Population Structure Analysis of Cashew (*Anacardium occidentale* L.) in Kerala -the Region of Introduction in India

VG Jayalekshmy*, K Rajmohan, Jayaprakash Naik, Gregoryzacharia, Dhanya Jayaseelan and Akhila Gopinath

Kerala Agricultural University, College of Agriculture, Vellayani-695 522, *Kerala*

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Cashew (*Anacardium occidentale*) entered the Malabar Coast of Kerala in 16th century along with the Portuguese. More than five centuries of existence in this area has acclimatized this crop to this region. The present study reveals the population genetic structure of cashew in Kerala through AFLP marker analysis. The germplasm accessions specific to the locality were used for the study. AFLP analysis gave moderately high Gst (0.23) and Fst (0.31) values which showed moderate to high genetic diversity. Genetic load developed by the cross pollination and use of non descript seeds have widened the genetic variation in addition to the few introductions. Population structure analysis confirmed the uniqueness of the Kannoore population of cashew in the northern districts of Kerala near the port of entry of the initial introductions. This study shows that moderate to high genetic diversity observed in cashew populations of Kerala has raised this region of introduction to the status of secondary centre of origin of cashew.

Key Words: AFLP, Cashew, Genetic diversity, Population structure

Introduction

Kerala had established itself as a major spice trade center since 3000 BC and had direct contact across the Arabian Sea with all the major sea ports in Red Sea and the Mediterranean, extending to the Far East (Menon, 1967). 'Muziris' the ancient port city of Malabar was said to be the greatest trading centre of the east in the ancient world. It had productions of pepper, cinnamon, cardamom, ginger and other spices. Through this port, south India had trade links with the West Asia, and Europe (Craig, 1997). Vasco da Gama's voyage to Kerala from Portugal in 1498 was largely motivated by Portuguese determination to break the Arabs' control over trade of spices grown in Kerala. In return the navigators provided new crops like cashew, rubber cocoa etc.to the west coast (Ravenstein, 1898).

Cashew is one of the few fruit crops normally grown from seeds. This is a native of eastern Brazil and introduced to the east along with other commercial crops like rubber, coffee, tea etc. by the Portuguese nearly five centuries back. The first introduction of cashew in India was made in Malabar coast from where it spread to other parts of the country. The vernacular name "kasu mavu" originated from the Portuguese name'caju'. Since the Portuguese entered kerala through the ports in Kochi and Kozhikode these areas are presumed to be the portals of cashew entry in Kerala. The trees were well adapted to the region, and became acclimatized. The commercial exploitation began from the early 1960's and due to the absence of high yielding varieties and suitable propagation techniques, non descript seeds and seedlings were used for planting purposes. Because of its adaptive ability in wide range of agro climatic conditions cashew has become a crop of high economy and attained the status of an export oriented commodity bringing considerable foreign exchange to the country. In addition to the few early introductions, import as seeds by the foreign traders - handed over to planters also contributed the genetic diversity of the cashew plantations in Kerala.

Success of the crop improvement programmes depend on the variability available and the genetic diversity existing in the populations. Germplasm maintained in a particular area includes all the variable accessions with unique characters prevailing in that locality (Frankel, 1984). Characterization and cataloguing of these types is a prerequisite to select the superior ones for further crop improvement programmes. Analysis of genetic diversity in germplasm collection can facilitate reliable classification and identification of core groups with possibility to use for specific breeding programmes (Brown, 1989).

*Author for Correspondence: E-mail: jayavgj@yahoo.com

In Kerala more than 50% of the cashew cultivation is concentrated in northern and central districts (Fig.1). In this state cashew germplasm is maintained at three locations under Kerala Agricultural University. The regional Agricultural Research Station at Pilicode in Kannoore district maintains the collection from northern districts of Kerala. The research station at Anakkayam in Malappuram district collects and maintains novel genotypes from the central districts. Cashew research station at Madakathara maintains germplsm collections from central and southern districts mostly from Kottarakara and Kollam. Germplasm accessions from these three field genebanks were undertaken for the study.

