FINAL REPORT

IFCPAR PROJECT No. 3000 B 1

Use of DNA Markers (AFLP) for Genetically Improving the Productivity, Palatability, Storability and Dry Matter Content of Tubers of Greater Yam

(Report covering the period from 1 August 2005 to 30 May 2009)

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Executive Summary

The project was funded by the Indo-French Centre for the Promotion of Advanced Research (IFCPAR), New Delhi for collaborative research work between the Central Tuber Crops Research Institute (CTCRI), India and the Centre de Cooperation Internationale en Recherche Agronomique pour le Developpement (CIRAD), France. The project duration was three years and ten months from 1 August 2005 to 30 May 2009. The main objectives of the project were, streamlining the conventional breeding of greater yam, integration of biotechnology tools for parent and progeny selection, generating basic genetic information on the crop and developing new, improved varieties of greater yam by hybridising genetically divergent stocks. The mode of operation of the project was to combine the conventional and biotechnological tools by the joint and complementary efforts of scientists of India (CTCRI) and France (CIRAD) by technology sharing and mutual visits to partner institutions for joint research work.

Under the project, several thousands of controlled pollinations were done in greater yam, both in CTCRI and CIRAD and seed production was accomplished. A large number of hybrid seedlings were generated and evaluated in subsequent clonal generations. Identification of fertile, flowering male and female clones and hybrid seed production in greater yam were accomplished in CIRAD for the first time, through this project. The CIRAD station (Guadeloupe) had the unique, fertile tetraploids of greater yam which were discovered for the first time under this project. Hybridization was done between tetraploids as well as between tetraploids and diploids, which resulted in the production of tetraploid and triploid hybrids respectively. The triploids and tetraploids were produced for the first time in the history of greater yam breeding. They were found have increased vigour and higher tuber yield than diploid hybrids which is a significant breakthrough in the genetic improvement of this crop.

Cytological studies were undertaken in the unique tetraploid males (2n = 80) for the first time. The meiosis showed that during metaphase I, there were mostly bivalents with 6-8 quadrivalents, indicating an autotetraploid nature of the 2n = 80 types which were previously considered as octoploids. The mode of inheritance of microsatellite markers in controlled crosses in 2n = 40 types indicated their diploid nature, which were hitherto believed as tetraploids. The discovery of fertile 2n = 80 types (hitherto considered as octoploids) with majority of bivalents during meiosis and the simple, Mendelian inheritance of the

microsatellite markers in 2n = 40 types (hitherto considered as tetraploids) have also led to a revision of the basic chromosome number of greater yam, establishing that the 2n = 80 and 40 types are tetraploids and diploids respectively as against the previous assumption of octoploids and tetraploids. It is a classical discovery, which came out of this collaborative project, changing the concept of the basic chromosome number in greater yam.

The genetic diversity of the CIRAD and CTCRI accessions (Pacific and Indian accessions respectively) of greater yam was assessed and compared. A large number of unique alleles were discovered in both the germplasm collections. The Pacific and Indian accessions formed two separate clusters indicating the genetic divergence of the accessions of the two geographical locations. Tetraploids were more frequent among Pacific accessions whereas diploids were prevalent among Indian accessions. The characterisation of germplasm accessions has led to the identification an allele (*Ats-1*) that could be linked to anthracnose resistance. These findings are of great practical applications in the genetic improvement of greater yam since combining the genetically divergent accessions would maximise heterosis and heterozygosity, both contributing to higher tuber yield.

The project has given a very good opportunity for the Indian and French scientists to undertake visits to the collaborating institutes to accomplish joint research work in India and France. The visits have imparted better exposure to the scientists, to different systems and techniques followed in the respective laboratories. As a result, the laboratory and field techniques were mutually shared for the progress of the project. The joint work was benefited by the complementary potentials of the partner institutes and scientists which led to the very important, new findings in greater yam breeding. **The new findings from the project work presented in the 14th Triennial Symposium of the International Society for Tropical Root Crops during November 2006 fetched the prestigious 'Pat Coursey Award' for the best yam research work presented in the symposium as well as the award for the best poster presentation.**

The project is concluded accomplishing all the objectives. The conventional breeding in greater yam was strengthened by selection of appropriate parents. Biotechnological tools (microsatellite markers) were used to assess and compare the genetic profile of Indian and Pacific germplasm for their effective utilization in conventional breeding and to link markers with desirable traits in the progeny. The project has resulted in the identification of higher yielding hybrid clones with desirable traits that are being tested in farmers' fields. Lastly, the most significant outcome of the project is the discovery of fertile tetraploids which led to the

revision of the basic chromosome number and started polyploidy breeding in this species, which holds tremendous potentials for its genetic improvement.

Introduction

The greater yam (*Dioscorea alata*) is a staple or subsidiary crop in many countries in Asia, Oceania, Caribbeans and Africa. The underground tubers are used almost exclusively for human consumption. It has great potential for commercial exploitation but the problems like low tuber yields, unacceptable or irregular tuber shape which makes harvest difficult, expensive staking and anthracnose disease make its cultivation less attractive. This traditional crop has been hardly improved by breeding in the past, except by clonal selection which is very slow and limited in results. Genetic improvement by hybridization and selection was not known in greater yam, until very recently, when CTCRI in India pioneered in this aspect.

The greater yam is considered to have originated in the Indo - Burmese region. The hilly forests of the North Eastern India has much genetic resources of this crop, occurring in natural state. By studies in a large number of genetic stocks collected from India, CTCRI has standardised the breeding methodology in this crop about two decades back. Apart from the North Eastern India, great genetic diversity of the crop occurs in the Pacific region. With hybridization and selection in greater yam made possible, it was envisaged to study both the Indian and Pacific genetic resources for bettering the results in the genetic improvement of this crop. Accordingly the project with the partnership of CTCRI in India and CIRAD in France was initiated with the following objectives.

