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Genomic and chromosomal organization of Ty1-*copia*-like sequences in *Olea europaea* and evolutionary relationships of *Olea* retroelements

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Abstract The Ty1-*copia*-like retrotransposon is one of the commonest class of transposable elements in the plant kingdom, often comprising several percent of the total DNA content. We aimed to study the evolutionary relationships of *Olea* retroelements, using part of the reverse transcriptase domain, as well as the genomic and chromosomal organization of these sequences in *Olea europaea* chromosomes and their transcription activity and copy number. Fourteen clones, that were isolated from four different species, were sequenced and a phylogenetic tree was constructed based on their predicted amino acids. Five clones derived from *O. europaea* were clustered together with a 87% nucleotide sequence homology and two *Olea oleaster* clones showed 98% sequence homology. The rest of the clones showed heterogeneity among them, leading to a common ancestral transposon that existed before the genus arose. The Ty1-*copia*-like sequences have a dispersed genomic organization, physically distributed on all chromosomes, showing minor clustering in some cases and low copy numbers in the smallest chromosome pair. The total copy number in the *O. europaea* genome was estimated by dot blotting to be 40,000 in a haploid nucleus, but a number of these are non-functional since the sequenced clones contained stop codons and frame-shifts. Some Ty1-*copia*-like copies, present in *O. europaea*, were found to be methylated, while no differences in methylation were observed between DNA isolated from young leaves and callus-suspension cultures.

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Introduction

A major part of the nuclear genomes in all higher plants is comprised of repetitive DNA sequences that are organized either in tandem arrays or in a dispersed fashion. The majority of the dispersed sequences are mobile genetic elements or their remnants, and can either move in the form of DNA or move and amplify by means of an RNA intermediate. The latter have been extensively studied during the last decade since they are widely spread within the plant kingdom and, in some cases, can occupy a considerable part of the genome (for a review see Kumar and Bennetzen 1999).

The cultivated olive, *Olea europaea* ssp. *sativa*, is considered one of the most important, both economically and culturally, crops of the Mediterranean basin. Its chromosome complement is $2n = 46$ and is considered a tetraploid with an unknown origin (Taylor 1945). Possible species involved in the evolution of the cultivated species include tropical and subtropical species, such as *Olea chrysophylla* Lam. and *Olea excelsa* Ait. The DNA content per haploid nulceus for two cultivars, 'Frantoio' and 'Leccino', was estimated by Feulgen cytophotometry to be 2.26 pg (2.18×10^3 Mb) and 2.20 pg (2.12×10^3 Mb), respectively (Rugini et al. 1996). Some highly repetitive DNA sequences have been isolated and their genomic and cytogenetic organization has been studied by Southern and in situ hybridizations (Katsiotis et al. 1998; Bitonti et al. 1999). These sequences were organized in tandem arrays, giving the characteristic ladder pattern in Southern hybridizations and were localized at heterochromatic regions of *Olea* chromosomes. No reports about the organization of dispersed sequences in *Olea* are currently available.

In the present study we have isolated sequences from Ty1-*copia*-like retrotransposons of *O. europaea* ssp. *sativa*, the olive tree, and investigated their genomic and

Table 1 Cloned *RT* PCR products, their size, and adenine and cytosine percent content

Species	Number	Size (bp)	% A+T content	GenBank number
O. europaea	Toe 7	265	66.0	AJ416428
O. europaea	Toe 8	263	56.4	AJ416429
O. europaea	Toe 9	263	56.4	AJ416430
O. europaea	Toe 14	264	58.3	AJ416431
O. europaea	Toe 15	268	58.4	AJ416432
O. europaea	Toe 19	262	57.8	AJ416433
O. europaea	Toe 21	264	61.6	AJ416434
O. oleaster	Too 10	265	59.2	AJ416435
O. oleaster	Too 15	261	56.7	AJ416436
O. oleaster	Too 16	260	56.9	AJ416437
O. chrysophylla	Toc 6	263	59.2	AJ416438
O. chrysophylla	Toc 8	264	62.7	AJ416439
O. chrysophylla	Toc 10	262	70.1	AJ416440
O. africana	Toa 3	255	51.9	AJ416441

chromosomal organization and estimated their copy number. The genomic organization of these dispersed sequences in other related genera of the Oleaceae family are also described. Furthermore, Ty1-*copia*-like sequences from other *Olea* species, including *O. europaea* ssp. *oleaster*, *O. chrysophylla* and *Olea africana* Lam., have been isolated and sequenced, and phylogenetic comparisons among these retrotransposons have been carried out.