Molecular markers had been proven as a powerful tool for analyzing genetic diversity in cashew (Mneney *et al.*, 2001; Dhanraj *et al.*, 2002; Archak *et al.*, 2003a, 2003b and 2009; Thimmappiah *et al.*, 2009; Jayalekshmy *et al.*, 2010). However the previous works were based on RAPD markers and have reported low genetic diversity (Mneney *et al.*, 2001; Dhanraj *et al.*, 2002). Archak *et al.*, (2003b) have compared RAPD, ISSR and AFLP markers for their efficiency in discriminating cashew genotypes and found AFLP markers to be the most efficient.

The major objective of molecular ecological research is to study the genetic structure of populations (Bensch and Akeson, 2005). Evaluating population structure is of considerable interest because it is a precursor to answering many other questions such as estimating migration, identifying conservation units and specifying phytogeographic patterns (Manel *et al.*, 2005). So far AFLPs have been used in a range of applications to assess population structure from small to large scales (Hardy *et al.*, 2006; River-Ocasio *et al.*, 2006; Archak *et al.*, 2009). Bayesian analysis of population structure using the model based approach of Pritchard *et al.*, (2000) provides a stratification pattern consistent with the observations derived from the traditional Fst approach.

Fig. 1. Location site of cashew populations under study

Archak *et al.* (2009) have utilized these markers to asses the population structure of Indian cashew. Kerala is the state with earliest histories of cashew introduction and popularization. Even now it has the largest area under cashew cultivation. In this study cashew germplasm of Kerala is being assessed based on AFLP markers to study the extent of variability and to know the population structure of cashew in Kerala which will throw light to the early introduction and spread of cashew in Kerala

Materials and Methods

Sixty cashew accessions maintained in the three research stations were selected for the study. Depending on the number of original collection 30 accessions were selected from Madakkathara and 15 each from Anakkayam and Pilicode (Table 1). The accessions from one location were considered as one population. These accessions were genetically analysed with AFLP markers generated from 5 primer pairs.

Plant DNA Isolation

A novel and efficient protocol for isolation of good quality DNA using CTAB (Cetyl trimethyl ammonium bromide) was standardized with extraction buffer (3% CTAB, 100mM Tris HCl (pH-8.0), 20mM EDTA,1.4M NaCl, 3%[w/v] Polyvinyl polypyrrolidone (PVP) and 1% [v/v] β-mercaptoethanol The DNA was pelletted in ethanol and dried pellet dissolved in100µl TE buffer. Good quality DNA, which could be used for AFLP was obtained by this cost effective method.

AFLP Analysis

Genomic DNA (60ng) was digested with *EcoRI & MseI* and double stranded adaptors legated to the fragments ends. This was followed by a pre-amplification step using non-selective primers. Selective amplifications were performed on the pre-amplified fragments mixture using a total of six primer combinations. Primers and other reagents provided in the AFLP Kit (Invitrogen) were used following their protocol. Only the *EcoRI primer* was radiolabeled with γ [32P] ATP. Amplified products were separated by denaturing 4% polyacrylamide gel electrophoresis (PAGE) visualized by autoradiography

Table 1. List of cashew accessions
(Accessions from Cashew Research Station Madakathara (1-30), Accessions from cashew research stations Anakkayam (31-45) and Pilicode (46-60))

Sl. No.	Name of accessions	Sl.No.	Name of accessions
1	Rajamundry	31	ALGD1-1
2	K-3-2	32	ABD-2-2
3	KTR-1-254	33	T-8-A
4	Anakkara	34	BRZ-18-1
5	Kottarakkara	35	NLR-1-1
6	P-10-1	36	K-12-1
7	Ullikkal-6	37	PTR-2-1
8	A/26/2	38	UL-3-2
9	Kelakam-1	39	CRPT-1-1
10	B-2-48	40	NL-2-1
11	Payam-1	41	K-27-2
12	Kankady	42	K-28-2
13	Peravoor-2	43	T-30-4
14	K-30-1	44	ABD-2-1
15	Pu-2	45	K-6-2
16.	Vettore-56	46	PCC-9
17	PU-8	47	Mdp-2
18	Rajapalayam	48	BLM-3
19	PTR-1	49	TDB-1
20	Payam-2	50	Thumbassery
21	P-3-2	51	PCK-1
22	Vapala	52	ADR-2
23	Ambayathode	53	CGR/1G-2
24	K-2	54	PU-1
25	B-2-44	55	AR-2
26	Ullikkal-1	56	PRL-1
27	K-3-1	57	MK-1
28	K-1	58	MLR-1
29	Anandappilly	59	KD
30	K-10-1	60	PT-1

