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Analysis of heterogeneity of *Copia*-like retrotransposons in the genome of Cassava (*Manihot esculenta* Crantz)

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Summary: Retrotransposons are ubiquitous in eukaryotic genomes and now proving to be useful genetic tools for genetic diversity and phylogenetic analyses, especially in plants. In order to assess the diversity of *Ty1/Copia*-like retrotransposons of cassava, we used PCR primers anchored on the conserved domains of reverse transcriptases (RTs) to amplify cassava *Ty1/Copia*-like RT. The PCR product was cloned and sequenced. Sequences analysis of the clones revealed the presence of 69 families of *Ty1/Copia*-like retrotransposon in the genome of cassava. Comparative analyses of the predicted amino acid sequences of these clones with those of other plants showed that retroelements of this class are very heterogeneous in cassava. Cassava is widely grown for its edible roots in the tropical and subtropical regions of the world. Cassava roots, though poor in protein, are rich in starch (makes up about 80% of the dry matter), vitamin C, carotenes, calcium and potassium. It has a great commercial importance as a source of starch and starch based products. Realizing the importance of cassava, it stands out as a crop to benefit from biotechnology development. Heterogeneity of *Mecop*s (*Manihot esculenta* copia-like Retrotransposons) showed that they may be useful for genetic diversity and phylogenetic analyses of cassava germplasm.

Keywords*:* Cassava, transposable-elements, retrotransposons, retroviruses, *Manihot esculenta*, *Ty1/Copia*

Abbreviations: RT (reverse transcriptase), TEs (transposable elements), *Mecop*s (*Manihot esculenta* copia-like retrotransposons)

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INTRODUCTION

There are two major super-families of transposable elements (TEs) based on their transposition intermediate and transposition mechanisms: DNA TEs and retrotransposons (Finnegan 1992). *Ty1/copia*–like retrotransposons are the best characterised of the group of retrotransposons in plants. They replicate by reverse transcription of RNA intermediates transcribed from the parental DNA copies and lack excision in their life cycle (Eickbush 1994).

The reverse transcriptase (*RT*) region of the *pol* gene is the most highly conserved sequence of retroelements, sufficient enough to permit unambiguous classification of the *RT* as being from or encoded by *Ty1/copia*-like retrotransposons (Doolittle *et al.* 1989, Xiong and Eickbush 1990). The use of PCR primers based on the highly conserved amino acid sequence of enzymes domains has proved highly successful in the survey of retrotransposons in many plants (Flavell *et al.* 1992, Hirochika and Hirochika 1993, Suoniemi *et al.* 1998, Vershhinin *et al.* 2002, Zhao *et al.* 2007, Ruas *et al.* 2008, Moisy *et al.* 2008, Karlov *et al.* 2010, Rajput and Upadhyaya 2010). It has made the study of their diversity, abundance and applications possible even in the species where full genome sequence data are not yet available.

There is dearth of information on *Ty1/copia*-like retrotransposons of cassava, a crop that is widely grown for its edible roots in the tropical and subtropical regions of the world where it is the staple food of over 500 million people. Apart from being used as a source of food, it has a great commercial importance as a source of starch and starch derived products. It is a very attractive source of renewable energy because of high starch content. There is now an increasing need to apply the findings from the so-called model plants to important food crops like

cassava. Knowledge of the presence and nature of *Ty1/copia*-like retrotransposons in cassava would contribute to the design and application of retrotransposon–based molecular markers. These have been used for the study of biodiversity in other crop plants including maize, pea, barley, oat and cashew (Purugganan and Wessler 1995, Kumar *et al.* 1997, Waugh *et al.* 1997, Ellis *et al.* 1998, Kalendar *et al.* 2000, Yu and Wise 2000, Syed *et al*. 2005, Kalendar and Schulman 2006). *Ty1/copia*-like retrotransposons also have applications in functional genomics research, such as gene tagging and reverse genetics, as demonstrated in rice (Agrawal *et al.* 2001, Hirochika 2001).

This study describes the isolation, cloning, sequencing and analysis of the *RT* fragment characteristic of *Ty1/copia*-like retrotransposons using degenerate PCR primers. The diversity of *Ty1/copia*-like elements within the cassava genome and their relationship to those of other plants were analysed. The nucleotide sequences described here have been submitted to the Genbank database and given the accession numbers **AY946085** to **AY946153**.

