [14]. Over time, there has been increasing interest in improving shea tree productivity to meet the looming domestic and international demand for shea products [15]. Some concerted efforts have been made in shea tree breeding at the University of Peleforo Gon Coulibaly (UPGC) in Korhogo, Côte d'Ivoire, where some elite trees were selected and propagated [16] by grafting to reduce the juvenile maturity period [17]. Other innovative approaches have been used in participatory plant breeding (PPB), using local knowledge to identify and select preferred traits by the communities. Such traditional and contemporary breeding and selection processes are important for tree species like shea trees, to generate new varieties with various desired properties [18].

Recent advances in shea tree genomic studies by Hale et al. [8] and Wei et al. [1] have provided insights on the new opportunities in genome-assisted breeding. Despite these advancements, the genomic resources remain underused for boosting production and improving oil yield and quality. Genome-wide association studies (GWAS) provide opportunities to identify genomic regions of an organism that are putatively associated with the traits of interest to plant breeders [8]. With the availability of affordable and economic modifications of genome sequencing approaches like genotyping by sequencing (GBS), discovering and using SNP markers has become a preferred way of genotyping. One of these technologies is the Diversity Arrays Technology Sequencing (DArTseq), where a genome is partially sequenced using a specific combination of restriction enzymes and the restriction tags are used for assembling and discovering the SNP markers [19]. The discovered SNPs are generally spread all over the genome and can be used in GWAS for

the study of a wide range of tree crop traits of economic importance [20]. This study was carried out to identify genomic loci associated with seed oil content, seed weight, seed length and width of the shea tree in Uganda. Determining the marker trait association shall enhance shea tree breeding by reducing on the time required to complete the breeding cycle.

2. Materials and Methods

2.1. Plant Materials and Leaf Sampling for DNA Extraction

A total of 374 shea genotypes from the germplasm collection (Breeding Seedling Orchard) Uganda were used in this study. A total of 3600 shea fruits/seeds were collected from 180 families (Supplementary Table S1) in the districts of Amuru, Arua, Katakwi, Moyo and Otuke. The seeds were then divided into two portions: for sowing and for oil extraction. A minimum of 10 seeds were randomly picked from each family for sowing to generate seedlings used in DNA extraction. Fifteen seeds from the remaining lot were processed and used for oil extraction.

The shea seeds were sown in a tree nursery at Ngetta Zonal Agricultural Research Development Institute (NgeZARDI), Lira—Uganda in the month of June 2018. The seedlings were managed in the tree nursery for 12 months until they developed between 4–6 leaves before sampling the leaf tissues for DNA extraction. Leaf samples of 374 seedlings were randomly picked for DNA extraction and analysis at Biosciences Eastern and Central Africa-International Livestock Research Institute (BeCA-ILRI). Only healthy and recently flushed leaves from the previous season were sampled and placed in DNA extraction kit and dried using Silica gel before shipping to BeCA-ILRI.

After leaf tissue sampling, the genotypes were further managed in the nursery for another 6 months to allow them to heal and later planted in a multi-locational trial (breeding seed orchard) located in Lira (NgettaZARDI) and Serere (National Semi Arid Resources Research Institute (NASARRI), using Random Complete Block Design (RCBD) in the month of October 2019. The trials were maintained as germplasm collection for future breeding programme in Uganda.

2.1.1. Shea Oil Extraction Procedure

Oil content was determined using Soxhlet extraction [21], the American Official Agricultural Chemists' method for determination of oil content in plant materials in the months of September and October 2020. Oil was extracted with continuous reflux of petroleum ether over crushed dried Shea nut powder in a Soxhlet extractor. The oil contents of each seed lot were extracted in triplicates and presented in percentage of its dry matter content.

2.1.2. DNA Extraction and SNP Discovery by DArTseq™ Technology

Total genomic DNA from silica dried leaf samples were extracted at BeCA-ILRI following the CetylTrimethylAmmonium Bromide (CTAB)/chloroform/isoamyl alcohol method [22]. DNA samples were processed in digestion/ligation reactions as described by Hale et al. [8]. The DNA was quality checked using standard processes involving 0.8% agarose gel electrophoresis, optical measurements for 260 and 280 nm using a NanoDrop 2000 spectrophotometer (ND-2000 V3.5, NanoDrop Technologies, Inc., Wilmington, DE, USA) and quantification using a Qubit™ 3.0 Fluorometer (Thermo Fisher Scientific, Grand Island, NY, USA). The libraries were prepared for 752 individuals using the PstI-SphI complexity reduction method [23] and partial-genome sequenced using proprietary DArTseq (1.0) methodology [19] on a HiSeq2500 Sequencer (Illumina Inc., San Diego, CA, USA) with 72 bases read length [24,25].

Sequences generated from each lane were processed using proprietary DArT analytical pipelines. DArT-Seq™ technology relies on a complexity reduction method using restriction enzymes that are sensitive to DNA methylated sites and repetitive DNA [24]. In the primary pipeline, the FASTQ files were first processed to filter poor-quality sequences, applying more selection criteria to the barcode region compared to the rest of the sequence. Approximately 2,500,000 (±7%) sequences per barcode/sample were used

in marker calling. Finally, identical sequences were collapsed into "fastqcall files." These files were used in the secondary pipeline for DArT P/L's proprietary SNP and SilicoDArT (Presence/Absence Markers in genomic representations) (present = 1 vs. absent = 0) calling algorithms (DArTsoft14). The analytical pipeline processed the sequence data. The reads were then aligned to the shea_V1 reference genome publicly available from the ORCAE database (https://bioinformatics.psb.ugent.be/orcae) (accessed on 30 December 2021), using BWA-MEM/VarDict mapper for mapping of reads against the reference genome [8].

2.2. Data Analysis

2.2.1. Seed Trait Data Analysis

The seed trait data were analysed using "*agricolae*" package in R software v 4.0 [26]. Analysis of variance (ANOVA) was performed to determine the variations within and among the genotypes. The "*corr*" function in R software v.4.0 (R Core Team, 2022) was used to calculate correlation coefficients between the studied traits and presented in graphical form.