Objectives of the Project

1. To overcome the constraints in greater yam improvement by streamlining and strengthening conventional breeding by hybridization
2. To develop and integrate biotechnological tools to facilitate efficient parent and progeny selection
3. To develop genetically improved varieties of greater yam having increased productivity, improved palatability, prolonged storability and enhanced dry matter content of tubers
4. To generate basic genetic information on greater yam for developing efficient breeding strategies and promoting interaction and technology sharing between CTCRI and CIRAD

Materials and Methods

The genetic stocks of greater yam (*Dioscorea alata*) maintained in the Central Tuber Crops research Institute (CTCRI), India and the Centre de Cooperation Internationale en Recherche Agronomique pour le Developpement (CIRAD), Guadeloupe, French West Indies formed the basic materials for the project work. In CTCRI, the natural germplasm collections from India and clones developed from sexual progeny were the materials, which were mostly diploids and some triploids. The CIRAD germplasm was mostly accessions from the Pacific which consisted of diploids, triploids and tetraploids. The selected flowered accessions were manually pollinated as well as left for open pollination for collecting seeds, during the flowering seasons. Pollination methods standardized at CTCRI were followed in CIRAD. Seedlings were germinated in nursery and later transplanted to field and the vines were staked. The seedling tubers harvested at the end of the season were used as planting materials to raise the clonal generation which were further multiplied to evaluate tuber yield.

Flowering date of clones was recorded as the day of opening of the first flowers on the respective clones. Meiosis was worked out by standard acetocarmine smear technique. Pollen fertility was estimated by stainability of pollen in 2 per cent acetocarmine.

For assessing the genetic variation of the collections and to compare the variation of Indian and Pacific collections, multiplex panels of microsatellite markers were used. CTCRI core collection comprising of accessions collected from Kerala, Assam, Meghalaya, Manipur, Nagaland and Andhra Pradhesh were used for the genetic diversity study. For the purpose of assessing genetic diversity leading to the preparation of a dendrogram, data were scored in binary format, with the presence of an allele scored as unity and its absence scored as zero. The binary data were used to compute pair-wise similarity coefficients (Nei and Li) and the similarity matrix thus obtained was subjected to cluster analysis using the UPGMA algorithm on NTSYS-PC version 2.0. A polymorphic index (PIC) was calculated as $PIC = 1- \Sigma P^2_i$, where P_i is the band frequency of the $_i$th allele (Smith *et al.,* 1997). Additionally, principal component analysis (PCA) was performed with the Ade4 module of R software (version 2.4.1; Department of Statistics and Mathematics, Wirtschaftsuniversität, Austria).

For joint work Indian scientists visited CIRAD, Montpellier and Guadeloupe and French scientists visited CTCRI, India.

Deviation in the methodology – and the reasons

The only deviation from the originally proposed methodology in this project was the use of microsatellite markers instead of AFLPs as DNA markers. When the project was formulated AFLP markers were used for assessing genetic variation and constructing genetic maps in many species including *D.alata,* but microsatellite markers were not reported in *Dioscorea* species and hence the AFLP markers was proposed. AFLP markers, despite their dominant nature, have been used increasingly in many applications in different plant species, mainly owing to the ability of the AFLP system to detect a very high number of polymorphisms in a single assay, good repeatability, reasonable coverage of the genome, and the possibility of automation. This technique, which is based on selective amplification of subsets of genomic restriction fragments, requires a high level of DNA purity. Later, when the project was started, microsatellite markers were reported in *Dioscorea* and DNA which had failed to give reproducible patterns with AFLPs produced very good results with microsatellites. The high polyphenol content of greater yam leaves hinder the extraction of good quality DNA needed for AFLP analysis. Even the use of Quiagen kit needs the pre isolation treatment to facilitate high quality DNA in greater yam. Hence a preliminary study was undertaken to study the usefulness of AFLP and microsatellites to fulfil the objectives of the project. Marker reproducibility was tested by analyzing different extraction per plant and different organs. The results of the pilot study showed that microsatellites are more reproducible than AFLP markers. In AFLP, the enzymatic digestion of nucleic acids constituted an additional source of experimental fluctuation and partial digestion could lead to erroneous profiles. Hence microsatellites which have many desirable marker properties and increasingly used in crop plants in genetic diversity studies were selected for detailed study mainly based on its codominant nature, high level of polymorphism and less stringent needs for high quality DNA as compared to AFLP. Microsatellite markers also combine several features of ultimate genetic markers, owing to their abundance and uniform dispersal in genomes, hypervariability, codominant nature, transportability among species, accessibility for other research laboratories and amenability to automation. Multi allelic codominant microsatellite markers are very useful for analysis of crop species since they allow individuals to be uniquely genotyped, which is important for cultivar identification and genetic variability

studies of germplasm collections. Hence there was the deviation of using microsatellite markers instead of AFLPs in this project.

Detailed report

Seed production by artificial pollination

The first objective of the project was 'To overcome the constraints in greater yam improvement by streamlining and strengthening conventional breeding by hybridization'. Hybridization in greater yam was standardised in CTCRI, for the first time, about two decades back. With the expertise of the CTCRI scientists the practical aspects of hybridization were familiarised by the French scientists by joint work during the flowering season. It was by this project that the hybridization and seed production in greater yam started in CIRAD station, Guadeloupe, for the first time. Seeds were produced by artificial pollination during the flowering seasons of 2005, 2006, 2007 and 2008 in CTCRI (India) and CIRAD Station (Guadeloupe). Pollinations were done individually in the two institutes as well as jointly by the Indian and French collaborators in CIRAD.

Pollination work in CTCRI (India)

Full sibs were produced by artificial pollination in CTCRI during four years. During 2005 a total of 11,472 pollinations were done in 110 parental combinations using 38 female and 31 male clones. Fruit set was recorded in 96 combinations (87.3 %) which ranged from 1.7 – 86.8 per cent. Seed set was recorded in 74 combinations (77.1 %) which ranged from 1.4 – 55.6 per cent. Seed germination was recorded in 22 combinations (29.7 %) which ranged from 0.8 – 81.3 per cent. A total of 3708 pollinated fruits were collected from which 4373 seeds could be obtained and 426 seedlings could be raised (annexure 1).

During 2006 a total of 6094 pollinations were done in 82 combinations involving 33 females and 41 males. Fruit set was recorded in 76 parental combinations (92.7%) which ranged from 6.7 – 95.6 per cent. Seed set was recorded in 70 combinations (92.1%) which ranged from 1.1 – 71.4 per cent. Seed germination was recorded in 32 combinations (45.7 %) which ranged from 2.8 – 88.3 per cent. A total of 3126 pollinated fruits were collected from which 5547 seeds were obtained and 1607 seedlings were raised. (annexure 2).