MATERIALS AND METHODS

Plant material and DNA isolation

Using the method of Dellaporta *et al.* (1983), DNA was extracted from young leaf samples of cassava cultivars grown in the tropical glasshouse at the University of Bath. The growth conditions include temperature at 22-28 ^{o}C, relative humidity of 40-80 % and a minimum light period of 12 h per day under day light, supplemented with 400 W Phillips high-pressure sodium lights when necessary.

PCR Amplification and cloning of cassava Ty1/Copia-like retrotransposon reverse transcriptase gene fragment

The PCR method used was as described by Voytas *et al.* (1992) with some modifications as described by Gbadegesin *et al*. (2008). Amplified DNA bands were gel purified (Qiagen, 'Qiaquik'), ligated into pGEM®-T Easy vector (Promega) and used to transform competent *Escherichia coli* DH5α according to standard procedures (Sambrook *et al.* 1989).

Sequence and phylogenetic analyses

DNA molecules were sequenced on an ABI 337 automated dye primer sequencer using universal primers for the cloning vector. The first line of sequence identification was by using BLASTN and TBLASTX searches against the GenBank non-redundant database at the default parameters (Altschul *et al.* 1990). The sequence fragments were assembled using the Vector NTI program. Consensus sequence data were aligned using CLUSTAL W (Higgins *et al.* 1994). The PHYLIP program package (Felsenstein 2004) and MEGA4 (Tamura *et al.* 2007) were used for phylogenetic analysis. In PHYLIP, consensus NEIGHBOR–joining trees (Saitou and Nei 1987) were derived from equally parsimonious trees using the extended majority rule in the CONSENSE while UPGMA method (Sneath and Sokal 1973) was adopted in MEGA4. Distance matrices for phylogenetic analyses based on nucleotide sequences data were computed using the Maximum Composite Likelihood method (Tamura *et al*. 2004). On the other hand, those based on amino acid sequences were computed using the Jones-Taylor-Thornton model (Jones *et al*. 1992) in the PROTDIST of the PHYLIP. Trees were drawn using TREEVIEW program version 1.6.6 available from the author, Roderic D.M. Page of the Taxonomy Unit, Department of Zoology, University of Glasgow.

RESULTS

PCR amplification of cassava Ty1/Copia-like retrotransposon reverse transcriptase gene fragment, cloning and sequencing

Amplified products were analysed by electrophoresis on ethidium bromide stained 1.0 % agarose gel. Approximately 0.3 kb cassava DNA was amplified (Figure 1).

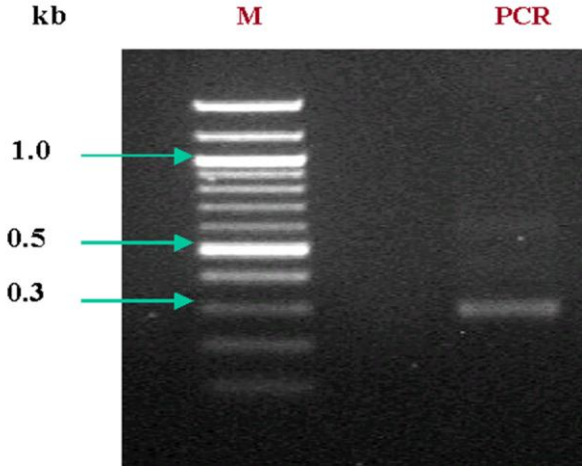

Figure 1:
PCR amplification of *Ty1/copia-like* RT fragment from cassava genomic DNA. PCR product was run on ethidium bromide stained 1.0 % agarose gel. The size marker (lane M) is Bioline DNA 100bp ladder while the PCR product is shown on the right lane.