2.2.2. Genome-Wide Association Analysis and Gene Annotation Identification

A multi-locus random-SNP-effect mixed linear model (mrMLM) [26] was implemented in R statistical software using the mixed model equation for GWAS presented in Equation (1), in accordance to Yu et al. [27], using additive, general; dominant alternative and dominant reference gene action models for trait association study [28]. This current study selected mrMLM method to avoid bottlenecks in stringent correction using other control measures (false discovery rate (FDR) and Bonferroni correction) against false positive rate [29]. The mrMLM uses a less stringent significance threshold considering a critical probability value or log of odds (LOD) making it possible to identify any possible loci of importance.

$$Y = Xb + Zu + e \quad (1)$$

where:

Y = the vector of the phenotypic observations estimated for the traits studied;
X = the SNP markers (fixed effect) matrix;
Z = the random kinship (co-ancestry) matrix;
b = a vector representing the estimated SNP effects;
u = a vector representing random additive genetic effects, and
e = the vector for random residual errors.

The phenotypic variation explained by the model for a trait and a particular SNP was determined using stepwise regression implemented in the "*lme4*" R package. The SNP loci in significant association with traits were determined by adjusted *p*-value using Bonferroni correction [30]. Quantile–quantile (QQ) plots were generated by plotting the negative logarithms (−log10) of the *p*-values against their expected *p*-values to test the appropriateness of the GWAS model with the null hypothesis of no association and to determine how well the models accounted for the population structure.

To account for the putative genes linked to traits, a window range of 5 kb (upstream and downstream) was defined [31]; and genes were searched from the *V. paradoxa* Whole Genome v2.0 Assembly and Annotation v2.1 [32] in the ORCAE database (https://bioinformatics.psb.ugent.be/orcae, accessed on the 30 November 2022) [3], with a search for candidate genes associated with oil yield traits. The gene name, description, and AGPv4 coordinates with their protein, were then retrieved from the *Vitellaria paradoxa* reference genome database. The putative functional candidate genes linked to the associated SNPs were then annotated in line with any initially annotated genes from other species.

A Linkage disequilibrium (LD) heat map was generated for the entire genome, with heterozygous calls ignored and a default sliding window of 50 used in tassel software. LD decay rate was then evaluated on a chromosome-by-chromosome basis. A measure of LD (r^2) and pairwise distance between SNPs were generated in TASSEL and exported to R version 4.3, where scripts were written to generate LD decay plots for significant

LD pairs. Mean LD per chromosome was calculated after every 20 kb interval, and the average genome-wide decay rate estimated by averaging LD in each interval across all chromosomes. A line graph was used to clearly display an overlay of chromosome-specific and the mean genome-wide LD decay rates.

3. Results

3.1. Phenotypic Variation for the Shea Tree Traits

The traits mean values, standard deviations and the phenotypic data range of a collection of 374 open pollinated seeds from 180 shea trees from Uganda's parklands are presented in Table 1.

Table 1. Summary statistics for the studied traits.

Traits	Mean ± (SD [a])	Minimum	Maximum
Kernel dry matter oil content (% [b])	53.53 ± 2.28	39.05	69.77
Kernel length (cm [c])	3.19 ± 0.34	1.90	8.43
Kernel width (cm)	3.61 ± 0.43	2.23	4.97
Kernel weight (mg [d])	10.30 ± 0.30	2.00	18.8

[a] Standard Deviation; [b] Percentage; [c] Centimetre; [d] Milligram.

The mean seed oil content of 180 shea genotypes was 53.53% with a range of 39.05–69.77%. A relatively heavy kernels (18.81) and very low weight genotypes were also observed (Table 1).

Analysis of variance showed that genotype, environment, and their interaction (genotype-environment) were highly significant for kernel oil content (Table 2). Variation in kernel weight and its axial dimensions were significantly influenced by genotype and the environment. However, the interaction of genotype and environment had no significant effect on kernel weight and its axial dimension (Table 2).

Table 2. Summary analysis of variance for the studied traits.

Source of Variation	Df [a]	KOC [b]	KL [c]	KW [d]	KWt [e]
Replications	2	4.81	0.01307	0.0249	0.08108
Environment	4	1840.82 ***	0.694 ***	0.82403 ***	0.90574 ***
Genotypes	373	60.42 ***	1.45026 ***	2.54701 ***	0.9112 ***
Genotype x Environment	1492	35.9 **	0.01524	20.69	0.01666
Residuals	3738	8.61	0.0159	0.01553	0.02156

[a] Degrees of freedom; [b] Kernel dry matter oil content (%); [c] kernel length (cm); [d] kernel width (cm); [e] Kernel weight (mg) and levels of significance '***' 0.001 '**' 0.01 '*' 0.05.

Seed oil content showed a significant positive correlation with kernel width ($r = 0.1$, $p \leq 0.001$). However, it negatively correlated with kernel weight (−0.01) and kernel length (−0.09) (Figure 1). The result further revealed a moderate (0.44) correlations between kernel width and kernel weight, and kernel width and kernel oil content (0.1), whereas oil content is negatively correlated with kernel weight (−0.1) and kernel length (−0.9) (Figure 1).

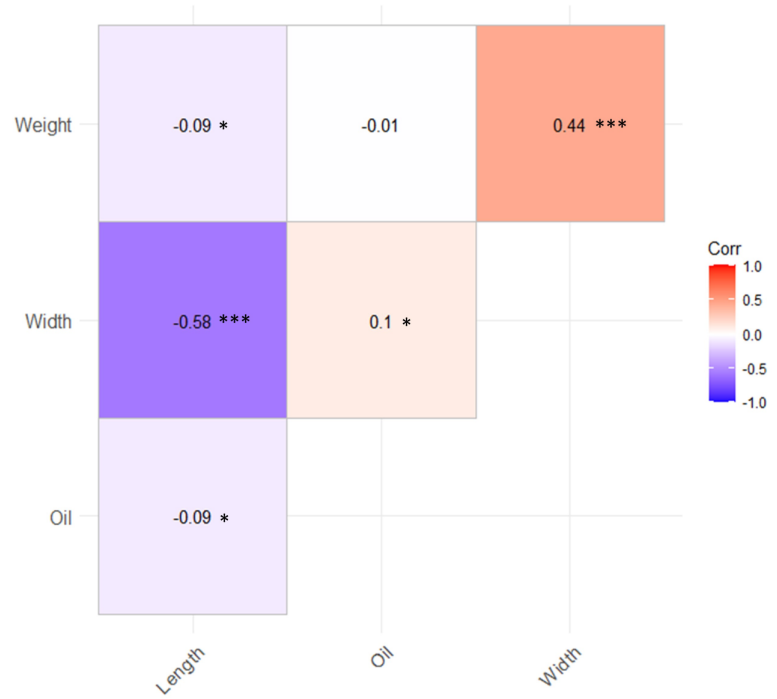

Figure 1. Correlation among four traits (Length = Kernel length, Width = Kernel width and Weight = Kernel Weight and Oil = Kernel oil content) of the 374 Shea tree lines. Colour in the boxes indicate proportion of correlations. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

