Research Article

Site-Specific Insertion Polymorphism of the MITE Alex-1 in the Genus Coffea Suggests Interspecific Gene Flow

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Miniature Inverted-repeat Transposable Elements (MITEs) are small nonautonomous class-II transposable elements distributed throughout eukaryotic genomes. We identified a novel family of MITEs (named Alex) in the Coffea canephora genome often associated with expressed sequences. The Alex-1 element is inserted in an intron of a gene at the CcEIN4 locus. Its mobility was demonstrated by sequencing the insertion site in C. canephora accessions and Coffea species. Analysis of the insertion polymorphism of Alex-1 at this locus in Coffea species and in C. canephora showed that there was no relationship between the geographical distribution of the species, their phylogenetic relationships, and insertion polymorphism. The intraspecific distribution of C. canephora revealed an original situation within the E diversity group. These results suggest possibly greater gene flow between species than previously thought. This MITE family will enable the study of the C. canephora genome evolution, phylogenetic relationships, and possible gene flows within the Coffea genus.

1. Introduction

Recently, a new type of molecular marker based on the insertion polymorphism of transposable elements (TEs) was shown to be particularly effective for plant diversity studies [1–4]. Because of their repeated nature and, in some cases, their great number [5, 6], these mobile genetic elements may be inserted at different loci in the genome where they lead to mutations or chromosomal rearrangements. Their activity is responsible for considerable natural polymorphism that can be used to study within and between species diversity and to identify possible population genetic structure and phylogenetic relationships [7, 8].

Among these mobile genetic elements, MITEs (Miniature Inverted-repeat Transposable Elements) form a particular group. MITEs are short (<600 bp) nonautonomous type II transposable elements that are often quite widely distributed in eukaryote genomes but at the same time are highly conserved, within a genome, in size and sequence, indicating that they might originate from a limited number of progenitors [9]. Their even distribution throughout plant genomes makes them an ideal tool for the study of genome evolution and genetic relationships [10–12].

Such elements could help to solve problematic phylogenetic relationships among species including those in the genus Coffea, which comprises 103 species originating from Africa, Madagascar, and several islands in the Indian Ocean [13]. A phylogenetic tree was constructed based on four plastid sequences: trnL intron, trnL-F IGS, rpl16, and accD-psi1 IGS and one nuclear repeated sequence: rDNA ITS [13]. The tree contains valuable information but also many unsolved relationships concerning the evolution of the genus and the speciation process, especially in Madagascar. Several approaches using TE could be used to solve this problem such as SSAP [14] and REMAP [15].
Both approaches enable estimation of the genome-wide TE distribution. A third experimental procedure reveals site-specific insertion polymorphism [16]. This method requires the identification of information on flanking sequences to facilitate the design of primers to detect polymorphism by PCR. Sequencing of a C. canephora BAC clone (46C02, accession no. EU164537) enabled identification of a new MITE, named Alex-1 (Figure 1), in the 12th intron of a gene (g3) of this BAC clone [17].

In this paper, we characterize this novel MITE family in the C. canephora genome. We also report the results of a study on the insertion polymorphism of the MITE Alex-1 at the g3 locus using PCR approaches on a representative set of Coffea species and a representative set of C. canephora diversity groups [18–21]. Our results revealed high insertion polymorphism of the Alex MITE at the g3 locus, which was not linked to the phylogenetic relationships of the Coffea species studied here. Taken together, these results suggest greater gene flow between species than previously thought.

2. Material and Methods

2.1. Plant Material. Twenty-eight Coffea species grown in tropical greenhouses at the IRD center in Montpellier (France) were used in this study. They represent the natural ecogeographical distribution of the genus. One plant from a related genus, Psilanthus, was included in the survey. One to 11 plants (genotypes) per species were analyzed (depending on the number of samples available in the collection) except for C. canephora for which 71 accessions were included (see Supporting Material available at doi: 10.4061/2011/3584122). For the latter species, DNAs from 12 plants from the Ugandan diversity group were kindly provided by P. Musoli from NARO/COREC (Uganda) and T. Leroy from CIRAD (France).

2.2. DNA Isolation. DNA was extracted and purified using Qiagen DNeasy mini Kits (Hilden, Germany) according to the manufacturer’s instructions. DNA quantification was performed on a NanoDrop TM 1000 Spectrophotometer (LabTech, France).

