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Posted Date: 25 June 2023

doi: 10.20944/preprints202306.1711.v1

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Characterization and Classification of Ty1-*copia* Retrotransposons in *Jatropha curcas* L.

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Abstract: *Jatropha curcas* is a potential energy crop and has been identified as a significant resource for biodiesel. Retrotransposons occupy the plant genome in a large proportion, so the specific retrotransposon characterization will help improve understanding of the *Jatropha* genome evolution and organization. This research aimed to characterize and classify Ty1-*copia* retrotransposons in *J. curcas* using specific gene sequence and to detect the distribution of Ty1-*copia* in *J. curcas* and *J. integerrima* chromosomes using florescence *in situ* hybridization (FISH) method. Ty1-*copia* sequences from *J. curcas* were isolated using three degenerate primer sets specific to the conserved Ty1-*copia* reverse transcriptase gene for polymerase chain reaction (PCR). FISH technique using the Ty1-*copia* probes was used to map physically on *J. curcas* and *J. integerrima* chromosomes. Altogether, 164 Ty1-*copia* sequences were obtained to use for the phylogenetic analysis and were classified into the four families of TAR, Angela, Ale and Bianca. Physical map using FISH method of Angela and Ale families on *J. curcas* and *J. integerrima* chromosomes exhibited biases for specific regions around the centromeres or at the chromosome terminal regions. The study indicates the high variation of the Ty1-*copia* elements in the genome of *Jatropha*. These results will enhance understanding of retrotransposon chromosomal distribution and evolution of the *J. curcas* genome and also promotion of retrotransposon possible utilization in *J. curcas* improvement projects using retrotransposon-based markers.

Keywords: florescence *in situ* hybridization; *Jatropha curcas*; phylogenetic tree analysis; Ty1-*copia* retrotransposons

1. Introduction

Jatropha curcas L. or physic nut is an energy crop potential in the use of renewable energy [1], yield since the first year, the oil is not consumed as food and a high oil content in seeds making it possible to use *J. curcas* as a feedstock to produce biodiesel. It is highly resistant to drought [2]; therefore, planting does not need to compete with food crops that require water and fertile soil. Moreover, in the process of oil chests squeezing, the remaining residue can be used as raw material in the manufacture of fertilizers, plastics, synthetic fibers and animal feeds [3-7].

It is commonly known that the activities of retrotransposons can be transferred in the genome, resulting in differences in the plant genome. Retrotransposons are found widely distributed in the plant genome as one of two types of TE (transposable elements). They can replicate themselves via RNA intermediates and lead to the regulation of different gene expression. These cause retrotransposons to be a significant element in the plant genome evolution [8].

There are two types of retrotransposons, that are long terminal repeat (LTR) and non-long terminal repeat (non-LTR). Approximately 40-70% of retrotransposons found in the plant genome are the LTR type which has two important domains, including group-specific antigen (Gag) domain and polyprotein domain (Pol) [9]. The Pol domain consists of 4 gene regulating enzymes, including protease, integrase, reverse transcriptase and RNase H which serves to cut Pol polyprotein and causes retrotransposon movement. LTR retrotransposons contain two large groups: Ty1-*copia* and Ty3-*gypsy* [9-10].

Fluorescent *in situ* hybridization (FISH) technique as well as its variation have been widely utilized in plant karyotype characterizations [11]. The techniques generally consist of matching the probes, which can be DNA or RNA, that have a specific sequence into the target genome. The objective is to specify the exact location in the chromosome. FISH uses various probes, either tandem repeats/rDNAs, centromeric/telomeric satellites or another specific DNA sequences of interest. FISH studies in plant chromosomes can identify the distribution of retrotransposons on plant chromosomes. Ty1-*copia* tends to be found at the distances away from the centromere of the plant chromosome, while *gypsy*-type is often found at the centromere or maybe opposite in some plants [12]. The study of retrotransposons in the *Jatropha* chromosome will be useful for understanding *Jatropha* genome, leading to successful breeding of this plant.

The aim of the present study was to isolate and characterize Ty1-*copia* retrotransposons in *J. curcas* collected from Thailand and other countries and to use the FISH technique to detect the distribution of Ty1-*copia* retrotransposons in *J. curcas* and *J. integerima* chromosomes. The results from the study will give more information on the diversity of Ty1-*copia* retrotransposons in *J. curcas* that will be useful for DNA marker development associated with breeding as well as improvement of the understanding of *Jatropha* genome evolution and organization.