3.2. Marker Coverage and SNP Distribution

The SNP calling pipeline generated 30,733 highly polymorphic SNP markers, of which 27,063 (88.1%) remained unmapped on the 12 *Vitellaria paradoxa* chromosomes. Only 7530 SNP markers (27.8%) of the mapped SNP markers were retained after filtering with >20% of missing data, <0.05 minor allele frequency (MAF) and utilized as input for the GWAS analysis.

Chromosome two had the highest number of markers (960 SNPs; Chr size = 74.5 Mb, ~13 SNPs/Mb) followed by chromosomes one (805 SNPs; Chr size = 82 Mb; ~10 SNPs/Mb), chromosome ten (780 SNPs; Chr size = 50 Mb; 10 SNPs/Mb), five and eight (650 SNPs; Chr size = 56.5 Mb; ~11 SNPs/Mb, and 645 SNPs; Chr size = 58 Mb; ~12 SNPs/Mb respectively). Meanwhile, chromosomes four (425 Chr size = 37 Mb; ~ 12 SNPs/Mb) and chromosome three (430 SNPs; Chr size = 38.6 Mb; 11 SNPs/Mb) had the lowest number of markers (Figure 2 and Table 3). This indicates a non-random distribution of SNPs with varying SNP frequencies on the 12 chromosomes of shea tree genome in Uganda. Further population structure and SNP data (Table 3) information are available in Odoi et al. [33].

Minor allele frequency (MAF) among the 7530 SNP markers varied from 0.03 to 0.50. The study further revealed a high level of heterozygosity within individuals (0.26) and markers (0.32) indicating a high non-random association of alleles at different loci that offer opportunity for association studies and allele transfer through marker-assisted selection of the population. The filtered markers were similar in their Polymorphic Information Content (PIC), ranging from 0.258 (chromosome 4) to 0.269 (chromosome 12) with a mean PIC of 0.26 across the chromosomes (Table 3).

There was a general high gene diversity (0.32) across the chromosomes with chromosome 12 being the highest (0.33) and chromosomes 4, 1 and 6 being the lowest (0.31 respectively). Structure analysis revealed that shea tree populations in Uganda are genetically grouped into two clusters of Eastern group and West Nile/Northern Uganda group. The Eastern cluster contributed the highest (57%) proportion of individuals and West Nile/Northern Uganda cluster (43%).

Out of the 12 chromosomes in the shea genome (Figure 2), only two (Chromosome 1 and 8) revealed significant loci. The result of Linkage disequilibrium (LD) indicated that 187,487 loci pairs in a physical distance of 605,450 bp. Of the total loci, 3.62% (6795) of them were in significant ($p < 0.01$) LD. The results further revealed that 87 (1.28%) loci pairs had $r^2 = 1$ (were in complete LD).

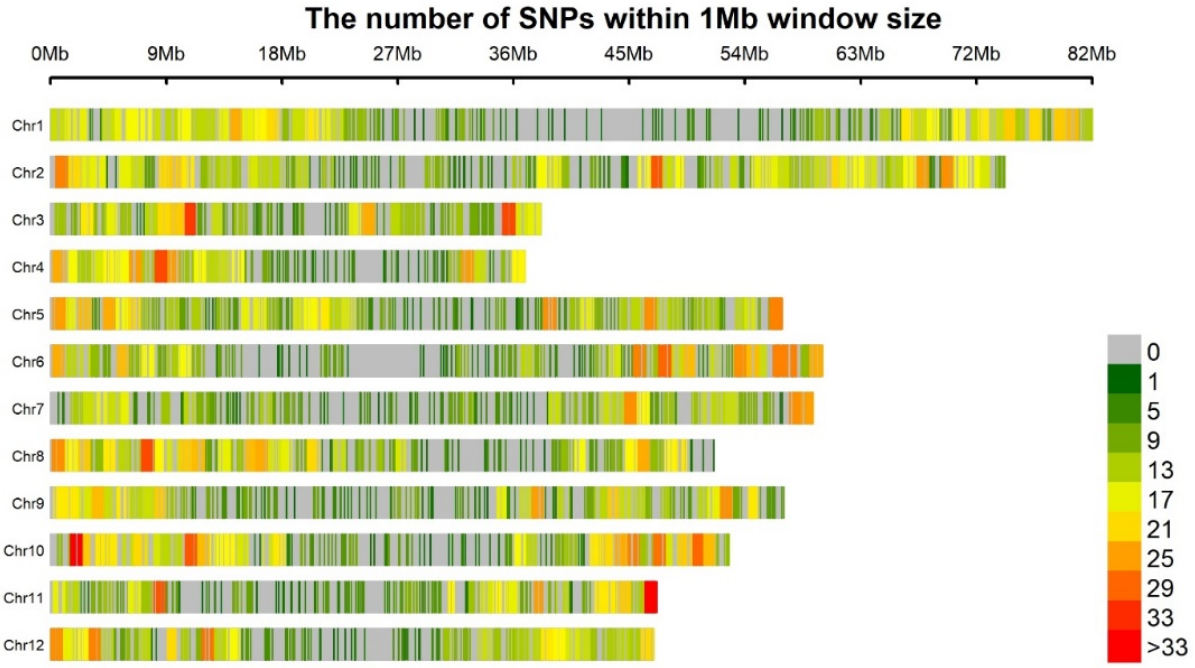

Figure 2. The number and size of SNPs within 1 Mb window size of *V. paradoxa* Subsp. *nilotica* genome.

Table 3. Number of SNPs for each chromosome before and after filtration and the average polymorphism information content for *V. paradoxa* subsp. *nilotica*.

