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Molecular characterization of Cassava with yellow-orange roots for beta-carotene improvement

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ABSTRACT - *Casssava (*Manihot esculenta *Crantz) is one of the main food and income sources of about 500 million people in the tropics. The crop is mainly cultivated by small farmers in tropical Africa, Asia and Latin America. Embrapa Mandioca e Fruticultura Tropical, based in Cruz das Almas, Bahia, maintains one of the largest cassava genebanks of Latin America. Among the accessions it contains, those with yellow-orange root color are particularly interesting. The objective of this study was to characterize 30 cassava accessions with yellow-orange root color by RAPD markers. The genetic distances of the 47 analyzed primers varied from 9.0 to 31.7 %, demonstrating the existing genetic variability to be exploited for the development of cassava varieties with higher beta-carotene contents.*

INTRODUCTION

Manihot esculenta Crantz is a species native to tropical America (Olsen and Schaal 2001), initially cultivated by native Latin Americans and later introduced into the African and Asian continents. The worldwide cassava production, on an area of 17.870.626 hectares is approximately 195.574.112 tons (FAO 2004). It is considered one of the most important sources of calories and is an inexpensive staple food in Latin America (Montero 2003), mainly in the northeastern region of Brazil (Mendes et al. 2006).

The genetic diversity of this species is wide (Nassar 2006), concentrated mainly in Latin America and the Caribbean. Approximately 8500 cassava accessions are maintained worldwide in different collections, of which 7500 in South America (Costa and Morales 1994). In Brazil, 4132 accessions have been collected and are maintained in genebanks across the country (Fukuda 2000). Carotenes (α-carotene, *b*-carotene, lycopene) represent the most multifaceted group of pigments in nature, with colors varying from yellow to red, found in photosynthetic and non-photosynthetic tissues, such as roots, seeds and fruits. Once ingested, *b*-carotene is transformed, in the liver, into Vitamin A. Vitamin A is a micro-nutrient with functions related to vision, cell differentiation, growth development, reproduction and the immune system. Vitamin A deficiency (VAD) can cause severe diseases, e.g., ocular syndrome, xerophthalmia, and advance to irreversible blindness (Underwood et al. 1999). Although the lack of vitamin A can be prevented, xerophthalmia is still a public health problem in many developing countries (Welch and Graham 2002).

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In general, staple foods are considered poor sources of micro-nutrients. Cassava genetic breeding may modify this situation, through the exploration of diversity encountered in yellow-orange root cassava accessions (Gregorio 2002, Welch 2002, Bedoya et al. 2003). Among the accessions of the Cassava Genebank of Embrapa Cassava and Tropical Fruits, those with yellow-orange roots, which have only recently become the subject of thorough studies, deserve special attention. The roots of these accessions possibly contain high b-carotene contents. Moreover, it is known that there is sufficient genetic diversity in the cassava genebanks that can be explored for this trait (Iglesias et al. 1997, Carvalho 2000). The inheritance for b-carotene concentration in cassava roots is controlled by few genes, i.e., the levels of b-carotene in cassava varieties can be improved through genetic improvement (Iglesias et al. 1997).

Although cassava contributes to ensure the food security of poor rural communities, little is known about the variability in nutritional and quality traits of the roots (Chavez et al. 2005) Since the crop grows well under harsh conditions, and such areas are increasing worldwide (El-Sharkawy 1993), this issue should not be overlooked

The objective of this study was to analyze the genetic variability of 30 yellow-orange root cassava accessions of the Cassava Genebank of Embrapa Cassava and Tropical Fruits using RAPD markers. This study is a first step towards the establishment of cassava improvement strategies to raise the b-carotene content in new cassava varieties, so that superior genotypes can be made available for farmers and later be released as new, b-carotene-rich cassava varieties.

MATERIAL AND METHODS

Plant material

Thirty cassava accessions with yellow-orange roots from the Cassava Genebank were evaluated by Embrapa Cassava and Tropical Fruits, in Cruz das Almas, state of Bahia, Brazil. Color and origin of the accessions are presented in Table 1.

DNA extraction

Young leaf samples from cassava cuttings were used for DNA extraction (Doyle and Doyle 1990). An amount of 300 mg leaf tissue was ground in liquid nitrogen and transferred to 2 mL Eppendorf tubes. The quality and quantity of the DNA was compared in agarose gel and later the concentration was adjusted to 20 ng mL^{-1} for the RAPD analysis.