Materials and methods

Plant material and DNA isolation

Young leaves from three *Olea* species (*O. europaea* ssp. *oleaster*, *O. chrysophylla* and *O. africana*; 2*n* = 46) and seven other genera of the Oleaceae family [*Phillyrea latifolia* L., 2*n* = 46; *Ligustrum vulgare* L., 2*n* = 46; *Forsythia europaea* Degen & Bald., 2*n* = 28; *Parasyringa sempervirens* (Franch.) W.W.Sm., 2*n* = 46; *Jasminum officinale* L., 2*n* = 26; *Fraxinus excelsior* L., 2*n* = 46; *Fontanesia phillyreoides* Labill., 2*n* = 26] were collected from the Diomides Botanical Garden of Athens at Dafni, Greece. Plant material from the cultivated olive *O. europaea* ssp. *sativa* clonal selection 'Koroneiki' was collected from the Agricultural University of Athens arboretum. Total genomic DNA was extracted from leaves using standard methods. For methylation studies, DNA was also extracted from cell-suspension cultures of 'Koroneiki' zygotic embryo explants incubated in MS medium, supplemented with 2,4-dichlorophenoxyacetic acid (0.5 mg/l) and kinetin (0.05 mg/l), and cultured for at least 9 months.

Polymerase chain reaction and cloning of PCR products

The internal region of the reverse transcriptase (*RT*) gene of the Ty1-*copia* retrotransposons was amplified by PCR using total genomic DNA of *O. europaea* ssp. *sativa*, *O. europaea* ssp. *oleaster*, *O. chrysophylla* and *O. africana* as templates and degenerate primers 5′-ACNGCNTT(C/T)(C/T)TNCA(C/T)GG-3′ and 5′-A(A/G)CAT(A/G)TC(A/G)TCNAC(A/G)TA-3′ (Flavell et al. 1992). Reactions were denatured at 94 °C for 1 min, followed by 35 cycles of 1 min at 94 °C, 1 min at 42 °C and 1 min at 72 °C, with a final elongation step of 5 min at 72 °C. Reaction mixtures were 50 µl, containing 150 ng of genomic DNA and 50 pmol of each primer. The products were re-amplified by PCR. Blunt ends were generated either by T4 DNA polymerase or by using *Pow* polymerase for the re-amplification PCR. The fragments were ligated into the *Sma*I site of pUC18 or pUC19. The DH5α strain of competent cells (GIBCO-BRL) was used as a bacterial host.

Sequence analysis, comparisons and phylogenetic trees

A total of 14 clones were sequenced on a 310 ABI Prism Genetic Analyzer (Applied Biosystems Elmer) using the dideoxy chain-terminator reaction. Search for homologous sequences of our clones was performed at the European Bioinformatics Institute (EBI) using the NewFasta3 software and the GenBank/EMBL database. Nucleotide sequences were translated for all six possible reading frames, using the ExPASy translation tool. The resulting sequences were compared visually with corresponding amino-acid sequences from other crops and frameshifts were introduced for optimal alignment where necessary. Optimal amino-acid sequences were aligned using CLUSTALW. An unrooted phylogenetic tree, using the predicted sequences, was constructed using the neighbour joining algorith and the TREEVIEW program.

Southern hybridization and dot-blot analysis

For Southern hybridization, DNA was digested with restriction endonucleases, separated on 1% agarose gels and transferred to positively charged nylon membranes (Biodyne B, Pall). Labeling and detection were performed with the non-radioactive ECL random primed labeling and detection system (Amersham), using the total *O. europaea RT* PCR product as a probe. Hybridization was performed at 55 °C overnight, while membranes were washed twice with 2 × SSC/0.1% SDS at 55 °C.