Chromosomes	All SNPs [a]	Filtered SNPs	Chr [b] Size (Mbs)	PIC [c]	Gene Div [d]
1	2893	805	82	0.262	0.32
2	3450	960	74.5	0.260	0.32
3	1545	430	38.6	0.261	0.32
4	1527	425	37	0.258	0.31
5	2336	650	56.5	0.261	0.32
6	2210	615	58	0.259	0.31
7	2088	581	57.3	0.262	0.32
8	2318	645	48	0.260	0.32
9	2124	591	56.5	0.262	0.32
10	2803	780	50	0.265	0.32
11	1791	498	47.1	0.265	0.32
12	1978	550	46.9	0.269	0.33
Total/Mean	27,063	7530	652.4	0.260	0.32

[a] Single Nucluotide Polymorphism; [b] Chromosome; [c] Polymorphic information content and [d] gene diversity.

3.3. Marker Association for the Studied Traits

The association analysis was performed on shea seed-related traits and 16 significant markers were identified on chromosomes 1, 2, 3, 5, 6, 7, 8, 9, 10, 11 and 12 (Table 4 and Figure 3). Quantile-Quantile plots produced by displaying −log10 *p*-values against individual *p*-values revealed suitability of GWAS for the trait's connection in the shea tree genotypes. The association analysis was performed for percent oil content of each shea tree line in a location using the *V. paradoxa* reference genome (https://bioinformatics.psb.ugent.be/orcae) (accessed on 8 March 2022). There were differences between the observed

and expected values of the target traits, indicating a link between the phenotypic and SNP markers as indicated in Quantile-Quantile plots.

The seven SNP markers linked to shea nut oil yield (S1_60237300, S3_14843482, S4_32032310, S5_6275145, S8_41696703, S9_32689981 and S11_43126044) were located on chromosomes 1, 3, 4, 5, 8, 9 and 11 (Table 4) and were associated with high nut percent oil content estimated on dry matter basis. These seven loci explained an overall phenotypic variance of 12.4%, however, makers S8_41696703 and S9_32689981 had negative effects on seed oil content, although they explained the most (13.31% and 11.52% respectively) of phenotypic variation.

This current study revealed six significant SNP markers linked with shea kernel length (S3_11153087, S5_15524578, S6_46530240, S8_11121701, S11_8320549 and S12_32853547) located on chromosomes 3, 5, 6, 8, 11 and 12 (Table 4; Figure 3). The proportion of phenotypic variance explained by significant QTNs ranged from 6.5% in marker S5_15524578 to 14.6% in S6_46530240. The total phenotypic variance expressed by the trait was 0.095.

The GWAS revealed 8 genomic regions that were significant associated with kernel width. The 8 significant SNP markers linked to shea kernel width (S1_32402910, S2_47786838, S2_64059706, S7_3025298, S9_43700743, S10_50604452, S12_32853547 and S12_7613999) were located on chromosomes 1, 2, 7, 9, 10 and 12 (Table 4). Marker S12_32853547 contributed most (13.14%) of the phenotypic variation compared to the rest (ranging from 4.5%to 9.75%) (Table 4). The total phenotypic variation explained by the trait was 0.17.

In two significant SNPs (S1_30720144 and S8_43605016) located on chromosomes 1 and 8. Marker S8_43605016 contributed most (15.79%) of the phenotypic variation compared to S1_30720144 (9.21%) (Table 4). The total phenotypic variance in this trait was 0.061 Table 4: Figure 3).

Table 4. List of significant markers in a panel of 374 *Vitellaria paradoxa* genotypes indicating the genomic regions associated with studied traits.

Trait	Pσ [a]	Marker	Chr [b]	Position (bp)	Alleles	QTN Effect	LOD Score	−log10 [c]	r^2 [d]	MAF [e]
Oil content	4.03	S1_60237300	1	60237300	AA	0.83	3.39	4.11	6.61	0.12
		S3_14843482	3	14843482	AA	−1.06	5.67	6.49	11.80	0.14
		S4_32032310	4	32032310	AA	0.74	3.07	3.77	6.76	0.19
		S5_6275145	5	6275145	AA	0.68	3.21	3.92	5.11	0.15
		S8_41696703	8	41696703	TT	−1.06	5.93	6.76	13.31	0.17
		S9_32689981	9	32689981	CC	−1.22	5.38	6.19	11.52	0.09
		S11_43126044	11	43126044	CC	0.81	4.28	5.05	8.18	0.31
kernel length	0.095	S3_11153087	3	11153087	TT	−0.13	3.44	4.16	8.19	0.12
		S5_15524578	5	15524578	AA	0.10	3.37	4.09	6.51	0.32
		S6_46530240	6	46530240	TT	−0.25	4.71	5.49	14.55	0.05
		S8_11121701	8	11121701	GG	−0.14	3.16	3.87	9.08	0.10
		S11_8320549	11	8320549	CC	−0.13	3.74	4.48	7.28	0.10
		S12_32853547	12	32853547	CC	−0.18	3.96	4.71	9.31	0.06
kernel width	0.169	S1_32402910	1	32402910	CC	−0.19	4.42	5.20	9.75	0.12
		S2_47786838	2	47786838	CC	0.16	4.99	5.79	9.01	0.26
		S2_64059706	2	64059706	AA	0.17	4.73	5.52	8.28	0.13
		S7_3025298	7	3025298	CC	0.15	3.22	3.92	5.29	0.10
		S9_43700743	9	43700743	AA	−0.18	3.30	4.01	7.77	0.11
		S10_50604452	10	50604452	GG	0.19	3.81	4.55	8.69	0.10
		S12_32853547	12	32853547	CC	0.29	7.02	7.89	13.14	0.06
		S12_7613999	12	7613999	TT	0.12	3.44	4.17	4.47	0.20
kernel weight	0.061	S1_30720144	1	30720144	CC	−0.08	3.06	3.76	9.20	0.22
		S8_43605016	8	43605016	CC	−0.11	3.29	4.00	15.70	0.18

[a] Phenotypic variance [b] Chromosome, [c] the negative logarithms (−log10) of the *p*-values [d] squared correlation coefficient [e] minimum allele frequency.