DNA amplification

The amplification reactions followed the protocol proposed by Williams et al. (1990) and the final volume of the samples was completed to 25 mL, containing: KCl 50 mM, Tris-HCl 10 mM (pH 8.3), $MgCl_2$ 2.4 mM, 100 mM of each of the dNTPs (dATP, dTTP, dGTP, dCTP), 0.2 mM of the primer (Operon Technologies, Alameda, CA, EUA), 20 ng DNA and 1.0 U *Taq* DNA polymerase.

Forty-seven primers were tested and the amplifications were carried out in a BIORAD-MyCycler thermocycler with the following amplification conditions: a denaturing step at 95 °C for 3 min.; followed by 40 cycles, each one consisting of denaturation at 94 °C for 1 min.; primer pairing with the DNA strand (35 °C for 1 min.) and fragment extension at 72 °C for 2 min, and after 40 cycles, a last extension step of 7 minutes at 72 °C. The samples were electrophoresed in agarose gel (1.2 %) at 90 V for approximately three and a half hours. DNA bands were captured by the Kodak Digital photo documentation system.

Genetic data analysis

The polymorphic and monomorphic bands originated from RAPD primers were used to calculate the genetic distances using Jaccard's similarity coefficient, which takes the presence (1) or absence (0) of bands into consideration. Only clearly identifiable bands were used in the genetic analysis. The software Genes (Cruz 2003) was used for the calculation of the distance matrix and for the construction of the dendrogram (UPGMA-Unweighted Pair Group Method with Arithmetic Mean). The node consistency in the dendrogram was verified by bootstrap analysis by checking if the number of polymorphic bands evaluated was sufficient for accurate genetic distance estimates (Felsenstein 1985). The Jaccard index of dissimilarity was used to calculate the genetic dissimilarity, where: I_{AB} = A / (A+ B+C); A = the same band for both individuals; B = presence of band in individual 1 and absence in individual 2; C = absence of band in individual 1 and presence in individual 2.

Table 1. Yellow-orange root cassava accessions evaluated of the Cassava Genebank, maintained by Embrapa Cassava and Tropical Fruits

Identification number	Root color*	Common name	Brazilian State of origin
BGM 1667	Yellow	Mandioquinha	Pará
BGM 1708	Deep yellow	Jabuti- IM 957	Amazonas
BGM 1757	Deep yellow	Rosa	Maranhão
BGM 1668	Yellow	Cacau amarelo	Pará
BGM 1456	Pinkish	Vermelha	Mato Grosso
BGM 1702	Yellow	Peixe Boi – IM 929	Amazonas
BGM 1666	Yellow	Manteiga	Pará
BGM 1669	Yellow	Amarela	Pará
BGM 1692	Deep yellow	Aipim Dendê	Bahia
BGM 1700	Deep yellow	Varejão – IM 924	Amazonas
BGM 1701	Deep yellow	Sem nome – IM 928	Amazonas
BGM 1703	Deep yellow	Olho verde	Amazonas
BGM 1704	Deep yellow	Caniço – IM 936	Amazonas
BGM 1706	Deep yellow	Amarelinha	Amazonas
BGM 1709	Yellow	Canela de Velho – IM 958	Amazonas
BGM 1711	Yellow	Arani – IM 962	Amazonas
BGM 1722	Deep yellow	João Velho (Abóbora)	Maranhão
BGM 1740	Deep yellow	Branca	Maranhão
BGM 1745	Deep yellow	Carema Branca	Maranhão
BGM 1751	Deep yellow	-	Maranhão
BGM 1752	Deep yellow	Girau	Maranhão
BGM 1776	Deep yellow	Seis Meses	Maranhão
BGM 1782	Deep yellow	Carga de Burro	Maranhão
BGM 1783	Deep yellow	Verdinha	Maranhão
BGM 1787	Yellow	Aparecida 1157	Maranhão
BGM 1795	Yellow	Mucurona 1165	Maranhão
BGM 0019	Deep yellow	Xingu	Pará
BGM 0021	Yellow	Cachimbo	Pará
BGM 0456	Pinkish	Cenoura Rosada	Bahia
BGM 1153	Deep yellow	Klainasik	Amazonas

* Root color according to the scale proposed by Echeverry et al. (2001)

In order to verify whether the number of polymorphic loci evaluated was high enough to provide accurate genetic distance estimates for each specific number of bands, the markers were submitted to 1000 random samplings with replacement (bootstrap samples) and genetic distances were obtained for each bootstrap sample (Felsenstein 1985, Halldén et al. 1994).