The copy number of the *RT* sequences was estimated using dot-blotting. Serial dilutions of *O. europaea* ssp. *sativa* genomic DNA (500 ng, 400 ng, 250 ng, 200 ng, 100 ng and 50 ng) and clone Toe15 (60 ng, 30 ng, 6 ng, 3 ng, 0.6 ng, 0.3 ng and 0.06 ng) were spotted on a plastic membrane. Hybridization and washing conditions were the same as described for Southern hybridization.

RNA isolation and Northern-blot analysis

Total RNA was extracted according to Suoniemi et al. (1996b) from *O. europaea* ssp. *sativa* young leaves and wounded young leaves. Leaves were wounded by cutting them into small pieces, incubated at room temperature for 2 h and followed by exposure to UV light (254 nm) for 3.5 h (0.2 J/cm^2). The RNA was mixed with a solution containing 1 × MOPS, 2.2 M formaldehyde and 50% (v/v) formamide, denatured by heating at 55 °C for 5–10 min and chilled on ice. The samples were fractionated in a 0.66 M formaldehyde agarose gel (1.2%) and transferred onto a positively charged nylon membrane, using 10 × SSC as a transferring solution. The non-radioactive ECL random primed kit was used for labeling and detection (Amersham), employing the total *O. europaea* ssp. *sativa RT* PCR product as a probe. Hybridization was performed overnight at 50 °C. The filters were washed twice in 2 × SSC/0.1 × SSC at 50 °C, prior to exposure to X-ray film.

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Toe8     TAFLHGDLDEEIYMHQPEFYKVEDKERQVCRLRKSLYRLKQSPRQWYSDFDSFMLEHGFSRSEYDCCVYIR-KLRGGNYIYLLLYVDDM
Toe9     TAFLHGDLDEEIYMHQPEFYKVEDKERQVCRLRKSLYRLKQSPRQWYSDFDSFMLEHGFSRSEYDCCVYIR-KLRGGNYIYLLLYVDDM
Toe19    TAFLSGDLDEEIYMHQPEFYKVDDKERQVCRLRKSLYRLKQSPRQWYK*FDSFMLEHGFSRSEYDCCVYIR-KLRGGNYIYLLLYVDDM
Toe14    TAFLSGDLDEEIYMHQLEFYKVDDKERQVCRLRNDLYGLKQSPRQWYQRLDSFMLKHGFSRSEYDCCVYIQ-KLREGNYIYLFLYVDDM
Toe15    TAFLSGDLDEEIYMHQLEFNKVDDKERQVCRLRNDLYGLKQSPRQWYKRFDSFMLKHGFSRSEYDCCVYIQ-KLREGNYIYLFLYVDDM
Toe7     TAFLHGELEEDIYMCQSQGFEERGKSDFVCKLQKSLYGLKQSPRQ*YKRFDTYV*KIGFRR-EYDHYLYYN-NSKTESKTYLLLYVDDM
Toc6     TAFLHGELDEDIYMSQPQEFKVKGREDYIYKLKNALYGLKQSSRQWYFRFDTYVLMIGFKKSEYDHSLCYN-NARAGKEVNYFLCVDDM
Toe21    TAFLHGELEEDLYMYQPQSFEVKGKANCVCKLEKSLYGLKQSPRQWYKHFDSLGTKNRFK-SDYDACLYYN-NSKRGHELYLLVYVDDM
Too10    TAFLHGELEEDIYMSQPEFFEVKGKTNCVANLERSLYGLKQSPRQWYIRFDTYVLKIGFKRSEYDACLYYN-DSKEGCEVYLALYVDDM
Toc8     TAFLHM*LLEKIFMDQPKFFENKQKSDHVCFLKRSLYGLKQSPRQWYKRFDSFVKTVGFKRSEFDHCLYIQGNAKNDNCVFFRLYVDDM
Toc10    TAFLHGDLEKDIYMHQAQFYVVTGKENMVCNS*KSLYDLK*AFRQWYSKFDNFMTNTGFHRCYGDPCCYYK--NLDTSYLILLLYVDDM
Too15    TALLHGTLNEEIYMIPPPFYCVKG-ETQVCRLRKSLYGLKQASRNWFFKLTSVFLNAGFVQSQADHSLFTL--ITATSLTIIVVYVDDM
Too16    TAFLHGTLNEEIYMIPPPFYCVKG-ETQVCRLRKSLYGLKQASRNWFFKLTSVFLNAGFVQSQADHSLFTL--ITATSLTIILVYVDDM
Toa3     IAFLHGDLHEVIYMTLPSFMPTSS-PHDVCMLRRSLYGLKQAPRAWFEKFRNTLITFSFAQNQYDSSLFFH--KSCSGIVLLLVYVDDM
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Fig. 1 Alignment of predicted peptide sequences, isolated from *O. europaea* ssp. *sativa* (Toe 7, Toe 8, Toe 9, Toe 14, Toe 15, Toe 19, Toe 21), *O. europaea* ssp. *oleaster* (Too 10, Too 15, Too16), *O. africana* (Toa 3) and *O. chrysophylla* (Toc 6, Toc 8, Toc 10). *Dashes* show gaps introduced for optimal alignment, frameshift mutations are *underlined* and *asterisks* indicate stop codons