& phosphor imaging methods. Five AFLP primer pair combinations were used in the study these are

1. E-ACG, M-CAT 2. E-AAG, M-CAA
3. E-ACA, M-CAT 4. E-ACT, M-CTC
5. E-AGG, M-CAC 6. E-ACT, M-CAC

Data Analysis

Each AFLP band was treated as a unit character and scored manually using independent binary codes (1 for presence and 0 for absence) sand 1/0 matrices were generated. Independently scorings were repeated and conflicting data were eliminated from the analysis. The resulting binary data matrix was used to compute a parwise distance matrix using the DICE coefficient utilizing the software WINBOOT (Yap and Nelson, 1996). The distance matrix was subjected to cluster analysis employing UPGMA using SAHN (Sequential Aglomerative Heirarchial and nested cluster) module of the software NTSYS pc (Rohlf, 2002). The robustness of the clusters was estimated by performing a bootstrap analysis on 1000 data replicates using WINBOOT (Yap and Nelson, 1996). Principal coordinate analysis was conducted using the EIGEN procedure of NTSYS pc in order to cluster the genotypes. The value of each of the five primer pairs was assessed using two indices PIC which is same as diversity index (DI) and Rp (Prevost and Wilkinson, 1999). PIC was estimated as PIC= $\sum(1\text{-pi}^2)/n$ where n is the number of band positions analyzed in the set of accessions, pi is the frequency of the i^{th} pattern. The ability of the primers to distinguish between accessions was assessed by calculating their resolving power as, Rp= $\sum$Ib where Ib is the band informativeness and Ib=1-(2x0.5-pi) where pi is the proportion of individuals containing the band (Prevost and Wilkinson, 1999). Marker Index was calculated as the product of DI and EMR (Effective multiplex ratio). EMR of a primer is defined as the product of the fraction of polymorphic loci and the number of polymorphic loci for an individual assay (Milbourne *et al.*,1997).

POPGENE software version 1.32 (Yeh *et al.,* 1997) was used to estimate the percentage of polymorphic loci, observed number of alleles, shanon information index (I) (Lewontin, 1972), Nei's gene diversity (h) (Nie, 1973) and geneflow (Nm) from the matrix generated from AFLP markers. Arlequin version 3.11 was used to evaluate the degree of genetic differentiation among populations using Gst statistics and Fst statistics.

The software Structure version 2.2 was used for inferring population structure (Pritchard *et al.,* 2003). This programme facilitates testing the veracity of the hypothesis about the number of populations by calculating the probability of the data for each hypothesis. The programme assumes that the markers are not in linkage disequilibrium and that they exhibit co-dominance, however there is a provision to estimate population structure based on the dominant markers as well. This was done by designating AA/Aa individuals as (1,-9), and an aa individual as (2,-9), where-9 was the value for the missing data. The programme" STRUCTURE" was run for many values of'k' for a burn in period of 10,000.

Results

A total of 277 amplicons were produced by the six primer pairs and of these 163 amplicons were polymorphic giving 58.84% polymorphism. The number of products produced by each primer pair combination and the number of polymorphic products is given in Table.2. Primer pair E-ACG+M-CAT gave highest number of products with

highest polymorphism. The total polymorphism ranged from 40 to 82.35 %. This primer pair gave the highest value for resolving power and marker Index (Table 2). So this primer pair can be effectively used for analyzing cashew genotypes with AFLP.

Table 2. AFLP primers, number of amplicons and percentage of polymorphism

Sl. No.	Primer Name	Number of products	Number of polymorphic products	Polymorphism (%)	PIC	Rp	MI
1	E-ACG+M-CAT	68	56	82.35	0.8432	29.94	29.94
2	E-AAG_M-CAA	66	28	42.42	0.8745	15.85	15.85
3	E-ACA+M-CAT	58	26	44.82	0.770	13.18	13.18
4	E-ACT+M-CTC	49	37	75.51	0.862	18.80	18.80
5	E-AGG+M-CAC	36	16	44.40	0.803	8.15	8.15
TOTAL		277	163	58.84			