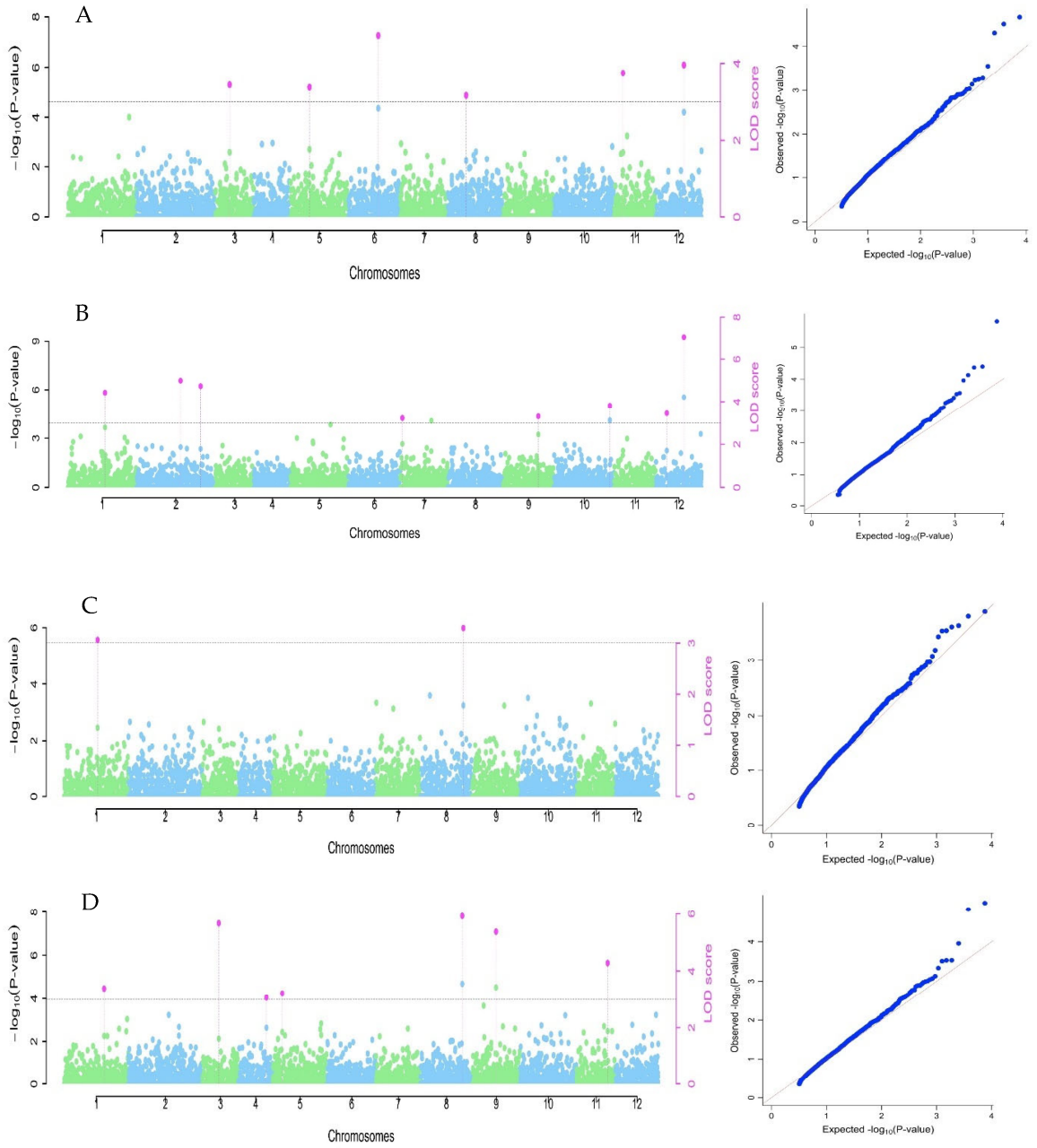

Figure 3. Genome-wide association of Kernel dry matter oil content in a panel of 374 *Vitellaria paradoxa* genotypes with 7530 SNP markers for kernel length (**A**); kernel width (**B**); kernel weight (**C**), and kernel dry matter oil content (**D**). The *y*-axis representing the *p*-value of the marker-trait association on a −log10 scale and the *x*-axis relates to the 12 shea tree chromosomes. The dots above the horizontal 5% Bonferroni threshold light dotted line indicates SNPs associated with QTL that condition the kernel traits.

Variations in the seed traits explained by the individual SNP markers (r^2) varied from 4.47% in kernel width to 15.79% in kernel weight for the significant SNPs, indicating that they represent major QTLs associated with oil yield and kernel physical parameters Alleles 'A' of marker S1_60237300; 'T' of marker S11_43126044; 'A' of markers S4_32032310 and S5_6275145 in oil yield, had the highest positive QTN effect (0.8255, 0.8098, 0.737 and 0.683 respectively) revealing higher association with increasing oil yield. Although most of the seed related traits indicated negative QTN effects, the allele which had the highest (0.2942) positive QTN effect was allele 'C' in marker S12_32853547.

3.4. Potential Candidate Genes

A total of 23 candidate genes were identified by linking the significant SNP regions with the *V. paradoxa* genome (Table 5). The annotation result revealed six putative genes associated with seed length traits. Among these were: Protein metabolism and gluconeogenesis on chromosome 12 and Protein translocation on chromosome 11. The proteins are well known to play important role in mediating plant seed oil biosynthesis [34] and early seedling morphogenesis and development.

From the kernel width, eight putative genes were discovered, of which three (Zinc Finger Transcription Factor located in chromosome 3, protein binding located on chromosome 9 and Protein metabolism and gluconeogenesis located on chromosome 12) had linkage with shea seed oil biosynthesis pathways. Zinc Finger has been associated with playing a key role in plant seed oil biosynthesis and accumulation [35]. All the two identified genes (ATP hydrolase located on chromosome 1 and Protein Kinase on chromosome 8) in kernel weight trait are important in the biochemical pathways of plant seed oil synthesis (Table 5). The hydrolysis process is performed by the FATB acyl-ACP thioesterase or by 3-ketoacyl-ACP synthase II (KASII).

Table 5. Gene annotation for the significant SNPs for shea seed related traits.