In situ hybridization

Chromosomes from olive root-tips were prepared according to Katsiotis et al. (1998). The total PCR product from the Ty1-*copia RT* gene of cultivated olive was labeled with biotin-11-dUTP (Roche) using PCR and detected with streptavidin-Cy3 conjugate (Sigma). Probe pOS218, a tandem repeat isolated from the cultivated olive (Katsiotis et al. 1998) localizing on heterochromatic regions, was labeled with digoxigenin-11-dUTP (Roche) and detected with the anti-digoxigenin-fluorescein Fab fragment (Roche). Slides were denatured in a MJ Research PTC-100 machine, followed by overnight incubation in a humid chamber at 37 °C. Slides were counterstained with DAPI (4′,6-diamidino-2-phenylindole, 6 μg/ml) for 8 min and mounted in antifade solution (AF1, Citifluor). An Olympus epifluorescence microscope with filters U-MUW, U-MSWG and U-MSWB was used for slide examination. Photographs were taken using Fujicolor Super G Plus 400 color print film. Adobe Photoshop software was used to optimize contrast of the whole image.

Results

All four *Olea* species used in the PCR amplification yielded a product of the expected size (approximately 270 bp). Since this product represents a pool of different Ty1-*copia*-like family fragments of the retrotransposon *RT* gene, the products were cloned into plasmids and bacterial hosts were transformed. The 14 clones that were sequenced ranged in size from 255 bp to 268 bp (Table 1). Nucleotide sequence homology within each species ranged from 60% to 100% for *O. europaea* ssp. *sativa* clones, 32% to 99% for *O. europaea* ssp. *oleaster* clones and 34% to 56% for *O. chrysophylla* clones, while overall homology, including *O. africana*, ranged from 10% to 100%. Nucleotide sequences, which were AT-rich, were translated into amino acids. To optimize alignment, frameshifts were introduced, where necessary, except for Too16 (Fig. 1). Stop codons were present in four clones (Toe19, Toe7, Toc8 and Toc10). Overall homology for the predicted amino-acid identity ranged from 31% to 100%. Searching through the nucleotide GenBank/EMBL database, all 14 sequenced clones showed high homology to Ty1-*copia*-like elements belonging to other plant species, including *Alstroemeria aurea*, *Vicia faba*, *Zea mays*, *Beta vulgaris*, *Lycopersicon esculentum*, *Arabidopsis thaliana* and *Gossypium hirsutum*. Using the neighbor-joining algorithm a phylogenetic tree was constructed from *Olea* and other genera retroelements, showing high homology between their predicted amino acids (Fig. 2).