During 2007 the total artificial pollination conducted were 5506 involving 23 female and 24 male clones in 77 combinations. Fruit set was recorded in 64 combinations (35.7 %) which ranged from 1.2 – 90.3 per cent. Seed set was recorded in 55 combinations (32.4 %) which ranged

from 3.2 – 70.9 per cent. Seed germination was recorded in 42 combinations (54.5%) which ranged from 1.0 – 57.7 per cent. A total of 1965 fruits were collected from which 3818 seeds were obtained and 752 seedlings were raised (annexure 3)

During 2008 the total artificial pollination conducted were 10037 involving 36 female and 26 male clones in 104 combinations. Fruit set was recorded in 86 combinations (82.7 %) which ranged from 2.6 – 76.1 per cent. Seed set was recorded in 82 combinations (78.8 %) which ranged from 4.2 – 83.3 per cent. Seed germination was recorded in 81 combinations (98.8%) which ranged from 10.5 – 98.1 per cent. A total of 2800 fruits were collected from which 5067 seeds were obtained and 2862 seedlings were raised (annexure 4).

A summary of the pollination results during the four years shows that the pollination efficiency has increased over the years (annexure 5 . Although the female parents were largely fruit setting and seed setting, the seed germinating parental combinations were initially very low (29.7%). By eliminating unproductive parental combinations, the seed germinating combinations could be enhanced to 98.8 per cent in the year 2008. Similarly the overall seed germination from the artificial pollinations was substantially enhanced from 9.7 per cent during 2005 to 56.9 in 2008 with the substantial increase of hybrid seedlings produced which provides better chances of isolating superior hybrids.

Production of half sibs

Open pollinated seeds were also collected from a number of females and several thousands of seedlings were raised as half sibs during all the four years for evaluation of their tuber yield and other attributes (annexures 6 - 9).

Identifying seedling producing parental combinations

The artificial pollination work during the four years has provided considerable practical information helpful for the future breeding work. It was observed that several parental combinations recording fruit set do not produce seeds or several combinations producing apparently normal seeds do not germinate to produce seedlings. Hence from the comprehensive pollination results during the four years, 50 male clones and 52 female clones which can eventually produce seedlings on hybridization were identified (annexures 10,11). Similarly a set of 26 male clones and 38 female clones which were not found to produce seedlings on hybridization, were also identified (annexures 12,13). These are useful for planning and selecting parents for future hybridization programmes for the improvement of greater yam. The flower opening dates of male and female clones were recorded during the years 2006 – 2008 which will

also be helpful in planning some of the desired crosses with particular parents as the male and female clones are to flower simultaneously for effecting hybridizations (annexures 14 – 19)

Pollen fertility studies

Pollen fertility was estimated from 96 diploid, male clones. The vast majority of the males had pollen fertility of above 70 per cent. There were only three clones which recorded pollen fertility of less than 50 per cent. It shows that, by and large, the diploid males of *D. alata* are highly pollen fertile (Anexure 20). Pollen of the few tetraploid males examined in Guadeloupe also showed a high fertility of above 85 per cent.

Pollination work in CIRAD Station (Guadeloupe)

In CIRAD station, Guadeloupe, successful pollination in *D. alata* was done for the first time in 2005. The pollination work was initiated jointly by CTCRI and CIRAD scientists at Guadeloupe, when Dr. K. Abraham, Principal Indian collaborator visited Guadeloupe from 28 November to 22 December 2005. The French partners could be familiarised with the field experience of artificial pollination and identifying fertile diploid female clones and sterile triploid female clones that were present among the flowered accessions. The fertile females could be visually identified on the basis of the ovary width and perianth size and pollinations were attempted only on fertile females so that wasteful efforts of pollinating sterile females could be avoided. About 12000 seeds were produced by controlled pollinations and open pollinations during the four years from 2005 to 2008. Crosses were carried out between elite male and female varieties chosen for their agronomic features (yield, tuber quality, resistance to anthracnose etc.) and their genetic distances in order to maximise heterozygosity and heterosis in the progenies.

Production of Tetraploid hybrids

There were tetraploid (2n = 80) male and female greater yam accessions available in CIRAD germplasm and the sexual fertility of those tetraploids was detected for the first time. The males were highly pollen fertile showing more that 85 per cent. Successful pollinations were carried out using a fertile tetraploid female (198 CTRT) and a fertile male parent (CTRT 148) that were genetically distant and viable seeds were produced for the first time. A total of 273 fruits and 670 seeds were produced. Six hundred hybrid seedlings were transplanted to the field for evaluation, which were subsequently propagated by vegetative multiplication and evaluated in the clonal generations.

Production of triploid hybrids

Triploid hybrids were produced for the first time in 2005 by hybridisation between diploids and tetraploids. A total of 701 controlled hybridisations were carried out between four diploid female clones (F5, F27, F53 and F74) and one tetraploid male clone (CTRT-148). The fruit set in the various combinations ranged from 45 to 56 per cent and the seed set from 33 to 38 per cent. Almost all of the seeds were found to contain embryos but with an abnormal development of endosperm tissue and no seedlings were obtained by normal germination of seeds. Hence, 50 embryos per cross were rescued by *in vitro* culture. The success of embryo rescue ranged from 15 to 30 per cent. During the 2006-2007 seasons, 4,000 seeds were obtained by open pollination between diploids and tetraploids and 1,000 immature embryos were rescued by *in vitro* culture.

Reciprocal crosses between tetraploid female clones and diploid male clones were attempted during the 2006-2007 seasons by manual pollination. Fruits were obtained but no seeds, which may be explained by parthenocarpy.

Production of diploid hybrids

Over 4,000 hybridisations were conducted by controlled and open pollination from 2005 to 2008 in Guadeloupe involving different diploid male and female clones. Since anthracnose is one of the main causes of economic loss in greater yam, different resistant males were used for natural pollination with distant females of high quality to produce resistant hybrids. A total of 1,000 seeds were obtained. The 800 hybrid seedlings obtained were evaluated in seedling and clonal stages.