PIC - Polymorphic information content; RP - Resolving Power; MI - Marker Index

The dendrogram constructed based on the AFLP markers (Fig. 2) indicated that the accessions selected for the study had below 83% similarity. The accessions from germplasm maintained at Pilicode were placed together. These accessions clustered with the accessions from other two stations only at 21% similarity. Five accessions from Anakkayam also clustered along with them with 40% similarity. Accessions from Madakathara and Anakkayam clustered at 30% similarity. Nearly 80% similarity was shown between the accessions IG-2 & M-1 (53 & 57) and KD & PT-1 (59 & 60). These accessions were from Pilicode. The study of genetic divergence with AFLP markers showed no duplicates in the germplasm maintained at three locations. Only below 41% similarity was noted among the different collections. Thumbassery is a dwarf accession with trailing branches and a unique morphotype but this variety showed a close association with TDB-1 another member of the same population. The bootstrap values above 50 given at the indices of the dendrogram. Principal coordinate analysis confirmed the clustering in dendrogram (Fig. 3).

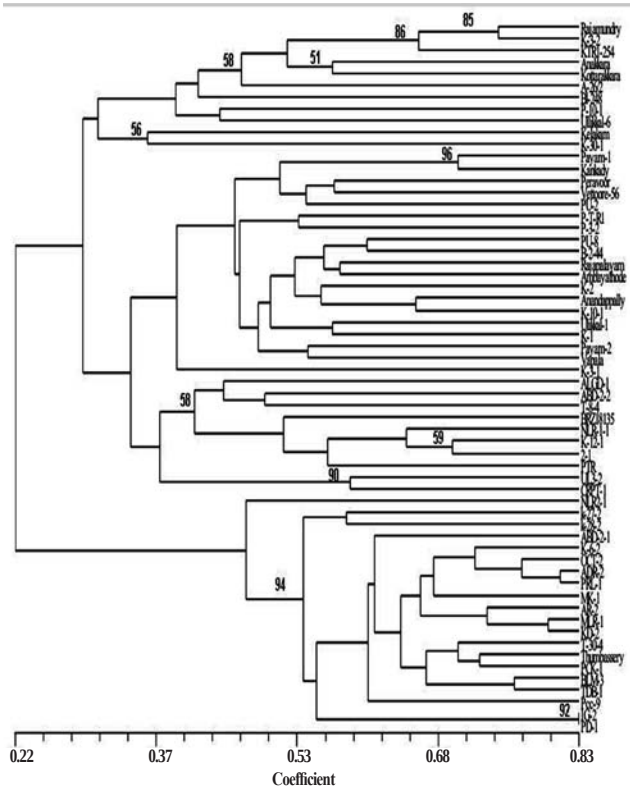

Fig. 2. Dendrogram constructed based on the AFLP scores (Bootstrap values above 50 at nodes)

In this study collections from the three centers were analysed as three populations, since they are the representatives from three distinct geographic zones of Kerala. Germplasm accessions from Madakathara (representing South and Central Kerala) was taken as population-I, accessions from Anakkayam (representing Central Kerala) as Population II and the accessions from Pilicode (representing North Kerala) was taken as Population III. Population analysis using Popgene was done and the effective number of alleles (Kimura and Crow, 1964), gene diversity (Nei, 1973), Shanons information index (Lewontin, 1972), the number of polymorphic loci,G_{st} and gene flow (Nm) across the population calculated using POPGENE and given in Table 3. Neis genetic diversity is highest for population III and Shanon information index was high for population I. Neis genetic identity and distance of the populations derived in the same analysis is given in Table 4. The AMOVA and Fst values calculated using the software ARLEQUIN is given in Table 5 and Table 6, respectively. Significantly high F_{ST} value is obtained in the analysis. Genetic difference among the three populations were significant with average F_{ST} 0.30. Largest pair wise