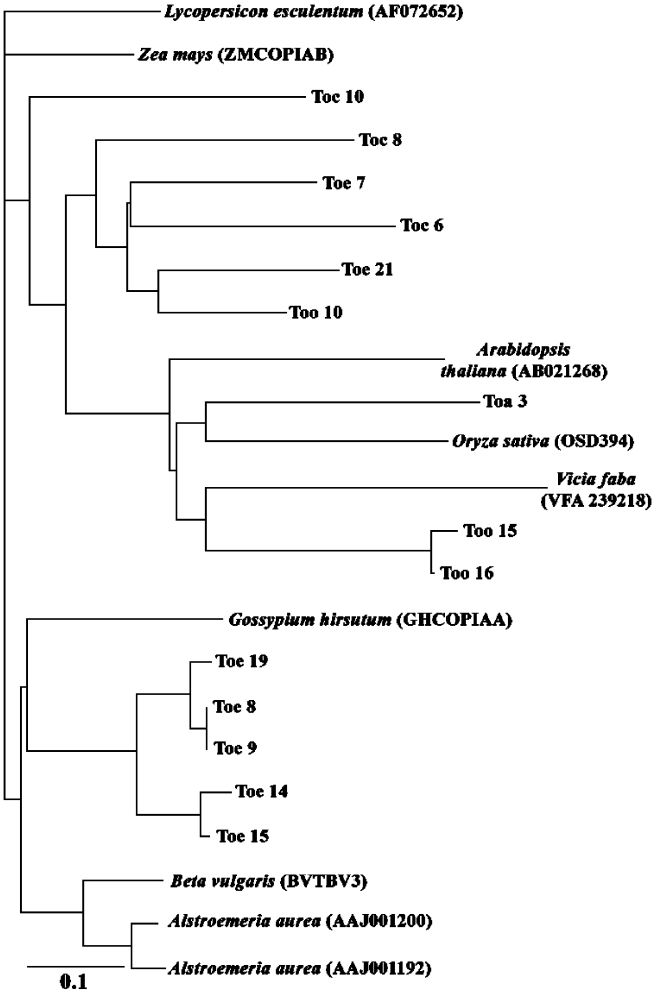

Fig. 2 Phylogenetic relationships among the predicted amino acids for the *RT* genes of *Olea* Ty1-*copia*-like retrotransposons and other related sequences, using the neighbor-joining algorithm and the TREEVIEW program

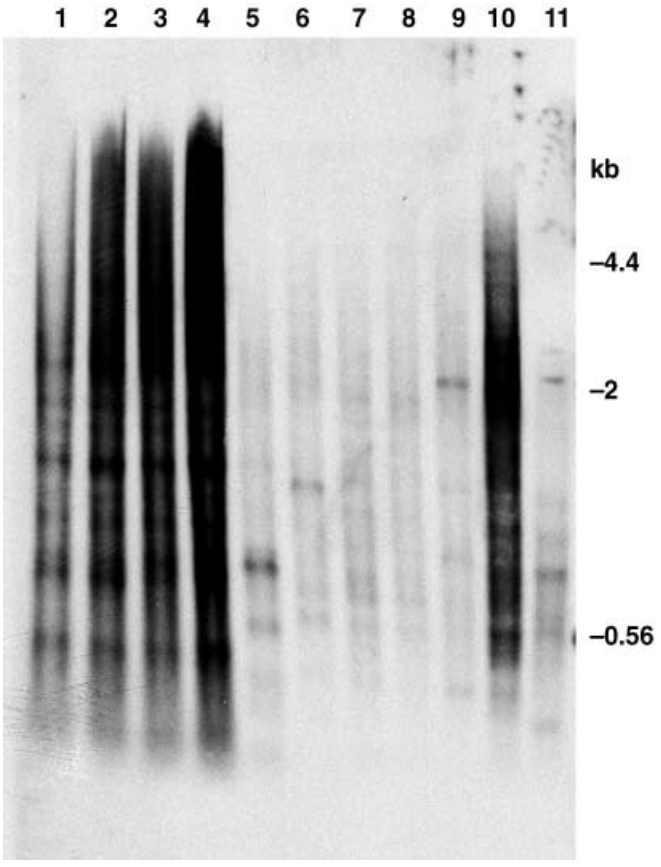

Fig. 3 Southern-blot analysis of *O. europaea* ssp. *sativa* (*lane 1*), *O. europaea* ssp. *oleaster* (*lane 2*), *O. africana* (*lane 3*), *O. chrysophylla* (*lane 4*), *P. latifolia* (*lane 5*), *F. europaea* (*lane 6*), *J. officinale* (*lane 7*), *L. vulgare* (*lane 8*), *P. sempervirens* (*lane 9*), *F. excelsior* (*lane 10*) and *F. phylliroides* (*lane 11*) genomic DNA, digested with *Sau*3A and probed with the total *RT* PCR product of *O. europaea* ssp. *sativa* amplification