Raising hybrid seedlings and evaluation of clonal plants

The first set of hybrid seeds were produced in November – December 2005 and the seedlings were produced in April – May 2006. Further seeds and seedlings were produced during the subsequent years, 2007 and 2008. The seedlings were carried over to the next generations as clonal plants as they were vegetatively propagated. The diploid and polyploid hybrids (triploids and tetraploids) produced during the 2005-2007 seasons in Guadeloupe and India, were evaluated for their performances in the field. At the time of the completion of the project, there were seedlings, first clonal and second clonal plants from which selections were made. Those produced in 2005 were evaluated over three consecutive years, whereas those produced in 2007 could only be evaluated for a single year. Ten tetraploid hybrids that

combined higher yields, tuber characteristics adapted to commercial production and anthracnose resistance were selected from the second clonal generation in Guadeloupe. They are now being evaluated in farm trials in Guadeloupe to ascertain their suitability for adoption by farmers. The diploid hybrids produced in CTCRI were evaluated in clonal generations and ten high yielding hybrids with high dry matter content of tubers were identified.

Cytological studies

Cytology of diploids

Meiosis was studied in diploid *D. alata* male clones to ascertain ploidy status and to check for any abnormalities. 22 males were studied in 2005 and 11 males were studied in 2006 at CTCRI. All the males checked were having 2n = 40 chromosome constitution with normal meiosis.

Cytology of tetraploids and triploids

Cytological investigations on the meiosis of tetraploid and triploid male clones of *Dioscorea alata* were also conducted. Very well spread meiotic stages (metaphase I) of tetraploids were prepared and photographed. The study clearly showed that there were majority of bivalents and 6-8 tetravalents in the tetraploids (Fig.1-2). These cytological stages in the tetraploid *D. alata* were worked out for the first time and the pictures are the first of its kind in *D. alata.* The chromosome behaviour clearly indicates that the 'so called octoploids' of *D. alata* with 2n = 80 chromosomes are actually autotetraploids. This is the first cytological evidence to establish that the basic chromosome number of *D. alata* is 20 which was also evidenced from the microsatellite studies conducted in this project. This finding differs from the commonly held view that the basic chromosome number of *D. alata* is 10 and the 2n = 80 types are octoploids.

Meiotic stages of 2n = 60 chromosome types were also worked out. Although the preparations were not very good they were found to have predominantly trivalents in the male meiosis. This is further evidence corroborating x = 20 as the basic chromosome number of the species.

Flow cytometry and ploidy determination

As the flow cytometry facility was available at CIRAD Guadeloupe, the work was undertaken there to determine the ploidy levels of *D. alata* clones. Measures were performed using a Bryte HS Flow Cytometer, that quantifies the fluorescence emitted by isolated nuclei

stained with a fluorochrome (Fig 3.). This method allows tetraploids, triploids and diploids to be identified. Two hundred clones from CIRAD and 72 accessions from India were analyzed for ploidy levels. Among them the great majority was diploids (2n = 40) followed by tetraploids and triploids (Annexure 21).

Polyploidy and tuber yield

Greater yam is a polyploid species with varieties having three different ploidy levels such as diploids (2n = 40), triploids (2n = 60) and tetraploids (2n = 80). Triploids and tetraploids were found to be more vigorous and recorded higher tuber yields than diploids (Fig. 4 & 5). Fifty five varieties of CIRAD's collection were evaluated in Guadeloupe from 2006 to 2008. Results showed that better tuber yield was obtained for varieties with the higher ploidy levels (Fig. 6 & 7). A significant correlation was also noted between tuber yield and the estimated heterozygosity of alleles. Triploid and tetraploid varieties have a higher number of alleles per locus which could also explain their superior tuber yield.

Development of a protocol for the long-term conservation of pollen by freeze-drying

Different conditions for freeze-drying of pollen were tested on two different species (*D. cayenensis-rotundata* and *D. alata*). Pollen grains were freeze-dried using different pressure, heating plate temperature and drying time parameters. A germination test was carried out for each treatment in order to verify pollen viability. The freeze-dried pollen of *D. cayenensis-rotundata* lost 97% of its water content and had a viability comparable to that of a fresh control at 0.200 mbar of pressure, a plate temperature of -5°C and a drying time of 23 hours. The pollen grains of *D. alata* could not withstand a rapid water loss. They required a lower heating plate temperature and, consequently, a longer drying time in order to have a significant germination rate. On the basis of the results obtained, the long-term conservation of yam pollen appears to be a good option for maximising the possibility of crosses.

Embryo rescue of triploids by *in vitro* culture

Following controlled hybridization between diploid females and tetraploid males of *D. alata,* the seeds were not germinating normally as the endosperm was abnormal, though the embryo was normal. Hence a method for rescuing immature embryos by *in vitro* culture was developed that could be used to obtain triploid hybrids from the non-germinable seeds. The effectiveness of different techniques and media were tested. Figure 8 shows embryos of triploid hybrids at different stages of development.

RAPD Analysis of CTCRI Germplasm

The protocol for the isolation of DNA from greater yam accessions was standardised. Thirty divergent landraces of greater yam were evaluated for genetic diversity using randomly amplified polymorphic DNA (RAPD) markers. The extracted DNA was subjected to RAPD analysis using different primers *viz.* OPA 19, OPB 4, OPC 19, OPE 19, OPI 16 and OPI 18. All the six random primers used in the study produced scorable, unambiguous markers. The primers produced a total of 244 polymorphic markers across 30 landraces of greater yam. The number of markers produced by different primers ranged from 26 to 72 with an average of 40.6 markers per primer. Among the primers used for RAPD analysis, the primer OPI 16 gave the highest number of fragments while the primer OPC 19 produced the lowest number of fragments (26). UPGMA clustering of the accessions generated from RAPD data analysed with DICE similarity coefficient resulted in grouping of accessions into two main clusters (Fig.9&10). In the first cluster 12 accessions were grouped into 8 sub groups while in the second cluster, 18 accessions formed 10 miniclusters. In the present study, geographical influence was not noticed on the clustering pattern among the accessions collected from different states of India. Da 144 from Manipur formed the same cluster with Da 28 from Eranakulam(Kerala) while Da 101 from Nedunkandam (Kerala) and Da 111 from Dandakaranya, (Orissa) were also grouped together. Also the accession Da 57 from Alanchery in Kerala was found to be highly divergent from others.