2.3. Identification of the MITE Alex. De novo identification of the MITE was performed using dot-plot alignments (DOTTER software, [22]) based on the presence of inverted repeats at both ends and target site duplication. Evaluation of the redundancy of the MITE in nucleotide sequences was

Figure 1: Structural characterization of the Alex-1 MITE. (a) Dot plot of the MITE against itself allowed the identification of the 18 bp terminal inverted repeats (in red). (b) Folding of Alex-1 revealed the typical stem loop structure of MITE elements.
conducted by BLAST searches using MITE Alex as a query against public databases of *C. canephora* (56,231 Expressed Sequence Tags (EST)) (with an e-value cut-off of $10^{-4}$).

2.4. PCR Amplifications. Two primers were designed in conserved regions according to the 46C02 BAC sequence (accession no. EU164537) to check the presence/absence of the MITE Alex-1 at the g3 locus in the different accessions used in this study. The forward primer was designed in the 11th exon of that gene and the reverse primer in the 13th exon (see Supporting Material 1). The primer sequences used were:

G3F: 5’ GTT-TGG-CTG-GGT-CTC-AT 3’ and G3R: 5’ CGA-CAA-GAG-GAA-AGC-CTC-AC 3’. The expected amplicon is 1093 bp long when the MITE Alex-1 is present and 916 bp when it is absent (see supporting Material 1).

The PCR conditions were 94°C for 1 min. followed by 35 cycles at 94°C for 1 min, 58°C for 30 sec, 72°C for 45 sec, and a final elongation period at 72°C for 4 min. PCR products were observed by electrophoresis in 1% agarose gel after staining with ethidium bromide.

2.5. PCR Product Sequencing. In order to check the absence of insertion—as oppose to excision—of a former inserted MITE Alex-1 at the g3 locus, several PCR products from different species were sequenced. After electrophoresis, the bands at 1093 or 916 bp were excised from the gel using a razor blade. DNA was purified using a Quiagen PCR purification kit according to the manufacturer’s recommendations and sent for sequencing to Eurofins-MWG (Ebersberg, Germany). Sequences were aligned using ClustalW software.

3. Results

3.1. Identification of Alex, a Novel MITE Family, in *C. canephora*. A BAC clone at the *CcEIN4* locus in *C. canephora* (BAC clone 46C02, accession no. EU164537) was recently sequenced [17]. This represented the first complete BAC clone ever sequenced in the *Coffea* genus. The typical structural features of a Miniature Inverted-repeat Transposable Element (MITE) were detected in the 12th intron of the g3 gene, encoding a putative protein (nucleotides 12468...12645 of the BAC clone). This element, named Alex-1, was flanked by the 3bp direct repeat AGT, generated upon the insertion of the element (Target Site Duplication, TIR) and had 18bp Terminal Inverted Repeats (TIR) at both ends of the element. The small sequence size (178 bp), rich A/T composition (73.4%), and the ability to form secondary structures characterized Alex-1 (Figure 1).

To further characterize this element, the nucleotide sequence of Alex-1 was compared with the public nucleotide sequences of *C. canephora* comprising 56,231 Expressed Sequence Tags. BLAST searches produced 42 significant hits, suggesting that Alex-1 belongs to a large family of MITE elements frequently associated with transcribed sequences. A BLASTN search performed on nonredundant (nr) public libraries did not produce any significant hit (length, percentage, identity, and e-value), except in *Coffea* genomic sequences.

3.2. Analysis of Genomic Polymorphism Associated with the MITE Alex-1 at the g3 Locus. Table 1(a) shows the presence or absence of the Alex-1 MITE at the g3 locus in the different plants analyzed. Within-species polymorphism in *C. canephora* is presented in Table 1(b) (see Supporting Material 3).

The majority of the species (18/28) displayed total absence of the MITE at the g3 locus, whereas 7/28 were homozygous for its presence and 3/28 displayed heterogeneous patterns. Only *C. liberica* var. *deewrei* and *C. canephora* displayed all three genotypes, homozygous +/+ , homozygous −/− , and heterozygous +/- .

Two species or taxons showed a majority of homozygous genotypes, +/+ (*C. sp N’Koumbala*) or −/− (*C. humilis*), few heterozygotes +/- , but not the reciprocal homozygous, −/− (*C. sp N’Koumbala*) or +/+ (*C. humilis*).