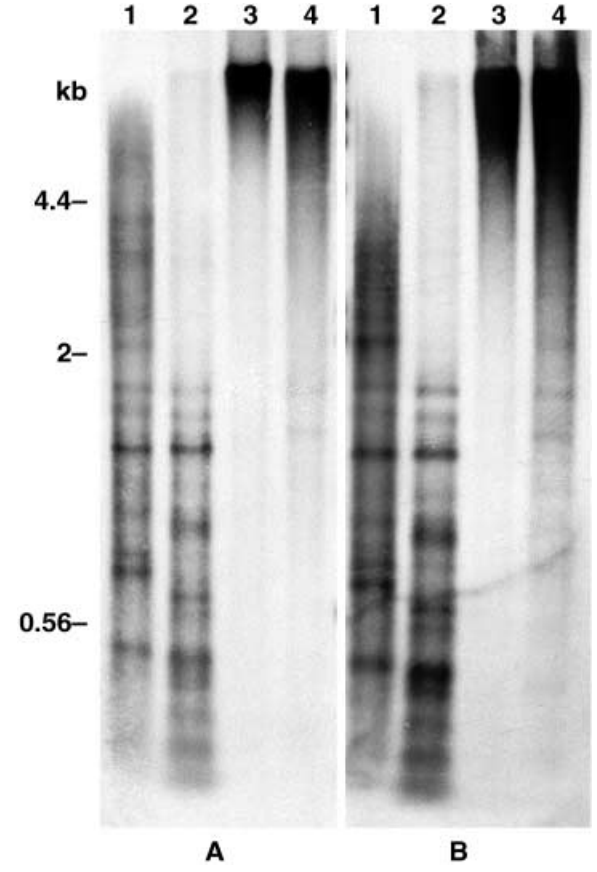

Fig. 5 Southern-blot analysis of *O. europaea* ssp. *sativa* genomic DNA isolated from young leaves (**A**) and cell suspension cultures (**B**), digested with *Sau*3A (*lane 1*) and *Mbo*I (*lane 2*), and *Hpa*II (*lane 3*) and *Msp*I (*lane 4*). Blots were hybridized with the total *RT* PCR product of *O. europaea* ssp. *sativa* amplification

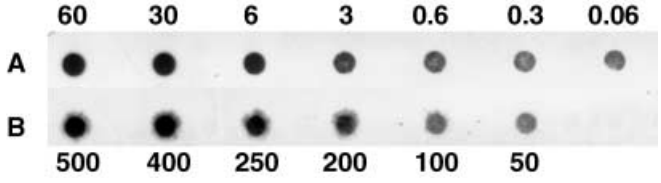

Fig. 4 Dot-blots for estimating the copy number of the Ty1-*copia*-like retrotransposons in the cultivated olive. Different amounts of clone Toe15 were dot-blotted on row *A*, while row *B* contains serial dilutions of *O. europaea* ssp. *sativa* genomic DNA (all quantities are in ng). Clone Toe 15 was used as a probe

The genomic organization of the Ty1-*copia*-like retrotransposons was revealed by Southern hybridization. Total genomic DNA from genera within the Oleaceae family were digested with the *Sau*3A restriction enzyme and probed with the total *O. europaea* ssp. *sativa RT* PCR product. The hybridization pattern of the probe was the same for all four *Olea* species, with distinct bands at 0.38, 0.75, 1.3, 1.6 and 1.9 kb (Fig. 3). The probe also hybridized to the other genera revealing the presence of sequences related to the *Olea* Ty1-*copia*-like probe. A strong hybridization signal was obtained for *Fraxinus excelsior* DNA, indicating the presence of high copy numbers of sequences homologous to the probe. The rest of the samples showed a lower intensity hybridization signal and thus the presence of lower copy numbers of sequences homologous to the probe. Common bands between *Olea* species and *P. latifolia* are present at 0.75 and 1.3 kb, and between *Olea* species and *J. officinale* at 1.3 and 1.9 kb (Fig. 3). The presence of a smear over the whole track indicates the dispersed organization of the analyzed retrotransposons, while the presence of superimposed fragments could be related to possible clustering of some sequences.