The fine clustering showed the grouping of accessions based on tuber shape. Compact shaped accessions with round and oval or oval oblong tuber shapes were not clustered together with accessions having long tuber shape. Further analysis of the RAPD profile of more accessions can lead to identification of molecular markers linked to tuber shape in Yams.

Assessment of the genetic diversity of CIRAD and CTCRI germplasm collections using microsatellite DNA markers:

The allelic diversity of 93 CTCRI varieties and 96 CIRAD accessions was characterised using nine selected microsatellite markers. In a first study, a set of six varieties was used to select the ten most suitable microsatellite loci out of the 40 available ones (Annexure 22). The selection was based on the degree of polymorphism detected and the quality of loci. We excluded those with stuttering effects. Primers selected were labelled with three different fluorochromes (HEX, FAM, TET), and PCR products were multiplexed. Migration was carried out with an automatic sequencer, ABI PRISM™ 3100 (Applied

Biosystems). This method is very efficient because several microsatellite markers can be pooled and it is highly reliable. Figure 11 & 12 shows typical electrophoregrams obtained with multiple samples loading and depicts allelic diversity among accessions.

The number of alleles recorded on the 189 varieties with the nine selected microsatellites, Da3G04, Da2F10, Da1F08, Da1F07, Dab2D08, Dab2E07, Dab2D11, Dpr3E10 and Dpr3B12, are 11, 15, 10, 3, 11, 14, 8, 5 and 8, respectively (Table 1). A total of 85 alleles were detected at nine loci. A larger quantity of unique alleles (defined as alleles present in one germplasm collection but absent in the other) was observed (Table 2): Da3G04 (two alleles: 296pb and 318 pb), Da2F10 locus (six alleles: 114pb, 123pb, 127pb, 143, 150 and 152pb), Da1F08 locus (seven alleles: 132pb, 164pb, 166pb, 174, 178, 180 and 197pb), Da1F07 (two alleles: 215pb 219pb), Dab2D08 locus (seven alleles: 316pb, 321pb, 325pb, 331pb, 339pb, 342pb, 346pb), Dab2E07 (four alleles:145pb, 174pb, 177pb, 190), Dab2D11 (two alleles: 232pb, 234pb), Dpr3E10 (three alleles: 180pb, 195pb, 197pb) and Dpr3B12 (one allele: 142pb).

Table 1. Distribution of alleles in Indian and Pacific greater yam germplasm

Locus No	Locus name	No. of polymorphic alleles	Band width bp	Unique alleles bp		Frequent alleles (bp)	
				India	Pacific	India	Pacific
Locus2	Da3G04	11	278-318	318	296	298 307	294,307
Locus3	Da2F10	15	114-157	-	114,123 127,143, 150	132,141	138,148
Locus 5	Da1F08	10	132-197	197	132,164,1 66,174,17 8,180	182	182,184
Locus7	Da1F07	3	213-219	-	215,219	213	213
Locus9	Dab2D08	11	316-349	316,342 346	331,339	323	323,329
Locus12	Dab2E07	14	131-190	145,190	174,177	163,165	147,165
Locus13	Dab2D11	8	228-243	-	232,234	236,238	232,234
Locus14	Dp3E10	5	180-197	180	195,197	184	186
Locus 15	Dpr3B12	8	138-157	-	142	138,155	138,150

Table 2. Comparison of allelic frequency in Indian and Pacific *D.alata* germplasm

	MICROSATELLITE MARKERS																					
	Da3G04			Da2F10			Da1F08			Da1F07			Dah2D08			Dah2E07			Dah2D11		
	Alleles	Frequencies		Alleles	Frequencies		Alleles	Frequencies		Alleles	Frequencies		Alleles	Frequencies		Alleles	Frequencies		Alleles	Frequencies	
n	10/10	INDE	CIRAD	10/14	INDE	CIRAD	4/9	INDE	CIRAD	1/3	INDE	CIRAD	9/6	INDE	CIRAD	12/12	INDE	CIRAD	6/8	INDE	CIRAD
1	**278pb**	0,043	0,028	**114pb**	-	0,009	**132pb**	-	0,104	**213pb**	0,914	0,726	**316pb**	0,011	-	**131pb**	0,247	0,019	**228pb**	0,011	0,104
2	**294pb**	0,054	0,283	**117pb**	0,129	0,047	**164pb**	-	0,094	**215pb**	-	0,132	**321pb**	0,312	-	**145pb**	0,366	-	**230pb**	0,011	0,406
3	**296pb**	-	0,019	**123pb**	-	0,132	**166pb**	-	0,038	**219pb**	-	0,028	**323pb**	0,452	0,443	**147pb**	0,032	0,274	**232pb**	-	0,292
4	**298pb**	0,624	0,028	**127pb**	-	0,019	**174pb**	-	0,019				**325pb**	0,086	-	**155pb**	0,097	0,019	**234pb**	-	0,198
5	**300pb**	0,097	0,019	**130pb**	0,172	0,226	**178pb**	-	0,019				**327pb**	0,194	0,038	**158pb**	0,075	0,019	**236pb**	0,484	0,311
6	**305pb**	0,022	0,132	**133pb**	0,581	0,038	**180pb**	-	0,057				**329pb**	0,161	0,651	**163pb**	0,312	0,047	**238pb**	0,527	0,217
7	**307pb**	0,624	0,642	**137pb**	0,108	0,274	**182pb**	0,677	0,717				**331pb**	-	0,075	**165pb**	0,323	0,274	**240pb**	0,258	0,047
8	**309pb**	0,054	0,142	**139pb**	0,183	0,415	**184pb**	0,086	0,632	-			**339pb**	-	0,113	**167pb**	0,215	0,113	**243pb**	0,140	0,028
9	**311pb**	0,011	0,170	**141pb**	0,516	0,028	**186pb**	0,011	0,075				**342pb**	0,011	-	**169pb**	0,043	0,151			
10	**314pb**	0,054	0,245	**143pb**	-	0,075	**197pb**	0,194	-				**346pb**	0,108	-	**171pb**	0,022	0,019			
11	**318pb**	0,086	-	**148pb**	0,032	0,340							**349pb**	0,065	0,264	**174pb**	-	0,038			
12				**150pb**	-	0,028										**177pb**	-	0,113			
13				**152pb**	0,032	-										**183pb**	0,065	0,038			
14				**155pb**	0,043	0,019										**190pb**	0,032	-			
15				**157pb**	0,118	0,085															
Genetic Diversity		**0,194**	**0,380**		**0,287**	**0,550**		**0,496**	**0,056**		**0,165**	**0,454**		**0,612**	**0,290**		**0,534**	**0,795**		**0,402**	**0,553**