2. Results and Discussion

2.1. Characterization of Ty1-copia sequences in the J. curcas genome

Three degenerate primer pairs related to the conserved sequence of the Ty1-*copia* reverse transcriptase genes in higher plants [13-15] were used by PCR-based method to increase the amount of DNA fragments of about 251-280 bp from six samples of *J. curcas* collected from Thailand and three samples of *J. curcas* obtained from Madagascar, Indonesia and Taiwan (Table 1). All of the obtained PCR products were cloned and selected for at least 40 clones from the individual of the nine *J. curcas* samples. The inserted clones were selected by preliminary grouping based on restriction patterns after cutting the inserted PCR products by restriction enzymes *Alu*I, *Eco*RI, *Hae*III, *Bcl*I and *Nco*I before sequencing. All three degenerate primer pairs could amplify various Ty1-*copia* elements. Altogether, 164 resulting sequences were used for the phylogenetic tree analysis. This study indicates the high diversity of the Ty1-*copia* elements in the genome of *Jatropha curcas*.

Table 1. Distribution of isolated Ty1-*copia* sequences.

***J. curcas* plant samples**	**Origin**	**TAR**	**Angela**	**Ale**	**Bianca**	**Total**
KUBP78-9	Thailand	11	8	7	-	26
KUBP74	Thailand	13	9	4	-	26
KUBP20	Thailand	11	4	2	-	17
KUBP16	Thailand	6	5	4	-	15
Salt-tolerant #1 from None-Sung district of Nakhon Ratchasima province	Thailand	4	10	-	-	14
Salt-tolerant #2 from Kham-Talay-Sor district of Nakhon Ratchasima province	Thailand	14	7	8	-	29
Madagascar	Madagascar	4	1	3	-	8
Taiwan	Taiwan	7	7	1	-	15

Indonesia	Indonesia	5	6	2	1	14
Total		75 (46.01%)	57 (34.97%)	31 (19.02%)	1 (0.61%)	164

Comparison of all 164 obtained sequences to the GenBank-NCBI database using the BLAST network service at http://www.ncbi.nlm.nih.gov/BLAST/ was performed and the analysis using SIB ExPASy Bioinformatic Resources portal at https://web.expasy.org/translate/ was done. Amino acid sequences of approximately 83-93 amino acids in length derived from all isolated Ty1-*copia* fragments, together with retrotransposons from other species, *Phaeodactylum tricornutum* (GenBank: EU432487.1) as an outgroup, were aligned and then used to construct a phylogenetic tree based on maximum likelihood (ML) method (Figure 1). Consistent with the high heterogeneity level reported among the reverse transcriptase (RT) sequences of Ty1-*copia* in plants [16], most of our isolated RT sequences were found different from each other and were found distributed into 12 clusters, which corresponds to Ty1-*copia* families of *Jatropha curcas*. The identified Ty1-*copia* sequences were classified in to four distinct groups (TAR, Angela, Ale and Bianca) with high bootstrap values, which indicated that they belong to at least four distinct Ty1-*copia* families in the *Jatropha* genome. Family TAR were found in a large number of isolated Ty1-*copia* clones (46.01%) that further classified into five subfamilies, followed by family Angela (34.97%) and family Ale (19.02%) which both could be further divided into 3 subfamilies, while Bianca possessed the smallest number of clone (0.61%) as shown in Figure 1. The isolated retrotransposon clone numbers in the individual family were counted and the patterns of retrotransposon family distribution were identified among the nine *Jatropha curcas* samples. We found that distribution patterns of Ty1-*copia* families were different among the nine *Jatropha* samples (Table 1).