Traits	Marker	Chr [a]	Pos [b]	Gene ID	GO. [c]	Function
Kernel length	S3_11153087	3	11153087	Vitpa03g07900	IPR006968	UVB-sensing and in early seedling morphogenesis and development
	S5_15524578	5	15524578	Vitpa05g09840	GO:0005515	ion transportation and signal transduction
	S6_46530240	6	46530240	Vitpa06g28930	PTHR23155	Disease resistance (R)
	S8_11121701	8	11121701	Vitpa08g10570	GO:0004017	Predicts residues in protein biosythesis
	S11_8320549	11	8320549	Vitpa11g07160	PTHR33052	Protein translocation
	S12_32853547	12	32853547	Vitpa12g19540	GO:0003824	Protein metabolism and gluconeogenesis
Kernel width	S1_32402910	1	32402910	Vitpa01g21080	GO:0005515	Consensus disorder prediction
	S2_47786838	2	47786838	Vitpa02g27300	GO:0043190	Glutathione synthetase ATP-binding
	S2_64059706	2	64059706	Vitpa02g39460		Zinc finger
	S7_3025298	7	3025298	Vitpa07g02460	GO:0005515	Calcium signaling
	S9_43700743	9	43700743	Vitpa09g19440	PTHR14859	Protein binding
	S10_50604452	10	50604452	Vitpa10g25960	GO:0003677	Chromosome cohesion
	S12_32853547	12	32853547	Vitpa12g19540	GO:0003824	Protein metabolism and gluconeogenesis
	S12_7613999	12	7613999	Vitpa12g07520	GO:0055114	Catalyze the oxidation of alcohols to aldehydes and ketones
Kernel weight	S1_30720144	1	30720144	Vitpa01g20620	GO:0003676	Hydrolyze ATP
	S8_43605016	8	43605016	Vitpa08g25310	GO:0004672	Predict protein residues as disordered

[a] Chromosome, [b] Marker chromosome position and [c] Gene ontology.

This study further identified seven gene/protein families associated with the percent dry matter oil content in shea nuts: Acyl-ACP Thioesterase Fat B (FATB); Acyl-CoA-binding protein (ACBP); Long Chain Acyl-CoA Synthetase (LACS); Fatty acid exporter (FAX2); (3-ketoacyl-ACP synthase II (KASII) and Fatty acid desaturases (FADs) on chromosomes 1, 3, 8, 9, and 11 (Table 6).

Acyl-CoA-binding protein (ACBP) was identified on chromosomes 3 at loci S3_14843482 and chromosome 5 at loci S5_6275145 that govern plant seed oil accumulation (Table 6). The genes are 1 Mbs from their respective SNPs. Candidate Gene (CG) selection for shea nut oil accumulation is presented (Supplementary Table S2). The genes were annotated with protein-coding genes, using GO.OBO v2.1. The functions of these genes in enhancing shea oil content are explained in Table 6.

Table 6. Gene annotation for the significant SNPs for oil content traits.

Traits	Marker	Chr [a]	Pos [b]	Gene ID [c]	GO. [d]	Function
Oil content	S1_60237300	1	62536299	Vitpa01g27780 (Acyl-ACP Thioesterase Fat B (FATB))	GO:0004553	Consensus disorder prediction
	S3_14843482	3	14843482	Vitpa03g10720 (Acyl-CoA-binding protein (ACBP))	GO:0005515	Protein binding
	S4_32032310	4	32032310	Vitpa04g14070 Long Chain Acyl-CoA Synthetase (LACS))	G3DSA	Oxidoreductase activity
	S5_6275145	5	6275145	Vitpa05g04280 (Acyl-CoA-binding protein (ACBP))	GO:0000160	Transcriptional regulation of oil biosynthesis in seed plants
	S8_41696703	8	41696703	Vitpa08g23790 (Fatty acid exporter (FAX2))	GO:0008168	methyltransferase activity
	S9_32689981	9	32689981	Vitpa09g14250 (3-ketoacyl-ACP synthase II (KASII))	GO:0004672	Early noduling
	S11_43126044	11	43126044	Vitpa11g24760 (Fatty acid desaturases (FADs))		abiotic stress reduction

[a] Chromosome, [b] Chromosome position, [c] Gene identification and [d] Gene Ontology.

3.5. Linkage Disequilibrium (LD)

The distance of the first part of the LD decay before correlation coefficient, r^2 values reach zero was 2,312,772 bp, comprising of 375,037 marker pairs (Table 7). The r^2, decayed within 1–2 Mbps to a value < 0.01.

Chromosome 2 had the highest (46,764 marker pairs) LD followed by chromosome 1 (39,461 marker pairs), while chromosome 4 had the lowest (21,698 marker pairs). The total number of significant marker pairs was 11,940, with chromosome 2 having the most (1330) marker pairs and chromosome 4 (626) having the list.

Table 7. Distribution of LD marker pairs according to chromosomes.

Chromosome	Chr1	Chr2	Chr3	Chr4	Chr5	Chr6	Chr7	Chr8	Chr9	Chr10	Chr11	Chr12
# marker pairs	39,461	46,764	22,990	21,698	34,482	30,834	28,187	32,786	30,232	35,784	24,736	27,091

"#" in Table 7 represents the word "number".

The association analysis of the 374 highly heterozygous shea trees and the 7530 quality SNPs resulted in two most significant SNPs. Variations in the seed traits explained by the individual SNP markers (r^2) varied from 4.47 to 15% for the significant SNPs. Allele 'A' of S1_60237300 marker had the highest allele effect (0.83) revealing higher association with increasing oil yield in shea tree, followed (0.81) by allele C in marker S11_43126044 and allele A (0.68) in S5_6275145 marker. Furthermore, for kernel width, allele 'C' in S12_32853547 also had a moderate effect (0.29). None the less, allele 'T' in marker S6_46530240 revealed the highest (−0.25) negative effect for the studied traits. The LD of significant SNP loci revealed six loci, three each on chromosomes 1 and 8, indicate that the markers had higher LD ($r^2 > 0.8$). The markers in the rest of the chromosomes had a considerably low LD ($r^2 < 0.5$).

4. Discussion

4.1. Phenotypic Data

Shortening juvenile maturity period for early fruiting and increasing oil yield per acre and quality aspects in shea oil are the major concerns in the shea industry. This study aimed at selecting shea parent materials for future breeding programme and bring about

farmer solutions by establishing Multi-location Breeding Seed Orchards as a short-term remedy to quality source of shea tree planting materials.