The copy number of the *RT* gene of the Ty1-*copia*-like elements was estimated by dot-blot hybridization. Serial dilutions of total genomic DNA from *O. europaea* ssp. *sativa* and the plasmid containing Toe15 were used to compare signal intensities (Fig. 4). Toe 15 was used as a probe since, according to the phylogenetic results (Fig. 2), it is a member of a retrotransposon subgroup present in *O. europaea* ssp. *sativa*. According to the signal hybridization intensity, the number of sequences homologous to

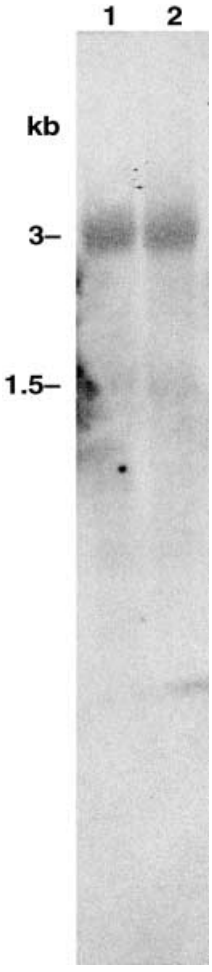

Fig. 6 Total RNA isolated from young leaves (*lane 1*) and wounded young leaves (*lane 2*) of *O. europaea* ssp. *sativa*, probed with the total *RT* PCR product from the *O. europaea* ssp. *sativa* amplification

Toe15 present to *O. europaea* ssp. *sativa* nuclear DNA is estimated to be 40,000 copies per haploid nucleus.

Methylation study was performed using the restriction enzyme pairs *Sau*3A/*Mbo*I and *Hpa*II/*Msp*I, recognizing sequences GATC and CCGG, respectively (Nelson et al. 1993). Enzyme *Mbo*I is insensitive to C methylation while the activity of *Sau*3A is blocked by C methylation. Similarly, *Msp*I is partially insensitive to the internal C methylation, while *Hpa*II is sensitive and does not digest the DNA at its recognition site. Genomic DNA from young leaves and from cell-suspension cultures of 'Koroneiki' was digested with the above enzymes. According to the results, some cytosines in GATC sites are methylated, since differences in the banding patterns between *Sau*3A and *Mbo*I are observed (Fig. 5). Furthermore, some internal cytosines in CCGG sites are also methylated, because additional bands are present in *Msp*I digestions (Fig. 5). The banding patterns between DNA extractions from young leaves and cell-suspension cultures are identical, indicating no differences in methylation pattern at these isoschizomeric sites (Fig. 5).

Forty micrograms of total RNA isolated from young leaves and wounded young leaves, were run on a denaturing gel. The RNA was transferred onto a nylon positively charged membrane and probed with the total *RT* PCR product according to manufacturer's recommendations (Fig. 6). A major band was present at a molecular weight of about 3 kb with some smear present along the lanes and an additional minor band at about 1.5 kb. No differences in signal intensities between the control and the wounded leaves were observed (Fig. 6).