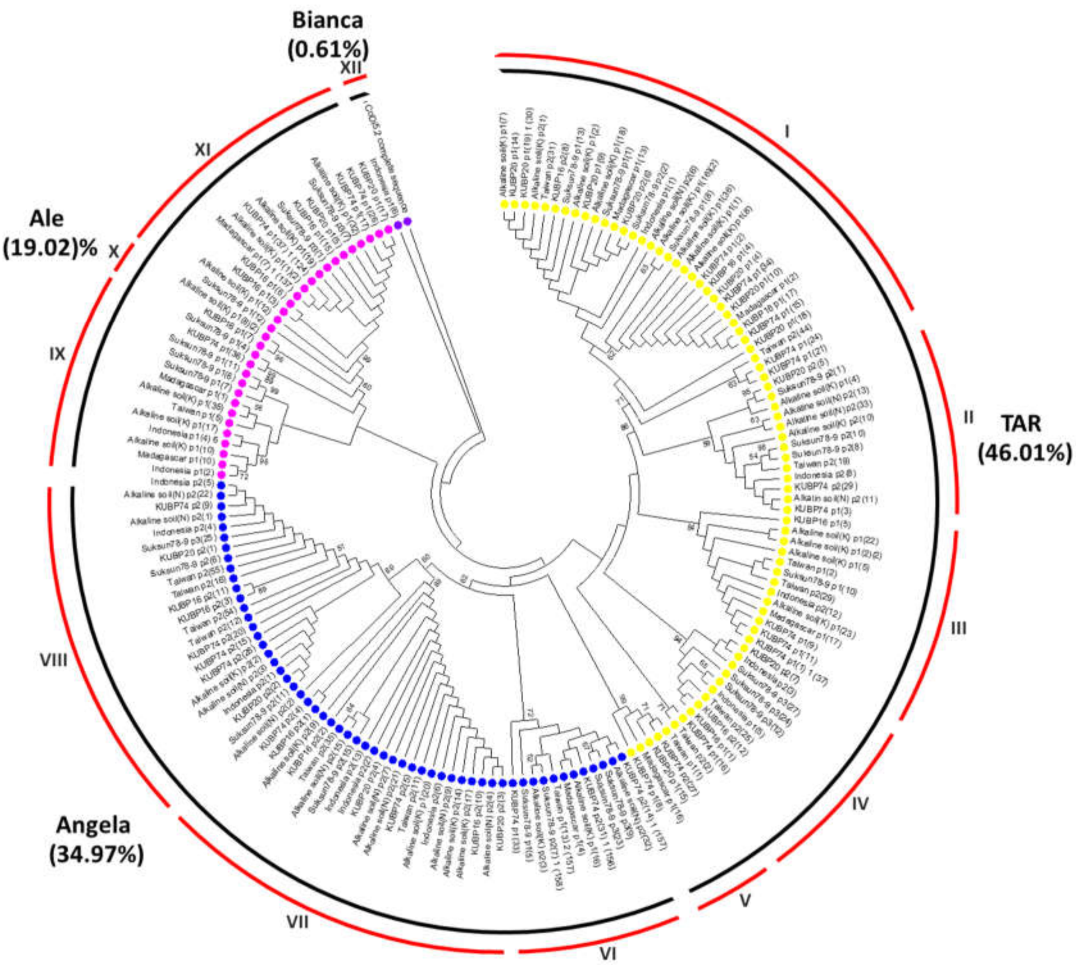


Figure 1. Phylogenetic tree based on reverse transcriptase gene sequences for Ty1-*copia* from nine *Jatropha* samples. TAR, Angela, Ale and Bianca sequences are marked with yellow, blue, pink and purple circles (I to V represent subfamilies of TAR; VI to VIII represent subfamilies of Angela; IX to XI represent subfamilies of Ale and XII represents family Bianca, containing one sequence belongs to *J. curcas* from Indonesia and the other sequence belongs to an outgroup: *Phaeodactylum tricornutum*, CoDi5.2).