RESULTS AND DISCUSSION

Screening of cassava of the genebank

It is well known that one condition for successful breeding programs targeting an increase of any favorable agronomic trait, is the availability of sufficient genetic variability in the plants under study (Iglesias et al. 1997, Chavéz et al. 2005).

Studies have shown that root color and b-carotene content are positively correlated; an important information that can help breeders in the early phases of breeding programs. On the other hand, the reports also demonstrated that quantitative evaluations should be taken into consideration. Iglesias et al. (1997) analyzed a sub-set of accessions of the global cassava genebank of the CIAT (International Center for Tropical Agriculture, Cali, Colombia) to determine the range of carotene concentration and variability by screening roots of cassava landraces. Results indicated considerable variation in the trait intensity of yellow, while some root parenchyma were closer to orange. Although the carotene transport and accumulation in cassava roots is governed by major genes, the quantitative variability of root color observed in clones suggests that a number of genes with less effect are involved in the accumulation process.

Furthermore, the five genotypes with the highest b-carotene concentrations were found in the Amazon Region of Brazil and Colombia. The highest value (2.55 mg 100 g^{-1} of fresh root) was measured in the genotype *Olho verde*, also studied here, which is promising for future recombination strategies. Another interesting result was the significant correlation (r = 0.82) between root color and carotene content, while 67 % of the total variability in carotene content could be explained by the variability in root color, demonstrating that in general, it is possible to improve carotene content by visual selection for color intensity (Iglesias et al. 1997).

Chavéz et al. (2005), in a continuation of the study carried out by Iglesias et al. (1997), evaluated improved clones (originated from breeding program at CIAT-Colombia, IITA – Nigeria and Rayong Experimental Station – Thailand) and landraces in the cassava germplasm maintained by CIAT. The carotene content and other nutritional and important agronomic traits of 2457 clones were evaluated. Carotene content in roots ranged from 0.102 to 1.040 mg 100^{-1} fresh tissue and also correlated positively ($r = 0.860$) with color intensity, indicating the potential of cassava clones with yellow roots to contribute to overcome vitamin A deficiency (VAD) in regions of the world where this disease is considered chronic.

Important research work regarding cassava b-carotene content has been done at CIAT, with a strong focus on the study of the genetic variability for nutritional quality traits in cassava, mainly on developing and identifying cassava germplasm with higher carotene root contents. The study was also carried out by the CIAT in cooperation with UNICAMP-Campinas-Brazil. This inter-laboratory partnership was created to evaluate cassava families for the total carotene content to elucidate the inheritance of carotene content in cassava roots (CIAT 2005). Based on preliminary results and in some contrast with earlier information, it was concluded that the mode of inheritance of the carotene content in cassava roots may be recessive; this fact is now being confirmed by self pollination (CIAT 2005). The cited paper is in agreement with previous studies, reinforcing the positive correlation between b-carotene content and root color intensity. The study was also conducted by the CIAT in partnership with Embrapa Cassava and Tropical Fruits, Cruz das Almas - Bahia, Brazil. A total of 1800 accessions from the genebank of Embrapa CNPMF were evaluated to identify those with high carotene root content quantitatively. The first selection was based on a color scale ranging from five (yellow) to eight (pinkish). This first screening selected 72 landraces from the genebank, which were evaluated for carotene quantification. The average carotene content was 6.6 mg total carotenoids per g of fresh root, demonstrating a wide genetic variation from 0.63 to 15.51 mg total carotenoids per g of fresh root (CIAT 2005).

Fukuda et al. (2005) evaluated the b-carotene content in 23 cassava genotypes of the cassava genebank at Embrapa-CNPMF and concluded that the percentage of total b-carotene (*cis* and *trans*) content varied from 12.12 to 79.8 % for the genotypes 1456 (*Vermelhinha*) and Hybrid 14-11, respectively. These results also indicate a broad range of variability. From these 23 cassava genotypes evaluated here, 11 were analyzed with RAPD markers (1700, 1704, 1711, 1153, 1456, 1667, 1668, 1692, 1721, 1722, and 0456). Their percentage of total b-carotene content varied from 13.06 to 69.74 %, for the genotypes 0456 and 1153, respectively (Fukuda et al. 2005). The combination of quantitative and genetic data are important for breeders, as orientation in the selection of promising parental genotypes.