At the sequence level, a closer look at the locus of insertion for the presence of Alex-1 in both positive and negative plants showed that only one of the negative individuals, *C. anthonyi*, displayed the remnant of a TSD (Target Site Duplication) sequence that indicates the former presence of a MITE and thus its transposition (Figure 2). This process of excision was precise since the whole element was removed from the site of insertion, and no large deletion occurred in the flanking regions.

Interestingly, in the African species, no link was found between the geographical origin of the species and the presence of the Alex-1 MITE. Indeed, Alex-1 was present in East, West and Central African species (Mozambicoffee and Eucoffea, resp.). However, all the Mascarocoffea species originating from islands in the Indian Ocean (Madagascar, Mauritius, and Comoros) lacked the MITE at the g3 locus. Most *Coffea* species appear to be homozygous since only seven plants out of 129 were heterozygous.

The situation of *C. canephora* is particularly interesting since the only diversity group E, originating from the Congo/Cameroon region, contains homozygous −/− genotypes (Table 1(b) and see Supporting Material 4). The hetero-zygotes detected in groups D, A, and C were previously identified as being intergroup hybrids all with a group E genotype in their pedigree [19]. Similarly, heterozygotes in group E turned out to be hybrids between group D and group E genitors [19]. The homozygous individual in group E was collected in RCI (Ivy Coast), far from the place of origin of that diversity group, which is in the Congo/Cameroon region. This particular plant was certainly introduced into RCI for improvement purposes quite a long time ago and has certainly undergone several crosses and backcrosses leading to an introgressed form bearing the inserted locus on both homologous chromosomes (+/+ ) (Accession 319, see Supporting Material 2 and 4). The diversity group E thus appears to be the only one among the *C. canephora* groups to be characterized by the absence of Alex-1 at the g3 locus.
Table 1

(a) Insertion polymorphism of the *Alex-1* MITE at the g3 locus among a representative set of *Coffeea* species and a close relative *Psilanthus ebracteolatus*.  

| Species analyzed       | No. of individuals | +/+ | −/− | +/− | Origin                |
|------------------------|--------------------|-----|-----|-----|-----------------------|
| *C. arabica*           | 3                  | 3   | 0   | 0   | E. Africa, Ethiopia   |
| *C. eugenioides*       | 9                  | 9   | 0   | 0   | E. Africa Kenya       |
| *C. pseudozanguebariae*| 11                 | 0   | 11  | 0   | E. Africa Kenya       |
| *C. racemosa*          | 11                 | 0   | 11  | 0   | E. Africa Tanzania    |
| *C. liberica var liberica* | 10            | 0   | 10  | 0   | W. Africa RCI         |
| *C. stenophylla*       | 10                 | 0   | 10  | 0   | W. Africa RCI         |
| *C. humidis*           | 10                 | 0   | 8   | 2   | W. Africa RCI         |
| *C. canephora*         | 71                 | 53  | 8   | 10  | W. and C. Africa      |
| *C. congensis*         | 5                  | 5   | 0   | 0   | C. Africa Cameroon    |
| *C. liberica var. dewevrei* | 10            | 4   | 3   | 3   | C. Africa RCA         |
| *C. liberica var. koto*| 3                  | 3   | 0   | 0   | C. Africa Cameroon    |
| *C. brevipes*          | 10                 | 10  | 0   | 0   | C. Africa Cameroon    |
| *C. heterocalyx*       | 1                  | 0   | 1   | 0   | C. Africa Cameroon    |
| *C. anthonyi*          | 7                  | 0   | 7   | 0   | C. Africa Cameroon    |
| *C. sp N’Koumbala*     | 10                 | 8   | 0   | 2   | C. Africa Cameroon    |
| *C. sp Mayombé*        | 3                  | 3   | 0   | 0   | C. Africa Congo R.    |
| *C. kapakata*          | 2                  | 2   | 0   | 0   | C. Africa Angola      |
| *C. myrtifolia*        | 3                  | 0   | 3   | 0   | Mauritius             |
| *C. resinosa*          | 1                  | 0   | 1   | 0   | Madagascar            |
| *C. tsirananae*        | 1                  | 0   | 1   | 0   | Madagascar            |
| *C. lancifolia*        | 1                  | 0   | 1   | 0   | Madagascar            |
| *C. perrieri*          | 1                  | 0   | 1   | 0   | Madagascar            |
| *C. sakarahae*         | 1                  | 0   | 1   | 0   | Madagascar            |
| *C. millotii*          | 1                  | 0   | 1   | 0   | Madagascar            |
| *C. dolchophyla*       | 1                  | 0   | 1   | 0   | Madagascar            |
| *C. heimii*            | 1                  | 0   | 1   | 0   | Madagascar            |
| *C. bertrandii*        | 1                  | 0   | 1   | 0   | Madagascar            |
| *C. humblotiana*       | 1                  | 0   | 1   | 0   | Comoros               |
| *P. ebracteolatus*     | 1                  | 0   | 1   | 0   | W. Africa RCI         |