There was variation in the seed trait characteristics in the shea accessions. The results for seed traits indicated a significant variation within the populations and a non-significant variation among the populations. Such non-significant variation observed among the populations is important in breeding for varieties that can easily be adapt across all the geographical range in Uganda. Furthermore, any newly bred variety shall be acceptable by all the communities within the shea parkland in Uganda. With the reliable heritability, selecting traits with marker association for high oil yield in shea tree will result in good genetic progress of the species. Earlier studies by Gwali et al. [36] and Okullo et al. [37] reported similar oil content (52.26%) in the species with this study (53.5%). The results of this study were slightly higher due to the participatory selection which suggests a potential genetic gain from selection given several genotypes with known higher yield (69.77%). Such results can be important in assessing the G × E interactions for some traits.

4.2. Candidate Gene Scan in the Oil Content Traits

The shea genome revealed associated SNP markers, important for identification of QTL regions controlling the variations of the quantitative traits [1]. Most important of this was the identification of the seven significant SNPs located close to genes that encode different proteins related to plant metabolic mechanisms and transport of biosynthetic products and materials.

GWAS can increase detectability of genomic association in plants [38]. In fact, GWAS has gained increasing popularity as a tool for analysing complex traits in plants [39]. It has been used to reveal the genes controlling polygenic traits including the genetic loci associated with the trait of interest in fruit trees [20]. Kumar et al. [40] used Mixed Linear Model statistical model for GWAS to study six commercial fruit traits in apple seedlings suggesting the potential of the tool in shortening the breeding cycle of tree species like shea.

The advances in omics technologies have enabled researchers to identify candidate genes that promote improvement of associated traits of commercial importance in plants. Earlier very few studies were conducted in determining these functional genes in shea tree [6,41,42]. However, recent sequencing of shea reference genome [8] and identification of genes in shea tree [1], has paved new avenues of genomic studies in the species. Studies for biochemical pathways of oil synthesis in plant seeds have been advanced [43] and several gene expression and enzyme activities in plant seed oil accumulation fronted [44]. Interestingly, 45 seed oil biosynthesis genes were reported in shea tree genome [8]. This study discovered 23 such genes that are potentially associated with shea nut oil biosynthesis pathways (Tables 5 and 6). Of all, acetyl-CoA carboxylase (ACC) is notably the major enzyme catalyst in shea oil biosynthesis [1]. Earlier studies revealed 9 gene copies of Ketoacyl-ACP synthase (KAS) in shea, 6 of these were also reported in *Theobroma cacao*, suggesting their contribution to the increased lipid content in shea than in cocoa. Other genes with higher number of copies in shea include: FAD2, FAD3, and LACS genes [34]. The biological effect of LACs genes discovered on chromosome 4 includes modification of fatty acids chain lengths along the plant oil biosynthesis pathways [45].

The validity of the interrelations among the traits of study was assessed using correlation matrix. It was observed that oil content in *V. paradoxa* was only moderately correlated with kernel width. As observed in the biochemical functions of the genes conditioning the seed related traits that condition seed development and seedling germination. In concordance with this study, Jasinski et al. [46] reported that plant seed oils in angiosperms act as an important reserve of carbon and energy soon after seedling germination until it starts photosynthesis. The presence of proteins suggests their role in promoting shea bioactive functions that condition high oil yield in the species. Previous studies in shea by Lovett and Haq [3] revealed similar proteins that play a major role in oil biosynthesis pathways in oil plant seeds. In fact, Wei et al. [1] predicted presence of more genes associated with oil metabolism in the shea tree genome. Another study Hale, et al. [8] predicted expansion

of gene families involved in stearic acid biosynthesis in shea tree which agrees with this current study.

The significant candidate gene for oil content in this study, Acyl-CoA-binding protein (ACBP) was located on chromosome 3 and 5 associated to markers S3_14843482 and S5_6275145 with annotated transcriptional regulation of oil biosynthesis in seed plants. The enzyme plays a role during early fruit formation and play multiple functions such as: tissue growth, cellular trafficking, and physiological processes [47]. The enzymes are usually in the nucleus, are expressed predominantly in developing seeds during maturation. Similar findings were also reported in Arabidopsis thaliana seeds [35]. Moreover, the strong association with annotated function and Acyl-CoA-binding protein (ACBP) genes could be taken advantage of to breed for high oil yield shea tree varieties in Uganda. The biological effect of ACBP includes lipid metabolism, cellular signalling for stress management and disease resistance in plants [48]. This gene encodes metal ion binding enzyme, mostly carbonic anhydrase and alcohol dehydrogenase enzymes that contain zinc as part of their molecule. This zinc finger gene family has been reported to play a major role in oil biosynthesis pathways in the oil palm [48].

The third significant candidate gene was Ketoacyl-ACP synthase (KAS) gene. The gene plays a major role in lipid biosynthesis pathways in shea nuts, thereby increasing oil content in the species [8]. Similar findings on Chinese seed oil shrub, *Paeonia lactiflora* have been advanced [49]. KAS II for example is key in the biosynthesis pathways of fatty acids in plant seeds [50] and early nudling. The Fatty acid exporter (FAX2) genes play a major role in biosynthesis transportation and significantly increases oil content in shea tree. In another study, Janik et al. [51] reported the involvement of FAX in *Chlamydomonas reinhardtii* oil synthesis, similar to this current study. On the other hand, Acyl-ACP Thioesterase Fat B (FATB) was also discovered in other plants like *Koelreuteria paniculata* known to be involved in the synthesis of saturated fatty acids in the species [52], which is in line with this current study. Further still, FADS genes reported in this study, is responsible for the synthesis of unsaturated fatty acids and important for plant development and response to biotic and abiotic stresses [53]. The report therefore confirms the findings in this current study for the role played by the genes in significantly controlling high oil yield in *V. paradoxa* Subsp. *nilotica*.

4.3. Candidate Gene Scan within the Seed Related Traits

The seed related traits with significant SNPs under this study were having linkage with oil yield in shea nuts. The proteins responsible for oil biosynthesis identified in kernel length trait was associated to marker S8_11121701 in chromosome 8. In kernel width trait, S1_32402910 marker discovered on chromosomes 1 had proteins which are linked with processes involved in plant seed oil biosynthesis pathways [24]. For kernel weight trait, S1_30720144 and S8_43605016 markers in chromosomes 1 and 8 were associated with the proteins responsible for oil biosynthesis. In fact, Wei et al. [1] reported similar results with QTLs identified at different locations of shea tree genome. The proteins play a major role in ATP hydrolysis and prediction of protein residues as disordered, during plant seed oil biosynthesis processes. The first evidence was reported by Botha et al [54] linking the functions of the genes to seed development and early seedling growth in *Ricinus communis* oil seeds. The genes reportedly play a major role during seed drying by concentrating inorganic phosphate while de-concentrating the extracellular pyrophosphate which inhibits formation of minerals [55].