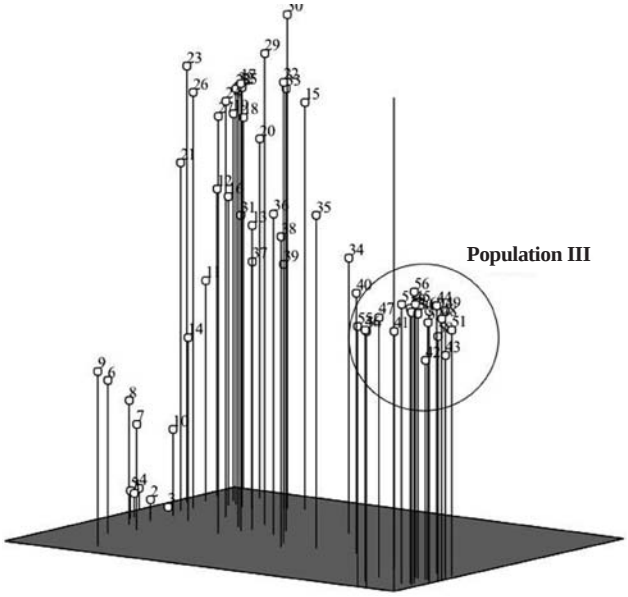

Fig. 3. Ordination projection of cashew genotypes showing the clustering of genotypes in Population III

F_{ST} was noted between population I and III. The lowest level was between I and II. (Table 4). The AMOVA from Population analysis showed that the within population variability was more than the inter population variability (Table 5). In structure analysis independent runs performed for different 'k' values (2, 3, 4) (Fig. 4) and in all the runs, population III was intact with least admixture. Fig.5 shows the triangular distribution at K=3 in structure analysis.

Discussion

The population analysis showed that the Neis genetic diversity across the populations was 0.23. Nies (1973) have reported that this value ranges from 0-0.5 for moderate diversity. Hence the diversity of Kerala cashew can be considered as moderate the same was reported by Archak *et al.*, 2003(b) with respect to Indian cashew.

AFLP based Neis genetic diversity estimates of some of the tree species in their native habitat were also found to be around this value as 0.225 for Belgian wild apple (Coart *et al.*, 2003) 0.243 in Chinese tree hibiscus (Tang *et al.*, 2003), 0.29 in Flemish oak (Coart *et al.*, 2002), 0.342 in Nicaraguan pines (Diaz *et al.*, 2001), 0.193 in Australian mangrove (Maguire *et al.*, 2002).

Table 3. Population analysis of the cashew genotypes

Population	h	I	NPL	PPL	Gst	Nm
I	0.241	0.3728	135	88.24		
II	0.1920	0.3008	110	71.90		
III	0.833	0.1282	43	28.10		
Across population	0.23	0.368	151	98.69	0.20	1.99

I- Accessions in Madakathara II- Accessions in Anakkayam III- accessions in Pilicode

h- Neis genetic diversity I- Shannons information Index

NPL- Number of polymorphic loci PPL- Percentage of Polymorphic loci

Table 4. Nei's Original Measures of Genetic Identity and Genetic distance

pop ID	1	2	3
1	****	0.9482	0.8753
2	0.0532	****	0.9488
3	0.1332	0.0525	****

Nei's genetic identity (above diagonal) and genetic distance (below diagonal).

Table 5. AMOVA of the populations

Source of variation	d.f.	Sum of squares	Variance components	Percentage of variation
Among populations	2	331.683	7.89637 Va	30.75
Within populations	57	1013.733	17.78480 Vb	69.25
Total	59	1345.417	25.68116	

Fixation Index FST : 0.30748

Fst measures the heterozygote defecit relative to its expectation under Hardy – Weinberg equilibrium (Hartl and Clark, 1997). For the interpretation of Fst it has been suggested that a value between 0.05 and 0.15 moderate differentiation 0.15-0.25 greater differentiation and values above 0.25 very great genetic variation (Wright, 1978; Hartl and Clark, 1997). In this study the fixation index was 0.3078 (Table 5). So the populations can be expected to have vast variation. Gene flow in this analysis is substantially high 1.9876 denoting a migration between sub- populations.

The dendrogram constructed based on the AFLP markers showed that none of the cashew accessions had a similarity beyond 80% which denotes that the collection doesn't have any duplicates. The cashew genotypes from the population III clustered together at 60% similarity this showed the uniqueness of the cashew in this part of Kerala (Kasargod and Kannoore districts) the clustering of the accessions from this region had a bootstrap value above 94 (Fig. 1). The other two populations did not show a separate clustering. Ordinate projection of the genotypes also shows the clustering of the genotypes of population III (Fig. 2).