Total               | 200                | 100 | 83  | 17  |

E. Africa: East Africa; W. Africa: West Africa; C. Africa: Central Africa.  
RCI: République de Côte d’Ivoire (Ivory Coast); RCA: République Centre Africaine (Central African Republic).

(b) Insertion polymorphism of the *Alex-1* MITE at the g3 locus among a representative set of the diversity groups of the *Coffeea canephora* species as defined by Gomez et al. [19].

| Diversity group | No. of individuals | +/+ | −/− | +/− | Origin          |
|-----------------|--------------------|-----|-----|-----|-----------------|
| D               | 29                 | 28  | 0   | 1* (DEA) | Guinea/RCI     |
| A               | 2                  | 1   | 0   | 1* (AE)  | Cameroon/Congo |
| B               | 3                  | 3   | 0   | 0   | RCA            |
| C               | 9                  | 8   | 0   | 1* (CE)  | Cameroon/Congo/RCA |
| E               | 16                 | 1   | 8   | 7* (DE)  | Congo/Cameroon/RCA |
| O               | 12                 | 12  | 0   | 0   | Uganda         |

Total               | 71                 | 53  | 8   | 10  |

+/+ & −/−: Homozygote for presence and absence, respectively; +/-: Heterozygote. *: intraspecific hybrids.  

[19] Gomez et al.
Analysis of the sequences in seven negative genotypes in the E diversity group and in negative genotypes in other diversity groups revealed no remnant TSD sequences, suggesting that the absence of Alex-1 at the g3 locus is more likely due to a lack of insertion than to postdifferentiation excision (Figure 3).

The closest relative to the genus Coffea, a plant from the Psilanthus genus, also lacked the insertion of the Alex-1 MITE at the g3 locus.

**4. Discussion**

In this paper we describe the first MITE characterized in the genome of a Coffea species that has no homologs in other published sequences, it appears thus as specific to the Coffea genus. We studied the insertion polymorphism of this MITE at the g3 locus. The distribution pattern of Alex-1 at this locus in the Coffea and Psilanthus species strongly suggests that insertion occurred early in relation with the evolution of these genera, although certainly after the divergence between Coffea and Psilanthus as Alex-1 was not found in Psilanthus. However, not enough Psilanthus species or genotypes were analyzed to confirm this hypothesis.

Because the distribution of the MITE Alex-1 does not corroborate previous phylogenetic studies [13] and was found in Coffea species independently of their eco-geographical distribution (Figure 4), its insertion at the g3 locus most probably occurred before the spread of the genus in Africa but probably after the colonization of Madagascar and the other islands in the Indian Ocean by one or several ancestral Coffea species if we consider the hypothesis that the genus originated in the African continent.

Its insertion certainly occurred before the formation of the C. arabica species, which is the only allotetraploid in the Coffea genus, originating from Southern Ethiopia and most probably resulting from a cross between C. eugenioides and C. canephora [23] both being only or mostly homozygous +/+.

When the insertion of a transposable element occurs, it is always in heterozygous form. The probability that the same TE is inserted at the same locus, at exactly the same spot on both homologous chromosomes, is almost nil. If the insertion does not modify a gene function leading to an advantage or disadvantage in terms of selection, its maintenance in the genome responds to a neutral model and may be conserved or eliminated in the following generations. In the present case, as no link was found between the presence or absence of Alex-1 at the g3 locus and the habitat type of the species, the neutral situation probably applies. It is still not clear why species then became preferentially fixed for the presence or absence of the TE, if this was not merely random.