Amplified DNA was purified and directly sequenced using the PCR primers. The sequence was submitted to BLASTN and TBLASTX searches to ascertain its identity. The searches confirmed that *Ty1/copia*-like reverse transcriptase fragment has been amplified in the PCR experiments. The result of the TBLASTX is shown in Figure 2. The cassava sequences were 71 % identical with RT of *Setaria verticillata Ty1/copia*-like retrotransposons *(Sv)*. The cassava sequence (*Me*) has some unresolved or ambiguous sequences represented as X in the amino acid sequence. The ambiguities suggest that the PCR band represent a population of individual members (with sequence variance) of *Ty1/copia*-like retrotransposons. The PCR-derived DNA band was ligated into pGEM-T Easy vector (Promega) and used to transform competent *Escherichia coli* as described in Materials and Methods section. Randomly selected clones were fully sequenced on both strands using the T7 and SP6 primers.

Sequence analysis

The sequences were assembled using the VECTOR NTI program. This allowed visualization and removal of vector sequences. The vector free sequence data were submitted to BLASTN and TBLASTX searches against the GenBank non-redundant database. The sequence data of cassava *Ty1/copia*-like RT sequence is summarised in Figure 3. Sixty-nine (89.6 %) of the clones showed clear homology to reverse transcriptase of *Ty1/copia*-like retrotransposons (most have E-value in the region of e^{-40}). Eight (10.4 %) did not show significant homology to any of the nucleotide or protein sequences that were registered in the databases and were not analysed further. The deduced translations of the clones with clear homology to *Ty1/copia*-like RT nucleotide sequences were obtained using ORF finder (www.ncbi.nlm.nih.gov/gorf/) (data not shown). Of these, 37 (54.5 %) contained neither a frame shift nor a nonsense mutation while 32 (35.1 %) did contain these mutations within the sequence analysed.

Phylogenetic analysis of cassava Ty1/copia-like retrotransposons RT

In order to determine the relatedness of the cassava *Ty1/copia*–like retrotransposons to each other the nucleotide sequences of the 68 *Mecop*s (minus one duplicate) were aligned using CLUSTAL W (data not shown). The aligned nucleotide sequences data of the 68 *Mecop*s were used to compute a distance matrix as explained in the Material and Methods section. Trees were then produced using the UPMA method and consensus-unrooted tree was derived from 1000 replicates. The consensus tree was drawn using TREEVIEW as shown in (Figure 4). Most members of the clade II sequences have an uninterrupted reading frame within the sequence in contrast to clade I family with many members bearing mutations within the sequence analysed. These members of the clade II whose sequences have an uninterrupted reading frame could be retrotranspositionally active as indicated by the amount of identities (97-99 % nucleotide sequence) they share. Clade II members were probably derived from the members in clade I. On the other hand, clade III members are highly diverged compared to members of the other clades. They have accumulated many mutations during their evolution. Many of its members bear stop codons and frame shift mutations within the sequence examined.

```
Me:3 EXDVXTAFLNGNLLEDVYMTQPEGFVIPXNXGKICKLXRSIYGLKQASRXWNLRFDXTVR 182
     + DV TAFLNGNL EDVYM QPEGFV P N GK+CKL RSIYGLKQASR WN+RFD V+
Sv:1 QMDVKTAFLNGNLAEDVYMIQPEGFVNPKNAGKVCKLQRSIYGLKQASRSWNIRFDEVVK 180

Me:183 DFGFIKNEDEPCVYKMVSGSAVXXLXLXVD 272
       F F KNE+E CVYK VSGS+V  L L VD
Sv:181 GFDFTKNEEESCVYKKVSGSSVIFLILYVD 270
```

Figure 2:
Alignment of cassava (*Me*) PCR product amino acid sequence with *Setaria verticillata* (Sv) *Ty1/copia*-like retrotransposon reverse transcriptase amino acid sequence (gi 17221911). The two sequences share 71 % identity.