Genetic variability analysis

A total of 30 accessions of yellow-orange root cassava of the Cassava Genebank of Embrapa Cassava and Tropical Fruits were analyzed using molecular markers (Figure 1). A dendrogram was obtained through cluster analysis for the 30 cassava accessions, where 47 RAPD primers generated a total of 282 bands (189 polymorphic and 93 monomorphic bands) (Figure 2), demonstrating that there is some genetic variability that can be explored in future breeding programs.

The closest accessions, according to the distance matrix were BGM-1722 and BGM-1666, with 9.1 % of dissimilarity; the dendrogram with a bootstrap value of 93.6 % clearly shows the proximity. The largest genetic distance was found between the accessions BGM-1740 and BGM-1692 (31.7 %), with 60.0 % bootstrap value. In order to verify if the number of polymorphic loci evaluated was high enough to provide accurate genetic distance estimates for each

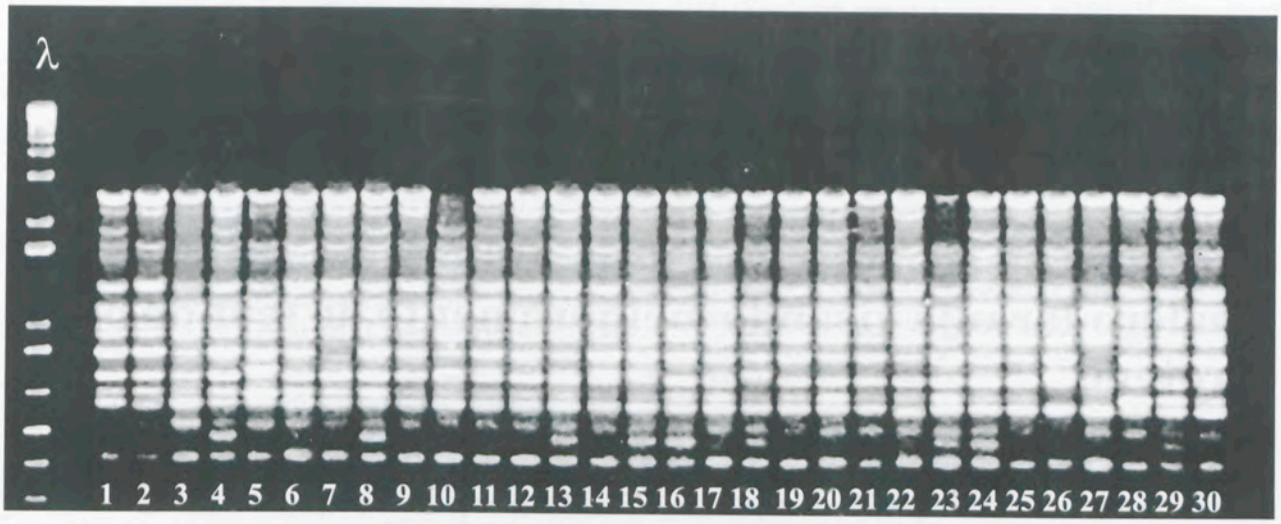

Figure 1. Eletrophoretic gel using RAPD primer OPF-05 of the 30 yellow-orange rooted cassavas studied of the Cassava Genebank, of Embrapa Cassava and Tropical Fruits. 1-30 BGM accessions: 1667; 1708; 1757; 1668; 1456; 1702; 1700; 1722; 1704; 1740; 1783; 1701;1709;1711;1752;1666; 1745; 1787; 1669; 0019; 1782; 1795; 1153; 1776; 1751; 1703; 1706; 0456;1692; 0021, respectively l = 1 kb ladder