For the purpose of assessing genetic diversity leading to the preparation of a dendrogram, data were scored in binary format, with the presence of an allele scored as unity and its absence scored as zero. The binary data were used to compute pair-wise similarity coefficients (Nei and Li) and the similarity matrix thus obtained was subjected to cluster analysis using the UPGMA algorithm on NTSYS-PC version 2.0. A polymorphic index (PIC) was calculated as PIC = 1- ΣP^2_i, where P_i is the band frequency of the $_i$th allele (Smith *et al.*, 1997). Additionally, principal component analysis (PCA) was performed with the Ade4 module of R software (version 2.4.1; Departement of Statistics and Mathematics, Wirtschaftsuniversität, Austria).

For CIRAD germplasm, the maximum genetic similarity ranged from 0,02 to 0,92, with a mean value of 0,47. PIC values ranged from 0.06 to 0.80 in the 96 genotypes surveyed, with an average PIC value of 0.43. The maximum genetic distance for CTCRI germplasm ranged from 0,04 to 0,92, with a mean value of 0.53. PIC values ranged from 0,16 to 0,61 in the 93 genotypes surveyed, with an average PIC value of 0.38.

The dendrogram prepared through cluster analysis is shown in Fig 13. suggesting a high level of diversity among the 189 genotypes surveyed. The UPGMA analysis clearly separated the Indian and Pacific accessions into two principal genetic groups. Both CTCRI and CIRAD accessions were divided into several subclusters corresponding to their geographic origin and their ploidy levels. The subcluster IIb2 includes the majority of CIRAD polyploid accessions (3x and 4x).

Furthermore, the results of a principal coordinate analysis (Fig 14) support those of a UPGMA cluster analysis. The PCA revealed that the first two axes clearly separated the Indian and Pacific accessions confirming the diversification of Indian and Pacific genetic stocks of greater yam. By exchanging such genetic widely divergent stocks between CTCRI and CIRAD, it will be possible to obtain by hybridization heterotic progenies.

Molecular marker heterozygosity determination using microsatellite markers

Heterozygosity was estimated by calculating the mean number of alleles per locus on 8 microsatellite markers. Test χ^2 showed that the number of homozygote and heterozygote clones is dependent on the ploidy levels. Diploid varieties are more homozygous than the triploid and tetraploid varieties. A significant correlation was observed between tuber yield and the estimated heterozygosity (Fig 15). Triploid and tetraploid varieties have a higher number of allels per locus which could explain their superior performance observed in the field. ***In conclusion, the production of triploids and tetraploids by crossing distant genotypes appears promising for the genetic improvement of the greater yam, making it possible to maximise heterozygosity and heterosis.***

Mode of inheritance of microsatellite markers in *D. alata* varieties, indicating the diploid nature of 2n = 40 chromosome types

The parents and progeny of four controlled crosses were used to determine the segregation patterns of microsatellites markers. The inheritance patterns of microsatellites were determined from a comparison of the distribution of progeny genotypes with the expected distribution based on the genotypes of the parents. We adopted a Bayesian procedure to discriminate among the different inheritance hypotheses (Table 3.). It is better suited to the requirement of testing complex segregation patterns than the χ^2 statistics. Inheritance of the genotypes in the progeny of four crosses was consistent with simple diploid, Mendelian inheritance at six loci tested (Annexure 23). ***Our results provide the first reliable evidence supporting the hypothesis that clones with 2n = 40 chromosomes are diploid and not tetraploid as previously assumed (Arnau et al., 2009).***

Table 3 Bayes factors testing the likelihood of a 2x versus a 4x inheritance pattern in four segregating populations of *Dioscorea alata* in six microsatellite loci.

Population	Locus	Parental phenotypes	Total number of different allele	Bayes factors	
				Hypothesis	
				A	B
24M X 27F (N = 60)	Da2F10	ab X bc	3	$6 X 10^{16}$	$5 X 10^{18}$
	Dab2E07	ab X b	2	1	1
	Da1F08	a X ab	2	1	1
17M X St. Vincent (N = 23)	Da2F10	bd X ac	4	$7 X 10^{13}$	$3 X 10^{20}$
	Da3G04	ab X bc	3	$4 X 10^{9}$	$4 X 10^{16}$
	Dab2D11	ab X b	2	$3 X 10^{2}$	$3 X 10^{4}$
	Dab2D08	a X ab	2	1	1
	Da1F08	a X ab	2	0,1	1
31M X 27F (N = 17)	Da2F10	bc X ab	3	$4 X 10^{2}$	$2 X 10^{4}$
	Dab2E07	ab X b	2	1	1
	Da1F08	a X ab	2	4	4
Pyramide X 27F (N = 10)	Da2F10	bc X ab	3	$6 X 10^{2}$	$6 X 10^{2}$
	Dab2D11	ab X ac	3	$1 X 10^{3}$	$2 X 10^{5}$
	Dab2E07	ab X b	2	4	20
	Da1F08	a X ab	2	1	1
	Da3G04	ab X b	2	0,4	2

Identification of markers linked to anthracnose resistance

The characterisation of germplasm collections using microsatellites markers made it possible to identify an allele that is only present in resistant varieties (*Ats-1*). Resistance evaluation within a breeding plan is extremely slow and costly. It requires a very complex experimental design. Analyses of the genotype and phenotype of several of the progenies produced are currently in progress and will make it possible to confirm whether or not the identified marker is linked to resistance. If it is the case, it could be used for the rapid and effective screening of resistant individuals from among the progeny produced by hybridisation.

Identifying high yielding clones with palatability

The mean tuber yield of the majority of the seedlings (202) ranged from 100- 300gm among the full sibs (Annexure 24). In the first clonal generation where the seedling tubers were vegetatively propagated, the highest mean tuber yield was recorded by the family, Das 295x 96-192 (Annexure 25). In the second clonal generation, the tuber yield was further increased and the mean tuber yield ranged from 20 – 8100g (Annexure 26). Earlier studies have shown that the tuber yield of sexual progeny of greater yam becomes stabilized by the

second clonal generation and hence the preliminary selection was done, identifying ten high yielding clones with good palatability.