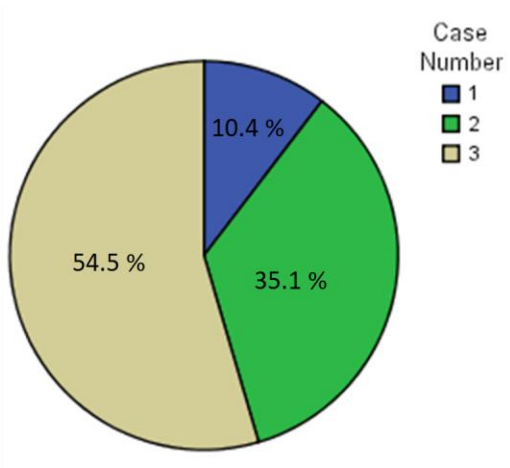

Figure 3:
The summary of the sequence data of 77 cassava putative *Ty1/copia*-like retrotransposons RT. The colour sectors represented are (dark yellow + green) for clones with strong homology to reverse transcriptase of *Ty1/copia*-like retrotransposons and blue for clones bearing no identity with *Ty1/copia*-like retrotransposons RT. Dark yellow sector represents clones with neither a frame shift nor a nonsense

mutation, while green sector represent clones with these mutations, within the sequence analysed.