4.4. Linkage Disequilibrium (LD)

The LD reveals the evolutionary and demographic events of a population and in mapping genes that are associated with quantitative traits. The implication of this association is that the marker loci contain a causal variant in LD with the identified marker by GWAS. This is further revealed by the small blocks in heat map where the causal variant(s) can be sought. Therefore, it is important to increase our understanding of co-evolution of linked

sets of genes. A wide range of LD ($r^2 > 0.2$) in the shea tree population used in this study, was also found in citrus [56]. Such a range of LD is expected in heterozygous outcrossing species like shea tree [56]. The mean r^2 (0.2) in the shea tree population indicated that the markers in the shea tree population is sufficient for genomic selection as LD is maintained by selection. This study describes the potential candidate genes associated with oil yield in shea tree. It further describes the locations of these significant genes in the chromosomes for any further verification. The significant association was discovered on chromosome 1 and 8 for seed related and oil yield traits, explaining 58% of the phenotypic variation.

Inbreeding creates LD owing to the recent common ancestry by increasing the covariance between alleles at different loci. This, therefore, offers opportunities to design association studies and allele transfer using marker-assisted selection [57,58]. LD therefore presents an opportunity in this study in that if an upper positive selection of preferred traits in shea tree is conducted, it will accelerate the frequency of alleles conferring the preferred trait during breeding. This is because as the linked loci strongly remain in LD with that allele.

4.5. Marker Assisted Selection in Shea Tree

The oil content candidate genes identified in this present study will be cross validated in the established multi-locational trials in NgetaZARDI and NASARRI to determine the ideal molecular markers for enhanced shea tree oil content breeding programs in the country. This is possible by stacking the novel genes into the shea tree genotypes with high oil content using marker-assisted selection. A combination of novel QTLs can further enhance oil content in the shea tree. Furthermore, determination of the allelic status at the markers with significant alleles for oil content will enable the selection of those significant markers for shea oil yield improvement in Uganda. The variations observed in the traits within the location but not across confirms that the species is highly outcrossing [42] or segregating population. The Analysis of variance (ANOVA) in Table 3 indicates a significant variation within the population and this further re-affirms the level of variation in the species. The result of this study points to potential QTNs that explain the genetic variations in the population. In this study, the putative major QTN for oil content explains up to 58% of the phenotypic variance in the species.

Developing MAS options that use the identified molecular markers linked to traits of interest is of importance for speeding the selection process in shea tree with high oil content [59]. The use of significant SNP markers identified through GWAS analysis are important for performing MAS for shea tree breeding. In fact, the application of MAS in shea tree breeding is now made easy with the availability of genomic information on the species [8] coupled with sequencing transcriptome that now makes it possible to align them with the identified markers of interest [1,8]. The six identified markers (S1_30720144, S1_32402910, S1_60237300, S8_11121701, S8_41696703 and S8_43605016) in this study could be applied in MAS for enhanced oil content in *V. paradoxa* Subsp. *niltica*. The MAS can play a very important role in this kind of trait useful for early nursery selection of late expressing traits in the species, and therefore, by performing MAS at seedling stage (far earlier than the juvenile maturity) will greatly reduce the breeding circle.

In this current study, the application of MAS will enable the selection of S1_30720144, S1_32402910, S1_60237300, S8_11121701, S8_41696703 and S8_43605016 markers linked with high oil content genes in the shea nuts. Selection of genotypes with a combination of preferred traits accumulated in one accession would therefore augment the process of shea tree improvement. More value to the communities as an upstream selection would also require prioritizing the genotypes with significant SNPs but from sweet pulped ethnovariety to meet the community's food and nutrition requirement [60,61]. The availability of markers linked to the identified genes will even make it possible to take the advantage of MAS in identifying heterozygous genotypes and therefore apply positive MAS selection for the alleles resulting in a very informative phenotypic traits selected for. On the other hand, MAS could also be applied in negative selection in order to introgress the target trait.

5. Conclusions

The study of marker trait association presents an important step towards identifying the genomic regions associated with the traits of interest to further marker-assisted breeding in shea tree. The current study identified 23 putative markers associated with oil accumulation in shea nut. Candidate genes located on chromosomes 1 and 8 were the most important genes in oil biosynthesis and accumulation in *V. paradoxa*. It is important to note in this study that the position of the seed traits related candidate genes were in agreement with the locations of the oil yield hotspots on chromosomes 1 and 8. This is in support of the need for application of MAS in shea tree and presents the first ever breakthrough in identification of chromosomes 1 and 8 hotspots in the improvement and breeding of shea tree in Uganda for increased oil yield. This study therefore presents the first ever genomic information on associated genes responsible for *V. paradoxa* Subspecies *nilotica* nut oil biosynthesis. The results therefore establish the foundation for explaining the molecular mechanisms of oil biosynthesis for *V. paradoxa* Subspecies *nilotica*. The markers and their linked genes provide a significant resource for improving oil content in the species. The study therefore sets pace for genomic assisted breeding in *V. paradoxa* Subsp. *nilotica* and also broadens our understanding in the role of genomic approaches in advancing yield component traits. The findings of this study will contribute to the initiation of shea breeding for increased oil yield in Uganda. This information could also be used for future gene pyramiding, increasing genetic gain, trait introgression, marker-assisted selection, and selection of parental lines for multiplication and generation of putative genotypes for shea tree breeding programs in Uganda. The study further presents gaps for future validation of the hot spot regions identified on chromosomes 1 and 8.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/horticulturae9070811/s1, Table S1: Passport data for the selected shea tree families used in this study, with details of their location, tree identification number, geographical coordinated and the details of the farmer on whose farm the tree is located. Table S2: Candidate genes at QTL region searched within approximately ± 20 Kb region of significant SNP markers. The identified genes is believed to be playing an important part in oil yield variation in shea tree.

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Conflicts of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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