Among the half sibs also the tuber yield increased from seedling to first clonal generation and from first clonal to second clonal generation and the highest tuber yield recorded was 7400g by the clone 6 – 197 (Annexure 27)

The selections made at CTCRI are diploids.In CIRAD, Guadeloupe, ten tetraploid hybrids that combined higher yields, tuber characteristics adapted to commercial production and anthracnose resistance were selected from the second clonal generation. They are now under evaluation trials in farmers' fields.

High dry matter types

Thirty eight hybrids from the second clonal generation of diploids were selected for higher dry matter content. The dry matter ranged from 30 – 36.6 per cent (Annexure 26).

Salient achievements - 5 points in English

1. Successful breeding work of greater yam was initiated in CIRAD station. The unique, fertile tetraploids were discovered and used for the production of higher yielding tetraploid and triploid hybrids by hybridization in greater yam for the first time.
2. The basic chromosome number of *D. alata* was revised from x = 10 to x = 20 by evidences from cytological studies and microsatellite markers inheritance, which is a fundamental discovery in this crop.
3. Comparative analysis of the allelic diversity of the Indian and Pacific greater yam accessions showed that they formed two different clusters indicating their genetic divergence and one allele that could be linked to anthracnose resistance (*Ats-1*) was identified.
4. A protocol was developed for the conservation of *D. alata* pollen grains by freeze drying and another protocol was developed to rescue immature embryos from diploid x tetraploid crosses.
5. Evaluation of the hybrid selections has identified six high yielding clones in India and ten high yielding clones in Guadeloupe.

Salient achievements - 5 points in French

1. Des travaux d'amélioration génétique sur les ignames *D. alata* ont été initiés avec succès, sur la station du CIRAD. Des tétraploïdes fertiles ont été découverts et utilisés pour créer par hybridation et pour la première fois, des hybrides tétraploïdes et triploïdes à haut rendement.
2. Le nombre chromosomique de base des ignames *D. alata* a été révisé de x = 10 à x = 20 par des études cytologiques et des analyses de ségrégation de marqueurs microsatellites, ce qui est une découverte fondamentale chez cette espèce.
3. Des analyses comparatives de la diversité allélique des accessions de *D. alata* de l'Inde et du Pacifique montrent que les variétés se séparent en deux groupes distants montrant leur divergence génétique, et ont permis d'identifier un allèle qui pourrait être liés à la résistance à l'anthracnose.
4. Un protocole a été mise au point pour la conservation du pollen d'igname par lyophilisation, et un deuxième protocole a été développé pour le sauvetage d'embryons immatures obtenus par croisement entre des diploïdes et tétraploïdes.
5. L'évaluation des hybrides sélectionnés a permis d'identifier six clones à haut rendement en l'Inde et dix clones à haut rendement en Guadeloupe.

Personnel

INDIAN SIDE	FRENCH SIDE
1. Dr. Abraham Principal Scientist & Head Division of Crop Improvement Central Tuber Crops Research Institute Trivandrum 695017, India	1. Dr. Gemma Arnau Yam Scientist, CIRAD – CA Station De Roujol 97170 Petit Bourg Guadeloupe, French West Indies
2. Dr. M. T. Sreekumari, Principal Scientist, CTCRI	2. Dr V. Lebot, Chercheur, Programme CALIM, CIRAD
2. Dr. M. N. Sheela, Senior Scientist, CTCRI	3. Dr. Jean Louis Noyer, Biomolecular Scientist, BIOTROP Unit, CIRAD
4. Ms. Sreeja Thankappan, Research Associate	4. Alice Nemorin, Research Associate

Exchange visits of scientists

India to France			France to India		
Name	Dates	Duration	Name	Dates	Duration
K. Abraham	28 Nov – 22 Dec 2005	24 days	Gemma Arnau	27 Jun – 7 Jul 2006	11 days
K. Abraham	20 May – 17 Jun 2007	29 days	Gemma Arnau	18 – 25 Nov. 2006	8 days
K. Abraham	31 Oct – 30 Nov 2007	30 days	Mary France Duval	11 – 20 Jun 2008	10 days
M. N. Sheela	8 – 31 Jul 2006	21 days			
M. N. Sheela	18 Nov – 18 Dec 2007	30 days			
M. N. Sheela	10 – 30 May 2009	21 days			

Funds received and utilised in India

DETAILS	Received	Utilised
SALARIES	Rs.4, 36,800	Rs.7,09,810
RECURRING	Rs.9,80,000	Rs.7,62,0335
EQUIPMENT	Rs.5,87,284	Rs.5,87,284
TOTAL	Rs.20,04,084	Rs.20,59,127

BALANCE AMOUNT TO BE REIMBURSED – Rs.55,043

Equipments purchased in India

NAME OF THE EQUIPMENT	VALUE	DATE OF RECEIPT
1. Scanner	Rs. 12,590	31 July 2006
2. Laser printer	Rs. 9,900	31 July 2006
3. Electronic balance	Rs. 2,644	2 Aug 2006
4. Digital camera	Rs. 57,938	4 Oct 2006
5. Laptop computer	Rs. 97,920	1 Dec 2006
6. Microscope with camera	Rs. 4,06,292	26 Feb 2007
Total	**Rs. 5,87,284**	

Publications

(Publications in journals & Presentations in conferences)

Publications in journals - Two

1. Lebot, V., Ivancic, A. and Abraham, K. 2005. The geographical distribution of allelic diversity, a practical means of preserving and using minor root crop genetic resources. Experimental Agriculture, 41(4): 475 – 489
2. Arnau G., Nemorin A., Maledon E., Abraham K**.** 2009. Revision of ploidy status of *Dioscorea alata* polyploid species by cytogenetic and microsatellite segregation analysis. Theoretical and applied genetics, 118(7):1239-1249.

Book chapter - One

1. Arnau G., Abraham K., Sheela MN., Chair H., Sartie A., Asiedu R. 2009. Yams. *In* Root and Tuber Crops, Handbook of Plant Breeding 7, (Springer Edition). In press.