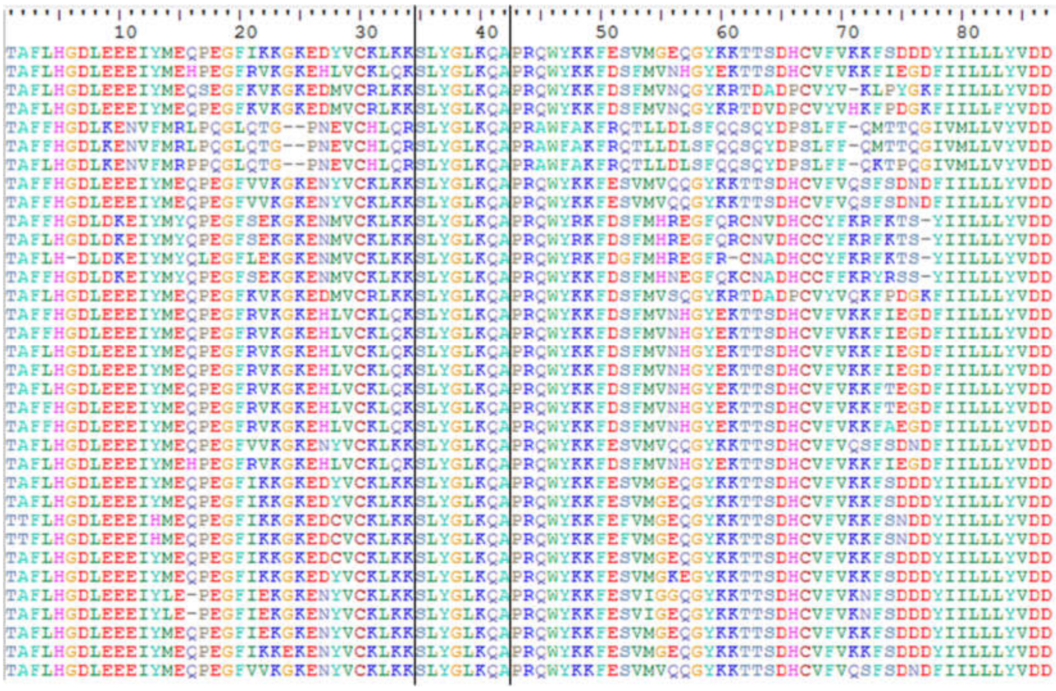
The consensus reverse transcriptase gene sequence of individual family was analysed and used to estimate relative copy number of the individual family in the *Jatropha* genome by the hit number of BLAST search in the database of *Jatropha* Genome at http://www.kazusa.or.jp/jatropha/ [17]. Average of Hit numbers of the TAR (75 clones), Angela (57 clones), Ale (31 clones) and Bianca (only 1 clone) were 61, 69, 64 and 11 respectively.

Molecular markers, for example SSR, AFLP and RAPD, have been widely used to evaluate genetic relationship among plants. Using these markers in *J. curcas*, almost very low genetic divergence was detected among the *J. curcas* varieties currently cultivated in Asia and America [18-19]. Therefore, other types of DNA markers, such as retrotransposon-based markers are of importance in order to use to identify and classify genetic characteristics of plants with narrow genetic base. This will increase the breeding efficiency in the *Jatropha* population.

Retrotransposons have been known to be one of the important components of the eukaryotic genome. This should be related to the variation and also evolution of the species. The differences within the inactive elements can contribute to plant genome evolution, gene duplication events and new properties in the retrotransposition mechanisms [20]. Detection of the retrotransposon presence or absence in an appropriate method will enhance characterization a population with low genetic diversity.

Sizes of plant genome are reported to be positively related to both differences and copy numbers of retrotransposon families [21]. The Ty1-*copia* in the plant kingdom have been characterized into six major families: lvana, Maximus, Ale, TAR, Angela and Bianca [22]. Ty1-*copia* families are reported to be more evolutionarily scattered and smaller in size than the Ty3-*gypsy* [23]. Different plant species carry different number of retrotransposon elements. Arabidopsis, having a small genome size of about 157 Mb, contains LTR retrotransposons in a very limited number of 5.60% [24], whereas rice with genome size of about 389 Mb is composed of 22% LTR retrotransposon sequences [25]. Maize, having large-sized genome of about 2045 Mb, has 74.6% of the LTR retrotransposons elements [26], while *J. curcas* with genome size of about 370 Mb contains 36% of transposable elements, comprising of Ty1-*copia* and Ty3-*gypsy* by 8.0% and 19.6%, respectively [27]. In this study on Ty1-*copia* characterization in *J. curcas*, we found four families of Ty1-*copia*, including TAR, Angela, Ale and Bianca, and distribution patterns of the four families were different among the nine *Jatropha* samples studied. TAR was found in a large number (46.01%), followed by Angela and Ale, whereas Bianca was found in a small number (0.61%), with only 1 sequence found from *J. curcas* from Indonesia. The result is consistent with previous reports on soybeans in which Ivana has the largest number of Ty1-*copia* families and Bianca was not found [28]. Tuntipaiboontana et al. (2018) [29] reported Ty1-*copia* in waterlilies and found Ale to be abundant and diverse, whereas Angela and TAR were found to be conserved.