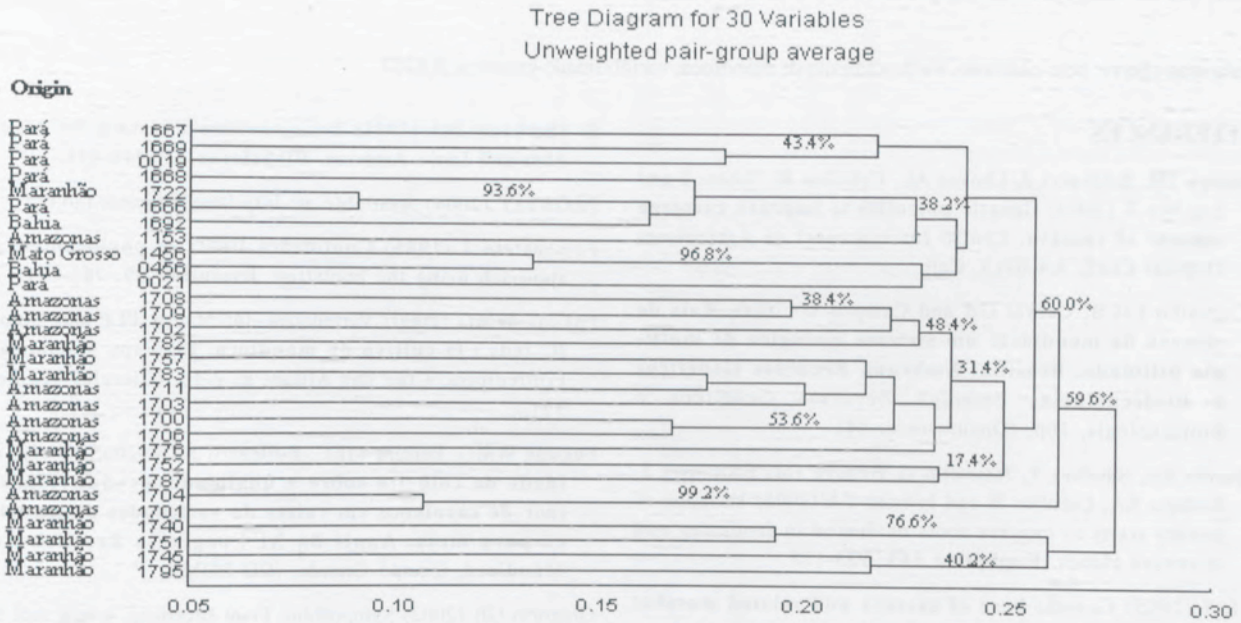

Figure 2. Dendrogram of 30 yellow-orange root cassava accessions of the cassava genebank of Embrapa Cassava and Tropical Fruits, in Cruz das Almas, Bahia, Brazil, based on data originated from 282 bands using RAPD markers. Percentage values are bootstrap

specific number of bands, the markers were submitted to 1000 random samplings with replacement (bootstrap samples) and genetic distances were obtained for each bootstrap sample (Felsenstein 1985, Halldén et al. 1994). The bootstrap values for the overall construction of the dendrogram reached a total average of approximately 60 % repeatability, which had been expected, based on the number of bands and the dominant nature of the RAPD marker.

Through the process of selection and recombination, the levels of important nutrient components can be improved to reach significant levels

in human nutrition (Iglesias et al. 1997). This preliminary evaluation using molecular markers was carried out to set the basis for future cassava breeding programs; it is also pioneering in terms of the molecular characterization of yellow-orange cassava roots in Brazil.

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Caracterização molecular de mandioca com raízes amalero-laranja visando melhoramento do teor de beta-caroteno

RESUMO - A mandioca (*Manihot esculenta* Crantz) serve como uma das principais fontes de alimentação e renda para aproximadamente 500 milhões de pessoas nos trópicos, sendo cultivada principalmente por pequemos produtores na África tropical, Ásia e América Latina. A Embrapa Mandioca e Fruticultura Tropical, localizada em Cruz das Almas, Bahia, possui um dos maiores bancos de germoplasma de mandioca da América Latina. Dentro dos acessos do banco, aqueles apresentando raízes de coloração amarelo-laranja merecem atenção especial. O objetivo do presente trabalho foi caracterizar 30 acessos de mandioca com raízes de coloração amarelo-laranja via marcadores RAPD. Quarenta e sete primers foram analisados. As distâncias genéticas variaram de 9,0 a 31,7 % demonstrando a existência de alguma variabilidade genética a ser explorada na obtenção de variedades de mandioca com maiores teores de beta-caroteno.