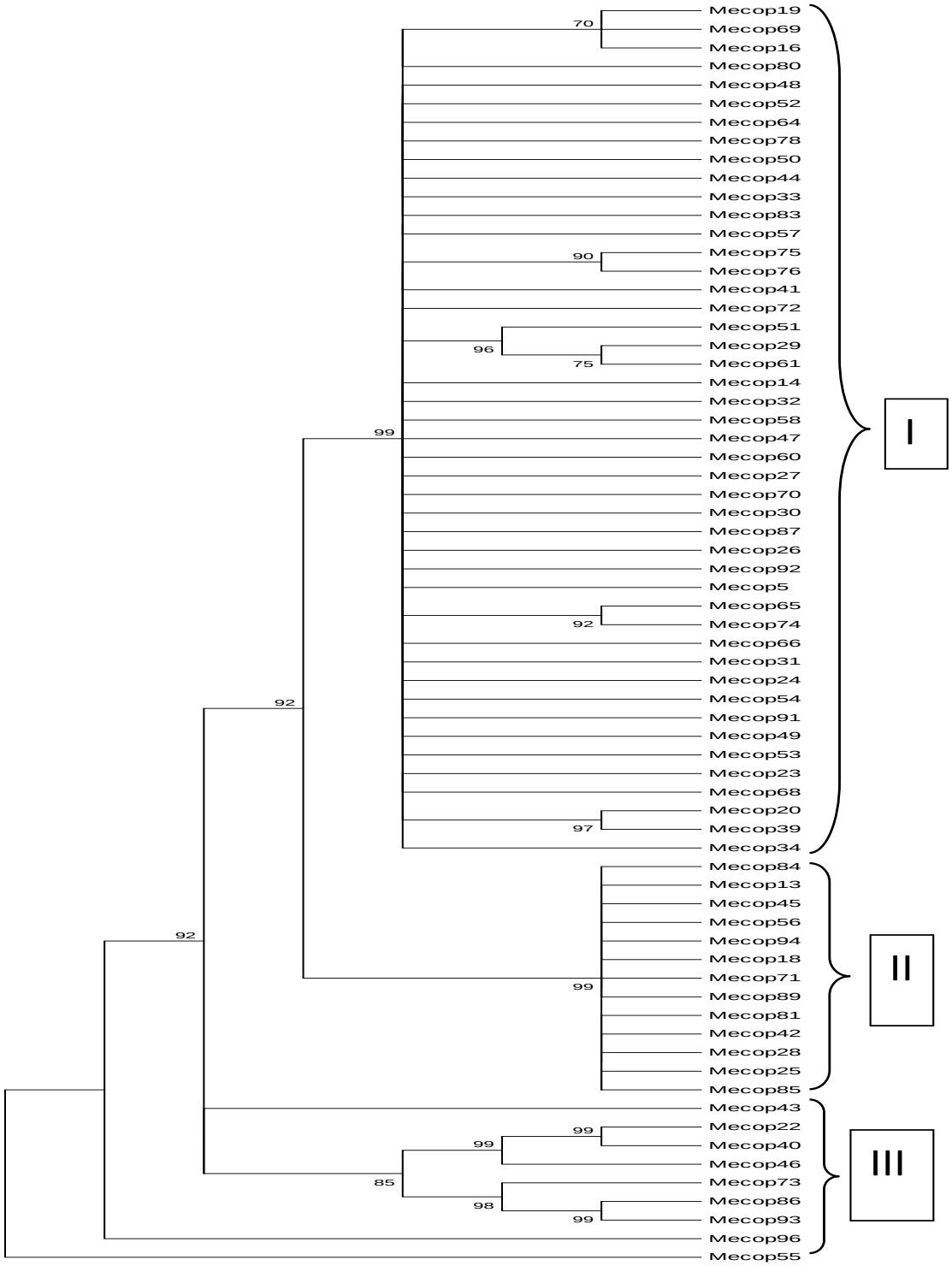

Figure 4*:*
Phylogenetic analysis of cassava *Ty1/copia*-like Retrotransposons. The phylogenetic relationship was inferred using the UPGMA method (Sneath and Sokal 1962). The optimal tree with the sum of branch length = 5.46569904 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches (Felsenstein 1985). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura *et al*. 2004) and are in the units of the number of base substitutions per site. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated from the dataset (Complete deletion option). There were a total of 181 positions in the final dataset. Phylogenetic analyses were conducted in MEGA4 (Tamura *et al*. 2007).The main clades identified are indicated with right brace and roman numerals.