Among LTR retrotransposons in plant species, the Ty1-*copia* superfamily has been reported as a multitude component of both angiosperm and gymnosperm genomes [30]. In our study, a degenerated primer method was used to evaluate the existence of Ty1-*copia* retrotransposons in *J. curcas*. The results indicated the existence of *copia*-related sequences in all genomes of nine *J. curcas* samples. This suggested that all of the nine *J. curcas* samples share this repetitive elements in their genomes. In addition, the deduced amino acid sequences support the presence of *J. curcas* Ty1-*copia* sequences, where the consensus domain SLYGLKQA (Figure 2), which is the Ty1-*copia* characteristic of plant genome, was found to be highly conserved.



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         10        20        30        40        50        60        70        80
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TAFFHGDLEEEIYMEQPEGFRVKGKEHLVCKLQKSLYGLKQAPRQWYKKFDSFMVNHGYEKTTSDHCVFVKKFTEGDFIILLLYVDD
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TAFLHGDLEEEIYLE-PEGFIEKGKENYVCKLKKSLYGLKQAPRQWYKKFESVIGEQGYKKTTSDHCVFVKNFSDDDYIILLLYVDD
TAFLHGDLEEEIYMEQPEGFIEKGKENYVCKLKKSLYGLKQAPRQWYKKFESVMGEQGYKKTTSDHCVFVKKFSDDDYIILLLYVDD
TAFLHGDLEEEIYMEQPEGFIKKEKENYVCKLKKSLYGLKQAPRQWYKKFESVMGEQGYKKTTSDHCVFVKKFSDDDYIILLLYVDD
TAFLHGDLEEEIYMEQPEGFVVKGKENYVCKLKKSLYGLKQAPRQWYKKFESVMVQQGYKKTTSDHCVFVQSFSDNDFIILLLYVDD
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Figure 2. Alignment of some amino acid sequences corresponding to the fragments of Ty1-*copia* reverse transcriptase gene isolated from *J. curcas*. Gaps are indicated as (-) while the consensus domain SLYGLKQA in amino acid sequences are indicated within the two black lines.

2.2. Molecular cytogenetic evaluation of the Ty1-copia in Jatropha based on FISH

DAPI-stained chromosomes in primary root of J. curcas at metaphase did not show clear constriction or distinguishing features among chromosomes in this study (Figure 3A). However, Kikuchi et al. (2011) [31] performed cytogenetic approach and reported that chromosomes of J. curcas are mostly metacentric or submetacentric and of similar size. In addition, in our study the relaxed prometaphase chromosomes of J. curcas showed obvious of centromere (primary constriction), secondary constriction and satellite (Figure 3B). The condensation patterns observed in chromosomes at the prometaphase stage of J. curcas were similar to those in the chromosomes of coffee, quinoa and lemon, in which the centromeric and telomeric regions mostly consist of the highly repetitive elements [32-34].