Papers presented in conferences - Five

1. Abraham, K., Sreekumari, M.T., and Sheela, M.N. 2006. Seed production strategies and progeny selection in greater yam breeding. In: Proceedings of 14th Triennial Symposium of the International Society for Tropical Roots Crops, Thiruvananthapuram, Kerala, India, p 51-52.
2. Arnau., G., Erick Malledon., Isabelle Bachand and Abraham, K. 2006. Production of interploid hybrids and molecular marker heterozygosity determination using microsatellite markers in the greater yam, *Dioscorea alata*: importance for the genetic improvement of greater yam. In: Proceedings of 14th Triennial Symposium of the International Society for Tropical Roots Crops, Thiruvananthapuram, Kerala, India, p 52-53.
3. Sheela, M. N., Gemma Arnau., Abraham, K. and Sreekumari, M. T. 2006. Comparative analysis of allelic diversity of Indian greater yam. In: Proceedings of 14th Triennial Symposium of the International Society for Tropical Roots Crops, Thiruvananthapuram, Kerala, India, p 79-80.

4. Arnau, G., Maledon, E., and Némorin, A. 2007. Genetic improvement of the greater yam *D. alata* through polyploidy breeding. In: Colon Wilfredo, Lugo Wanda I. (eds.). Marketing opportunities for agriculture and forestry products in the Greater Caribbean - A challenge for the 21st century. Caribbean Food Crops Society, Annual Meeting Caribbean Food Crops Society, 16-22 September, San José, Costa Rica, p. 112.
5. Arnau, G., Némorin, A., Maledon, E., and Lambert, F. 2007. Morpho-agronomic, cytogenetic and molecular characterization of the CIRAD yam collection for their enhancement and utilization in genetic improvement programs. In: Colon Wilfredo, Lugo Wanda I. (eds.). Marketing opportunities for agriculture and forestry products in the Greater Caribbean - A challenge for the 21st century. Caribbean Food Crops Society. Annual Meeting Caribbean Food Crops Society, 16-22 September, San José, Costa Rica, p. 43.

Posters presented in conferences - Three

1. Arnau., G. and Maledon, E. 2005. Genetic improvement of yam: what strategy and objectives? Poster presented to the 41st Annual Meeting of the Caribbean Society of Food Crops. July 10 – 16, 2005. Gossier, Guadeloupe, France.
2. Abraham K., Nemorin A., Lebot V., Sheela MN., Sreekumari MT, Arnau G. 2009. 7. Polyploidy breeding in greater yam for tuber yield improvement. Poster presented in ICPHB, International Conference on Polyploidy, Hybridization and Biodiversity; 17-20 May 2009, Saint Malo, France.
3. Arnau G., Nemorin A., Maledon E., Abraham K**.** 2009. Revision of ploidy levels of *Dioscorea alata* polyploid species by cytogenetic and microsatellite segregation analysis. Poster presented in ICPHB, International Conference on Polyploidy, Hybridization and Biodiversity; 17-20 May 2009, Saint Malo, France.

Achievements & benefits of the project

Achievements: One of the most significant achievements of this project was the initiation of conventional breeding of greater yam in the CIRAD station, Guadeloupe, French West Indies and the discovery of unique, fertile tetraploids of greater yam that could be utilized in yam breeding for the first time. The tetraploids were used for hybridization with other tetraploids as well as with diploids for the production of new tetraplolid and triploid hybrids. These higher ploids were higher yielding types and thus a new line of breeding – polyploidy breeding - could be initiated in greater yam improvement.

The meiosis of the tetraploid males of greater yam was studied for the first time and new information was gathered on the chromosome behaviour. The meiotic metaphase I showed mostly bivalents with only 6 – 8 quadrivalents, indicating an autotetraploid nature. The inheritance pattern of microsatellite markers futher confirmed the diploid nature of the 2n = 40 types which were considered to be tetraploids. These information gained from the project work has changed the concept of the basic chromosome number of *D. alata* which was revised from x = 10 to x = 20.

Comparative analysis of the allelic diversity of the Indian and Pacific greater yam accessions showed that they formed two different clusters indicating their genetic divergence and scope for selection of unique parents for hybridization and improvement. A protocol was developed for the conservation of *D. alata* pollen grains by freeze drying so that planned hybridization can be made in this dioecious crop, even if the required male parent does not flower in a particular season. The evaluation of the hybrid selections has identified six high yielding clones in India and ten high yielding clones in Guadeloupe. **The poster presentation - 'Comparative analysis of allelic diversity of Indian greater yam' which was from the findings of the project fetched the prestigious 'Pat Coursey Award' for the best yam research work and the best poster award during the 14th Triennial Symposium of the International Society for Tropical Root Crops (ISTRC).**

Likely benefits: The discovery of fertile tetraploids and the initiation of polyploidy breeding are breakthroughs in greater yam breeding. The production of tetraploid and triploid hybrids can substantially increase tuber yield in this crop. The comparative analysis of Indian and Pacific genetic stocks of greater yam has given very valuable information for the selection of parents in hybridization programmes to maximise heterosis and heterozygosity that can contribute to higher tuber yields.

Self assessment of the project

The project was highly fruitful due to the collaborative work, which could bring together different technologies by the scientists of CTCRI and CIRAD, to study the genetic stocks from two different geographical locations such Asia and Pacific. Such a study was undertaken for the first time. The Indian and French expertise could be complemented in the programme and far better results, than the individual accomplishments, could be obtained. As envisaged among the objectives, conventional and molecular breeding could be integrated for the genetic improvement of greater yam and most of the work plans were accomplished. The new discovery on the polyploidy breeding holds great potential for the greater yam improvement and technique for pollen preservation would facilitate hybridization even when male plants do not flower. The revision of basic chromosome number of the species is a fundamental discovery on this crop which is important in the practical breeding aspects. The provision for the international visits and working together of scientists in the partner institutes in India and France has improved the faculty of the individual scientists by exposure to different institutions and environments. The project has brought out results that are not only valuable for India and France, but also for the greater yam breeding in any country.

The carrying out of the project, though mostly individually in the partner institutes, which was augmented by international visits, gave excellent opportunities to work together, gain new insights, personally involve in discussions, share the experiences and get newer exposure, and that was perhaps the best part in the implementation of the project. There had been no hitches during the tenure of the project as the IFCPAR was very supportive and responsive.

Signature

(Indian Principal Collaborator)

Signature

(French Principal Collaborator)