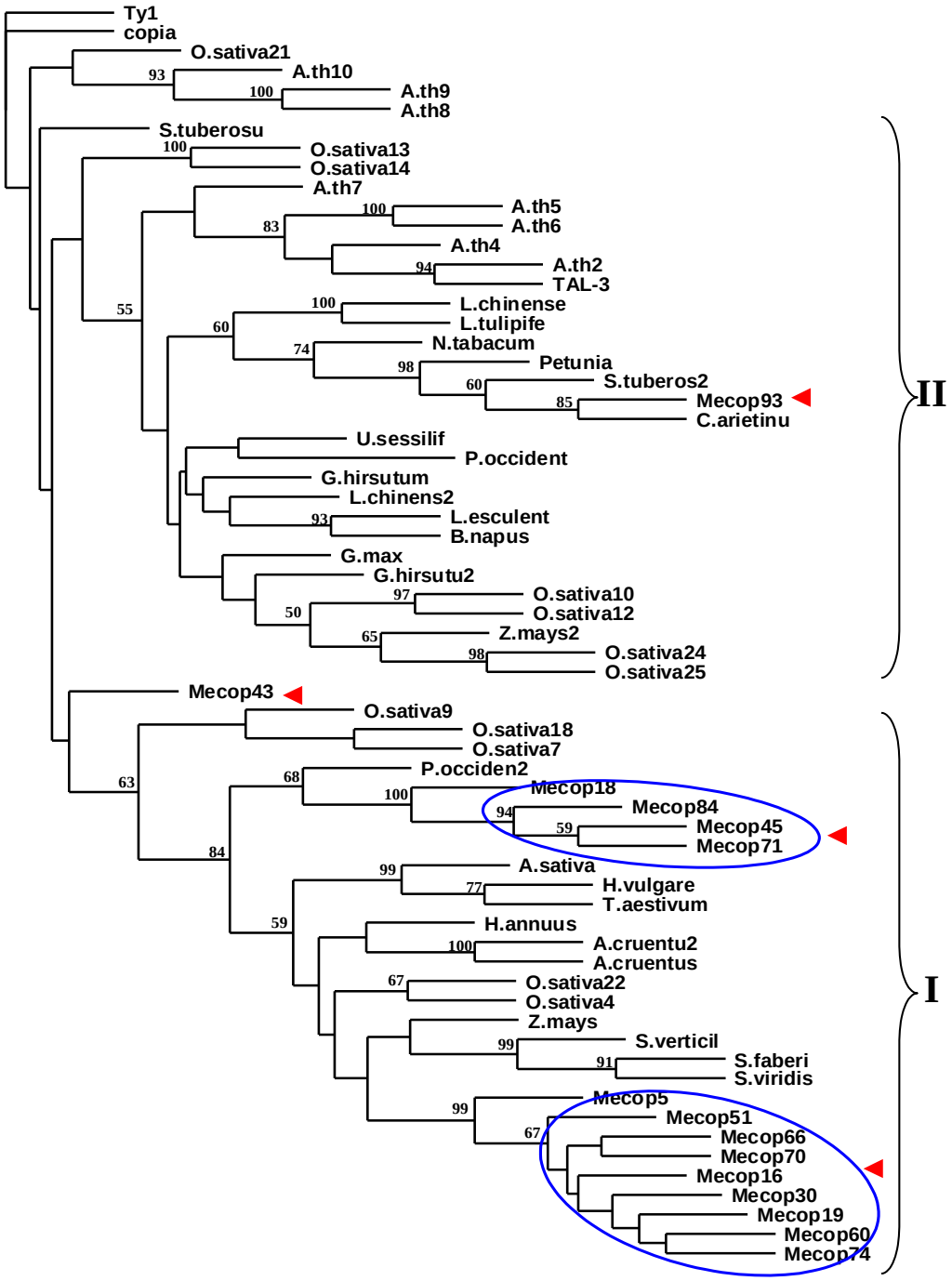

Figure 5:
Comparative phylogenetic analysis of cassava and other plants *Ty1/copia*-like retrotransposons. The tree is based on amino acid sequences of 15 *Mecop*s, 48 other plants *Ty1/copia*–like retrotransposons and sequences of *Ty1* of yeast and *Copia* of *Drosophila* included as outgroup sequences with *Ty1* as root. Each taxa is represented by fragment of the reverse transcriptase. The tree is produced by NEIGHBOR-joining program using the distance matrix method of Jones *et al*. (1992). The positions of the *Mecop*s are indicated with arrowheads. Bootrap values (1000 replicates) >/=50 are shown. The main clades identified are indicated with right brace and roman numerals.

Table 1
The reverse transcriptase amino acid sequences of *Ty1/copia* retrotransposons used in the comparative phylogenetic analysis with 15 representative *Mecop*s. The table shows the names used for the elements in the multiple alignments, the corresponding botanical name and the geninfo (GI) number.

Name used	Source species	NCBI GI number	Name used	Source species	NCBI GI number
Z.mays	*Zea mays*	15148823	N.tabacum	*Nicotiana tabacum*	100342
Z.mays2	*Zea mays*	478218	L.tulipifera	*Liriodendron tulipifera*	478084
Ty1	*Saccharomyces cerevisiae*	1945315	L.esculentum	*Lycopersicon esculentum*	478263
U.sessilif	*Uvularia sessilifolia*	478189	L.chinense1	*Liriodendron chinense*	478188
T.aestivum	Triticum aestivum	477749	L.chinen2	*Liriodendron chinense*	478147
S.viridis	*Setaria viridis*	17221919	H.vulgare	*Hordeum vulgare*	477070
S.verticillata	*Setaria verticillata*	17221911	H.annuus	*Helianthus annuus*	478085
S.faberi	*Setaria faberi*	17221874	G.max	*Glycine max*	478148
S.tuberosum	*Solanum tuberosum*	478243	G.hirsutum1	*Gossypium hirsutum*	477976
S.tuberos2	*Solanum tuberosum*	100442	G.hirsutu2	*Gossypium hirsutum*	477748
Petunia	*Petunia x hybrida*	478217	C.arietinum	*Cicer arietinum*	26985780
P.occident	*Platanus occidentalis*	477975	Copia	*Drosophila melanogaster*	1491679
P.occiden2	*Platanus occidentalis*	477747	B.napus	*Brassica napus*	477069
O.sativa25	*Oryza sativa*	169782	A.th9	*Arabidopsis thaliana*	539018
O.sativa24	*Oryza sativa*	14164956	A.th8	*Arabidopsis thaliana*	539017
O.sativa22	*Oryza sativa*	14164964	A.th7	*Arabidopsis thaliana*	539016
O.sativa21	*Oryza sativa*	14164966	A.th6	*Arabidopsis thaliana*	539015
O.sativa18	*Oryza sativa*	1419106	A.th5	*Arabidopsis thaliana*	539014
O.sativa14	*Oryza sativa*	1419118	A.th4	*Arabidopsis thaliana*	539013
O.sativa13	*Oryza sativa*	1419120	A.th2	*Arabidopsis thaliana*	539011
O.sativa12	*Oryza sativa*	1419123	A.th10	*Arabidopsis thaliana*	539010
O.sativa10	*Oryza sativa*	1419127	A.sativa	*Avena sativa*	478264
O.sativa9	*Oryza sativa*	1419129	A.cruentus	*Amaranthus cruentus*	12005128
O.sativa7	*Oryza sativa*	1419133	A.cruentu2	*Amaranthus cruentus*	12005119