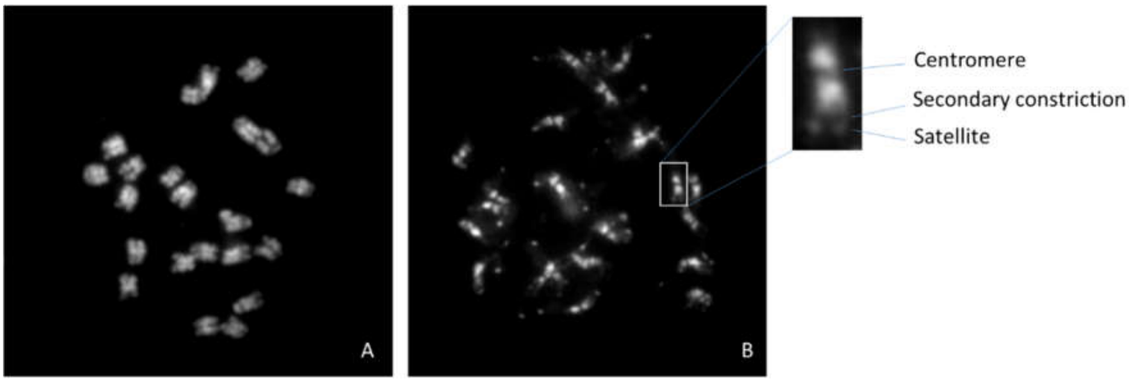
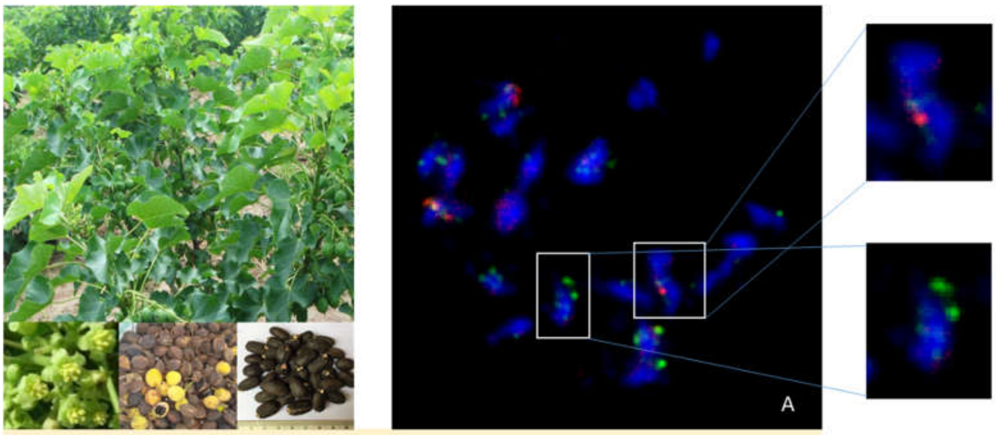
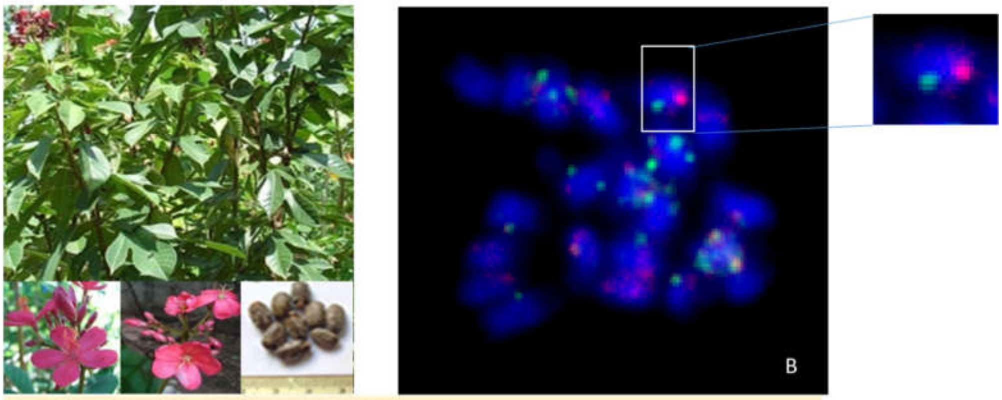


Figure 3. DAPI-stained somatic *J. curcas* chromosomes in primary root (2n = 22). (A) Metaphase chromosomes are mostly observed without clear constrictions. (B) Relaxed prometaphase chromosomes exhibit obviously of centromere, secondary constriction and satellite.

The chromosome distribution of our two identified families of Ty1-copia, Ale and Angela, was evaluated using FISH technique on mitotic chromosomes of both J. curcas and J. integerrima, Results suggested that Ale and Angela Ty1-copia families are dominantly clustered around the centromeres or at the chromosome terminal regions of Jatropha chromosomes (Figure 4A and 4B). The FISH result confirms the existence of Ty1-copia, Ale and Angela, in genomes of both J. curcas and J. integerrima in this study. Patterns of Ty1-copia distribution in the chromosomes of Jatropha were the same as those in slash pine, lemon and oat, in that telomeric regions contained mostly repetitive retrotransposons [34-35]. In addition, the results were supported by the analysis of flanking regions in the EMBL database under AJ269530 and AJ269531 accession numbers [36].



J. curcas



J. integerrima

Figure 4. Chromosomes of *J. curcas* were counterstained with DAPI in light blue color. Red and green color signals represent Ale and Angela Ty1-*copia* with Cy3-dUTP and Dig-dUTP, respectively. (A) *J. curcas*, Ale and Angela are found on different chromosomes. (B) *J. integerrima*, Ale and Angela are found on the same chromosome.