Palavras-chave: beta-caroteno, melhoramento de mandioca, variabilidade genética, RAPD

REFERENCES

Bedoya JM, Echivecci J, Chavez AL, Ceballos H, Tohme J and Sanches T (2003) **Genetic potential to improve carotene content of cassava**. Centro Internacional de Agricultura Tropical CIAT, AA 6713, Cali,

Carvalho LJCB, Cabral GB and Campos L (2000) **Raiz de reserva de mandioca: um sistema biológico de múltipla utilidade. Brasília: Embrapa Recursos Genéticos e Biotecnologia**. Embrapa Recursos Genéticos e Biotecnologia, 16p, (Documentos, 44).

Chavéz AL, Sánchez T, Jaramillo G, Bedoya JM, Echeverry J, Bolãnos EA, Ceballos H and Iglesias CA (2005) Variation of quality traits in cassava roots evaluated in landraces and improved clones. **Euphytica 143**: 125-133.

CIAT (2005) **Genetic base of cassava and related *manihot* species evaluated and available for cassava improvement: higher nutritional quality**. CIAT Annual Report, Output –1, p. 1-44.

Costa IRS and Morales EAV (1992) Cassava Genetic Resources in south America. In: **1st Meeting of the International Network for Cassava Genetic Resouces**, Calli, p. 17-21.

Cruz CD (2003) **Programa Genes, versão Windows**. UFV, Viçosa, Brasil.

Doyle J and Doyle JL (1990) Isolation of plant DNA from fresh tissue. **Focus 12**: 13-15.

El-Sharkawy MA (1993) Drought tolerant cassava for Africa, Asia and Latin America. **BioScience 43**: 441-451.

FAOSTAT (2004) Available at: http://www.faostat.fao.org

Felsenstein J (1985) Confidence limits of phylogenies: an approach using the bootstrap. **Evolution 39**: 783-791.

Fukuda WMG (2000) Variedades. In: Mattos PLP and Gomes JC (eds.) **O cultivo da mandioca**. Embrapa Mandioca e Fruticultura, Cruz das Almas, p. 7-10, (Circular Técnica, 37).

Fukuda WMG, Pereira MEC, Follegatti MIS (2005) **Efeito de idade de colheita sobre a qualidade, produtividade e teor de carotenos em raízes de variedades de mandioca para mesa. Anais do XI Congresso Brasileiro de Mandioca**, Campo Grande, (CD-ROM).

Gregorio GB (2002) Symposium: Plant Breeding: A new tool for fighting micronutrient malnutrition. Progress in breeding for trace minerals in staple crops. **Journal of Nutrition 132**: 500-502.

Halldén C, Nilsson NO, Rading IM and Sall T (1994) Evaluation of RFLP and RAPD markers in comparison of *Brassica napus* breeding lines. **Theoretical and Applied Genetics 88**: 123-128.

Iglesias C, Mayer J, Chaves L and Calle F (1997) Genetic potential and stability of carotene content of cassava roots. **Euphytica 94**: 367-373.

Mendes RA, Andrade AP, Fukuda WMG, Mascarenhas MM, Valle TL and Galera JMSV (2006) Mapeamento da distribuição geográfica das espécies silvestres brasileiras de *Manihot* com vista à conservação dos parentes silvestres e das variedades crioulas das mandiocas. In: **Ministério do Meio Ambiente-Parentes silvestres de espécies de plantas cultivadas**. Brasília, 44p.

Montero WR (2003) Cassava: Biology, Production and Utilization. **Crop Science 43**: 448.

Nassar NMA (2006) Indigenous and wild cassava: A rich source of genetic diversity in Brazil. **Chronica Horticulturae 46**: 20-21.

Olsen KM and Schaal BA (2001) Microsatellite variation in cassava (*Manihot esculenta*, Euphorbiaceae) and its wild relatives: Further evidence for a southern Amazonian origin of domestication. **Annuals of Journal of Botany 88**: 131-142.

Underwood BA and Smitasii S (1999) Micronutrient malnutrition: Policies and Programs for control and their implications. **Annual Review of Nutrition 19**: 303-324.

Welch RM (2002) Breeding strategies for biofortified staple plant foods to reduce micronutrient malnutrition globally. **Journal of Nutrition 132**: 495-499.

Welch RM and Graham RD (2002) Breeding crops for enhanced micronutrient content. **Plant and Soil 345**: 205-214.

Williams JGK, Kubelik AR, Livak KJ, Rafalski JA and Tingey SV (1990) DNA polymorphism amplified by arbitrary primers are useful as genetic markers. **Nucleic Acids Research 18**: 6531-6535.