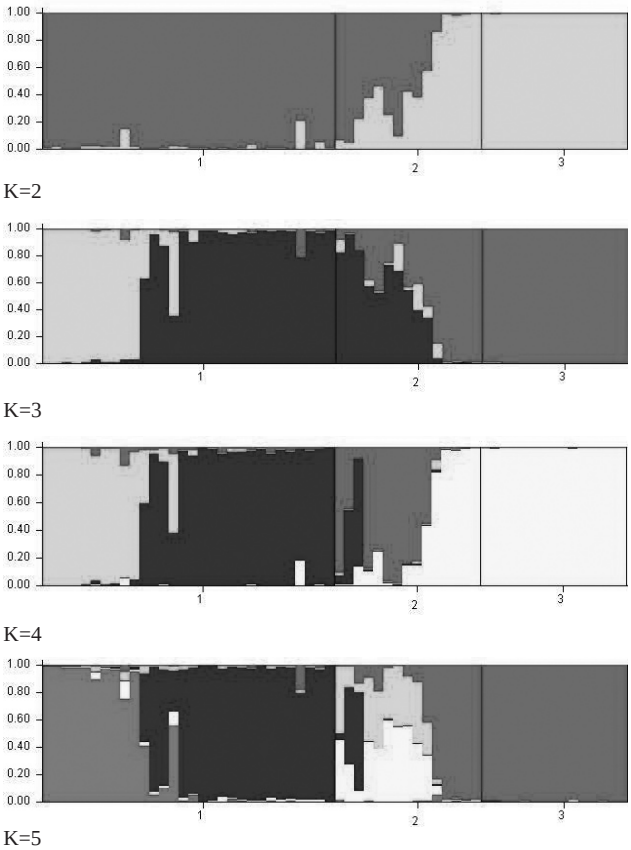

Fig. 4. Estimated population structure of Kerala cashew at different values of 'k'

Intactness of the population III in all the 'k' values shows the uniqueness of the cashew of that region. Archak *et al.*, (2009) reported no population differentiation while studying Indian cashew in different states and they also found shared alleles by all the populations. In this study shared alleles were seen only between two populations. Gst value was also high and the AMOVA showed considerable variation among the populations. The third population which did not have any shared alleles had accessions mostly from Kannoore and Kasargod districts which were the regions of first introduction. The other two populations include collection from the central districts (Pop II) and from southern districts (Pop II). These collections would have included introduced material. Cashew came to Kerala as an introduced crop but over the decades of cultivation it has well acclimatized to the Kerala climate and soil. In addition to the few initial introductions at the Malabar coast by the portugese there would have been more entries through the other ports on the west coast of Kerala and the import by the industrialists. Cashew being a cross pollinated crop the recombinant seedling progenies would have raised the genetic divergence from low to moderate or even high status.

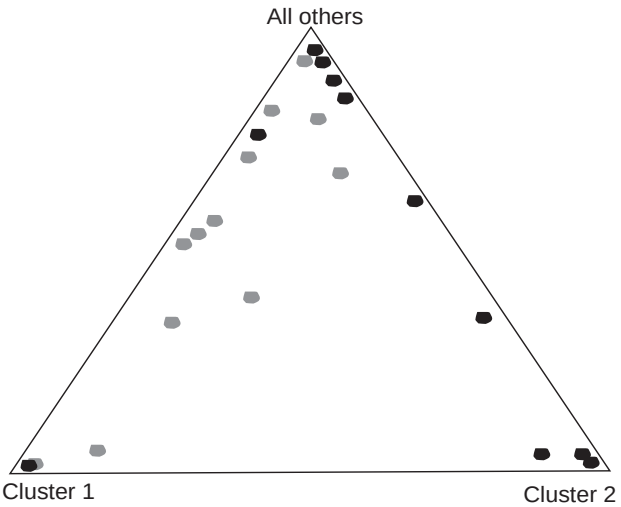

Fig. 5. Triangle plot of the population structure at k=3

Earlier studies have revealed the existence of wide morphological diversity in cashew accessions in India Rao *et al.* (1998), Swamy *et al.* (1998), Jayalekshmy and John, 2004). Archak *et al.* (2009) indicated the existence of substantial genetic diversity in India to raise it to the status of secondary centre of cashew diversity. This study also supports that view and the secondary centre of origin can be the 'Malabar coast' to be more precise. Cashew in kerala especially in Malabar Coast is a typical example of a crop introduction raised to the status of secondary centre of origin near the port of entry through years of acclimatization.

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