During normal development, transposable elements have been known to be silenced, but under abiotic and biotic stress, they will be motivated. The stimulators of stress, for example wounding, pathogen attack, allopolyploidization tissue culture conditions and unfavourable environmental conditions, including suboptimal temperature or water and nutrient availability, can cause transposon activation [37-38]. Under stress, transcripts of plant retrotransposons have been reported to be found in many plants, for example pine, Orobanche and Phelipanche [27,39]. This supports the genomic reorganization role predicted by McClintock for transposable elements in stress response [40]. Stress-activated transposable elements can provide plants more variability, making them possible to survive in stressful environments. This may be due to various genes can be amplified and combined by the activity of transposable elements. New genetic variability induced by the action of transposable elements can facilitate quick adaptation [41]. Therefore, transposable elements are the potential source of genetic variation that can contribute to differences in both genome structure and gene expression. They are an important factor for genome plasticity and genome evolution in plant [42].

3. Materials and Methods

3.1 Isolation and characterization of Ty1-copia retrotransposons in J. curcas

Genomic DNA was extracted individually from young leaves of nine *J. curcas* accessions, including four high yield accessions from KU Bio-diesel project (KUBP) and two accessions from salinity soil area, representing salt-tolerant cultivars, from Thailand and 3 accessions of *J. curcas* obtained from Madagascar, Indonesia and Taiwan. DNA extraction was performed following the cetyl trimethyl ammonium bromide (CTAB) extraction method with some modifications [43]. The quality and quantity of the DNA were evaluated based on 0.8% agarose gel electrophoresis.

Three degenerate primer sets corresponding to the highly conserved peptide sequence of Ty1-*copia* reverse transcriptase (RT) gene were used in this study. The primers include F: 5′–ACNGCNTTYYTNCAYGG–3′ and 5′– ARCATRTCRTCNACRTA–3′ (13), V: 5′–CARATGGAYGTNAARAC–3′ and 5′–CATRTCRTCNACRTA–3′ (14), H: 5′–GAYGTNAARACVGNTTYY–3′ and 5′–AYRTRTCNACRTANARNA–3′ (15). PCR reaction was performed in a volume of 25 µL reaction mixture, containing final concentrations of 100 ng genomic DNA, 2mM MgCl2, 200 µM dNTPs, 50 pmol forward and reverse primer and 2 U *Taq* DNA polymerase (Invitrogen Life Technology, Brazil). PCR amplification was done in a thermal cycler (PCR-100TM; MJ Research, Inc.; USA) with a PCR program of initial denaturation at 94 °C for 4 min, 35 cycles of denaturation at 94 °C for 1 min, primer annealing at 45 °C for 30 s, extension at 72 °C for 2 min and the final extension at 72 °C for 7 min.

The amplified products were electrophoresed on 1% agarose gel using 1X TBE buffer at 120 V for 35 min. Then, the gel was stained with ethidium bromide (0.5 µg/mL) and was recorded using the Gel Doc XR+ imaging system. PCR products were purified from the agarose gel, cloned into the pGEM®-T Vector (Promega, USA) using T_4 DNA ligase (400,000 U/µl, Fermentas, USA), and then the recombination plasmids were transformed into *E. coli* strain (XL1-Blue) followed by screening white colonies in selective LB/IPTG/X-gal/Ampicillin/agar plates. The transformed clones were screened by PCR analysis (colony PCR) using corresponding M13 primers and were grouped based on restriction patterns produced by five restriction enzymes (*Nco*I, *Bcl*I, *Hae*III, *Eco*RI and *Alu*I). The colonies containing various groups of DNA fragments were selected for extraction of their plasmids using a High-Speed Plasmid Mini Kit (Geneaid, Taiwan). Then the plasmids were sent for sequencing at 1st BASE, Malaysia.

The obtained nucleotide sequences were trimmed and amino acid sequences were deduced using SIB ExPASy Bioinformatic Resources portal [44] with a consideration of spontaneous frameshift mutations. Multiple sequence alignments were performed using Clustal Omega online software [45]. The phylogenetic trees were constructed using maximum likelihood method.

3.2 FISH technique for study of chromosomal distribution of Ty1-copia elements

3.2.1. Chromosome preparation

Seeds of *J. curcas* and *J. integerrima* were germinated at 25 °C in a growth chamber. Root-tips of about 0.3-0.5 mm long were collected and pretreated with 2mM 8-hydroxyquinoline at 20 °C for 3 h and then fixed in 3:1 ethanol: acetic acid for 16 h. Root-tips were washed with distilled water and incubated in enzymatic mixture (1% cellulase, 2.5% pectolyase) at 37 °C for 1 h 30 min to 2 h. They were washed for 1 min in distilled water. The meristem was excised and squashed in ethanol: acetic acid (3:1), then exposed to steam and dried in air.