Comparative Phylogenetic analysis of cassava with other plants Ty1/copia-like retrotransposons RT

Fifteen cassava *Ty1/copia-like* clones bearing neither stop codons nor frame shift mutations (*Mecop*5, 16, 18, 19, 30, 43, 45, 51, 60, 66, 70, 71, 74, 84 and 93) were randomly selected around the tree (Fig 4) as representative of the clades for comparative analysis with *Ty1/copia*-like retrotransposons from other organisms. The amino acid sequences for *Ty1* ofyeast, *Copia* of *Drosophila* and selection of *Ty1/copia*-like retrotransposons from dicotyledonous and monocotyledonous plants were chosen (Table 1). These were aligned with amino acid sequences of the 15 representative *Mecop*s using the CLUSTAL W program. The multiple alignments were used to compute distance matrix using the Jones-Taylor-Thornton model in the PROTDIST of the PHYLIP. Trees were then produced by NEIGHBOR-joining method. The unrooted tree drawn in TREEVIEW is shown in Figure 5.

DISCUSSION

Using PCR degenerate primers, cassava reverse transcriptase fragments characteristic of *Ty1/copia*-like retrotransposons have been isolated, cloned and sequenced. Sequence analyses including the deduced translations of cassava *Ty1/copia*-like RT nucleotide sequences revealed that 54.5 % contained neither a frame shift nor a nonsense mutation while 35.1 % did contain these mutations within the sequence analysed. The latter group are most probably non-functional enzymes, while the former may include functional enzymes. Alignments of the nucleotide sequences of cassava *Ty1/copia* clones (*Mecop*s) lead to the identification of highly diverse members of this group of elements. In addition, the alignments of the putative peptide sequence of representative *Mecop*s with known *Ty1/copia* retrotransposons of other plants revealed identity at most positions that were conserved in the majority of the elements compared.

Phylogenetic analysis of cassava and other plants *Ty1/copia* RT revealed that the cassava elements are highly heterogeneous, with the cassava elements forming clusters with elements from other plants. The finding here is in line with those of the population of *Ty1/copia*-like group retrotransposons in some other plant genomes examined (Flavell *et al.* 1992, Hirochika and Hirochika 1993, Marillonnet and Wessler 1998, Zhao *et al*. 2007, Zhou *et al*. 2010).

The clustering of *Mecop*93 with *Ty1/copia*-like retrotransposon of *Nicotiana tabacum*, *Cicer arietinum, Solanum tuberosum* and *Petunia x hybrida* could be called a congruent relationship because this correlates with plant divisions thus supporting the hypothesis for the vertical transmission of *Ty1/copia* retrotransposons. The similarity between cassava and the other plants' reverse transcriptases implies that the retrotransposons were present prior to the separation of the species and they have undergone little change since that time. Two subgroups of closely related cassava sequences (*Mecop*s 5, 16 19, 30, 51, 60, 66, 70 and 74) and (*Mecop*s 18, 45, 71 and 84) grouped with subgroups of elements from monocotyledons in what could be termed a non-congruent relationship because they did not reflect the evolutionary relationships of their host (cassava). They appear to share a common ancestor with elements from considerably distant taxa. One plausible source of incongruence could be horizontal transmission between two distantly related species in the more recent past. Many other plants *Ty1/copia*-like retrotransposons studied also shown characteristics similar to the observed in cassava (Flavell *et al*. 1992, Voytas *et al.* 1992, Hirochika and Hirochika 1993, Marillonnet and Wessler 1998, Zhou *et al*. 2010).

In addition, the presence in many of the *Mecop*s of numerous stop codons, and frame shift mutations interrupting the reading frame suggest that these cassava elements are defective and probably have lost the ability to transpose. The abundance of the defective retrotransposon in plants may also be related to the genome size of the plants. The larger genomes of plants may have a capacity to maintain the large copies of retrotransposons (Hirochika and Hirochika 1993).

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