Four species displayed the presence of heterozygous genotypes and only two (C. canephora and C. liberica var dewevrei) showed the three possible patterns (Table 1(a)),

**Figure 2**: Sequence alignment of Alex-1 insertion site in different Coffea species. C. anthonyi is the only negative sequence displaying the presence of a Target Site Duplication (TSD) (empty boxes in the figure). Black arrows indicate the presence of the Terminal Inverted Repeats (TIRs).
homozygous (+/+ and −/−) and heterozygous (+/−). It is very likely that because of the size of the sample, all possible situations have not yet been identified in all the species. It is also possible that some fixation and/or divergence events are actually still underway. The most intriguing example is C. liberica var dewevrei, which displays the three genotypes (homozygous and heterozygous), while C. liberica var liberica is fixed for the absence of Alex-1 (homozygotes −/−) and C. liberica var koto is fixed for the presence of Alex-1 (homozygotes +/+).

C. liberica var dewevrei may still be in the fixation process but this could take quite a long time, as the three genotypes are encountered with equal frequency. In the cases of C. sp N’Koumbala and C. humilis, no −/− or +/+ homozygotes were identified (Table 1(a)), which does not mean that these types of homozygotes do not exist but simply that they were not present in the sample we analyzed. The presence of heterozygotes (+/−) can result from an allelic equilibrium with a low frequency of positive alleles in C. sp N’Koumbala and of a negative allele in C. humilis, but in such a situation, the reciprocal homozygote would also be expected to be present, and this was not the case in our sample. Another possible explanation for this low allelic frequency is that interspecific crosses, even if very rare, may happen throughout the Cameroon/Congo region and in RCI, which are hot spots of diversification and secondary centers of speciation for Coffea species [24].

The C. canephora group E insertion pattern suggests possible interspecific hybridization and gene flow. Indeed, it is the only group in this species that lacked Alex-1 at the g3 locus. The absence of TSD in the sequenced amplicons (Figure 3) indicates that Alex-1 has never been inserted at that locus, and consequently, that its absence is not the result of transposition to another site. All the other genotypes, whatever diversity group they belong to, are +/+ homozygotes, it is thus highly likely that the common ancestor of C. canephora underwent the insertion of Alex-1 at the g3 locus and then evolved towards the fixation of the insertion (+/+ pattern). Group E, and certainly other unidentified genomic sequences, could then result from an introgression following a cross with a neighboring (sympatric) species and backcrosses to recover C. canephora properties.

Interestingly, under this hypothesis of introgression, C. sp N’Koumbala is a possible candidate to be the provider of the absence of insertion if an allelic equilibrium remains in this species. Indeed, this taxon grows in the same region as plants from diversity group E, but additional comparative sequencing of the g3 locus, including flanking regions, is necessary to confirm or reject the hypothesis.

It is also possible that this particular group derives from a sister plant to the plant that integrated the MITE, the latter...
resulted in the full *C. canephora* lineage except for group E. In this case, group E may derive from a population that lived in sympatry with *C. canephora*, from which it has never completely genetically separated due to cross hybridization. If this is the case, the genome region that contains the *Alex-1* MITE was preserved from recombination, which should have led to +/− and +/+ genotypes. However, genotypes that are found in artificial intraspecific hybrids make this hypothesis unlikely.

5. Conclusion

The insertion pattern of the *Alex-1* MITE at the g3 locus in *Coffeea* species indicates an original path of speciesdifferen-
tiation including gene flows between ancestral forms that happened before the present. Recent collecting missions very occasionally identified natural interspecific hybrids or sympatric populations of Coffeea species. However, it is known that such events can happen in the wild (C. arabica being the best example), or in displaced populations in functioning or abandoned coffee plantations [25]. Changing environmental conditions and habitat modification could certainly have led to cohabitation of two or more species in limited areas where their specific phenology was disturbed, thus allowing cross pollination. Subsequent environmental changes could have led to the expansion of favorable habitats, resulting in the isolation of the newly formed species.

MITEs thus appear to be a powerful tool to analyze these speciation events and to trace the phylogenetic relationships between species and if the number of specific insertion sites is sufficient to enable the establishment of an event chronology [8].

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