3.2.2. Probe labeling

Total genomic DNA was extracted from young fresh leaves of *J. curcas* (2n=2x=22) and *J. integerrima* (2n=2x=22). Based on sequence alignment, the primers specific to the two lineages of Ty1-*copia* retrotransposons; Ale: 5′-AYGGAGTGTTAAAGGAAGATG-3′ and 5′-CRTACAGAAGCAATACTGCC-3′ and Angela: 5′-AYGGTGAYTTAGAGGOAGGAAG-3′ and 5′-ACTAACATRATGAGCTTGCTC-3′, were developed. PCR products were labeled with Cy3-dUTP (Cytiva) for Ale and digoxigenin-dUTP (Roche) for Angela by nick translation, according to the manufacturer’s protocol. FISH was conducted according to the procedures of Ohmido and Fukui (1997) [46] and Ohmido et al. (1998) [47] with minor modifications. After the cell spreads were dried on slides, they were post-fixed in 1% formaldehyde/1X PBS buffer for 5 min at room temperature, followed by washing the slides with distilled water and then the slides were dried before hybridization.

3.2.3. Hybridization

The hybridization mixture contains labeled probes in hybridization buffer, consisting of 50% formamide, 2X SSC and 10% dextran sulfate. The hybridization mixture was denatured at 80 °C for 10 min and immediately cooled down on ice. 100 µL of 50% formamide in 2X SSC was applied to the chromosomal DNA, covered with a glass cover slip (24 x 45 mm) and then denatured at 70 °C for 4 min. Slides were dehydrated in the ethanol series (70% and 100%) at -20 °C for 5 min each and then the slides were dried. 10 µL of hybridization mixture was applied to each slide, covered with a cover slip (20x20 mm) and sealed with sealing film. Then, slides were incubated in a moist chamber at 37 °C for 18-48 h. After hybridization, the cover slips were removed carefully and washed three times in 20% formamide/2X SSC and 0.1X SSC respectively for 5 min each, at 42 °C. After washing, 100 µL of anti-digoxigenin-fluorescein isothiocyanate (FITC) (Roche) in 4X SSC was applied for 1-2 h at 37 °C in a humid dark box. The slides were washed in 2X SSC at room temperature three times for 5 min each, then air-dried. 70 µL of DAPI (4′6-Diamidino-2-Phenylindole, Dihydrochloride; 0.5 µg/mL) was added and the slides were mounted with a parafilm for 10 min. The slides were washed in water for 5 min and then dried. After washing, 10µL of Vectashield (mounting medium, Vector) was added to slides and the slides were mounted with a glass cover slip (18 x 18 mm).

4. Conclusions

In the present study, Ty1-*copia* sequences from *J. curcas* were isolated and characterized using three degenerate primer sets corresponding to the conserved Ty1-*copia* reverse transcriptase gene. In total, 164 Ty1-*copia* amino acid sequences were used for the phylogenetic tree construction and were classified into four families of TAR (46.01%), Angela (34.97%), Ale (19.02%) and Bianca (0.61%). FISH technique was used to map physically Angela and Ale families on to *J. curcas* and *J. integerrima* chromosomes. They tend to exhibit biases for the specific regions around the centromeres or at the chromosome ends. This study indicates the high diversity of the Ty1-*copia* elements in the *Jatropha* genome.

Funding: This research was funded by the Research and Researcher for Industry (RRi) PhD level, grant number PHD57I0043 and by Ladda Co., Ltd.

Data Availability Statement: Data available within the article.

Acknowledgments: We would like to thank Mr. Suksan Suttipolpaiboon and Mr. Pichai Maneechote for supplying *Jatropha* samples. The Petroleum Authority of Thailand (PTT) funded research on breeding *Jatropha* using interspecific hybridization under the KU-Biodiesel Project and the Kasetsart University Research and Development Institute (KURDI), Bangkok, Thailand supported research on *J. curcas*. This work was also supported by JSPS Bilateral Program Number JPJSBP 120203507 and JPJSBP 120223504 to N.O.

Conflicts of Interest: The authors declare no conflict of interest.

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