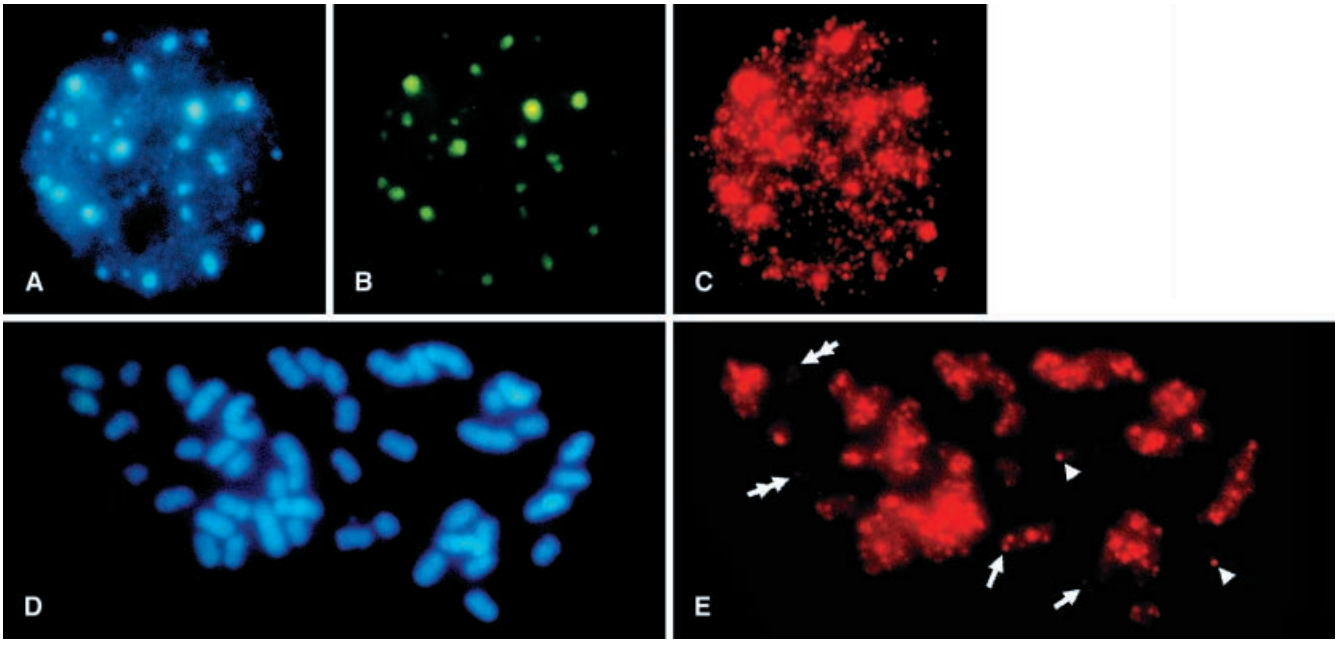

Fig. 7A–E Localization of Ty1-*copia*-like retrotransposons sequences on root-tip interphase nuclei and metaphase chromosomes of *O. europaea* ssp. *sativa* by fluorescence in situ hybridization; nucleus and metaphase chromosomes stained with DAPI (**A** and **D**). Total *RT* PCR product from the *O. europaea* ssp. *sativa* probed on interphase nucleus (**C**) and metaphase chromosomes (**E**). The same interphase nucleus was probed with the tandemly repeated sequence pOS218 (**B**), hybridizing at heterochromatic regions. *Arrows* on metaphase chromosomes (**E**) indicate minor clustering of Ty1-*copia*-like sequences at subtelomeric regions, *double-arrows* indicate the smallest chromosome pair showing weak hybridization and *arrowheads* indicate chromosomes showing signal accumulation on restricted areas

To study the physical distribution of the Ty1-*copia*-like sequences on interphase nuclei and metaphase chromosomes of *O. europaea* ssp. *sativa*, fluorescent in situ hybridization was used. From the interphase nuclei it is evident that the Ty1-*copia* sequences are widely dispersed over the chromatin, with clustering in some areas. The same is evident on metaphase chromosome spreads; discrete clusters on both chromatids are present on a number of chromosomes (Fig. 7E, arrows) or are accumulated in restricted areas (Fig. 7E, arrowheads). A weak hybridization signal indicates the presence of a low copy number of these sequences in the smallest chromosome pair (Fig. 7E, double arrows). On most metaphase chromosomes the probe also shows a dispersed organization (Fig. 7E).

Discussion

Retrotransposons comprise a significant proportion of nuclear genomes, making them one of the most important components affecting the structural evolution of genomes. The present study is an attempt to investigate the sequence heterogeneity, the copy number, the transcriptional activity and the chromosomal and genomic organization of the Ty1-*copia*-like retrotransposons in the *O. europaea* genome. Fourteen clones isolated from four *Olea* species were sequenced, translated, aligned and used to derive phylogenetic relationships among them. Although this is considered a small number of samples showing variable homogeneity (ranging from 10% to 100%), nevertheless useful phylogenetic relationships can be drawn. In similar studies in cereals a total of 102 Ty1-*copia*-like sequences were used (barley:24, maize:5, wheat:30, rice:26, rye:16 and oat:1), revealing clustering in subgroups and supergroups (Gribbon et al. 1999). In the present study, five clones from *O. europaea* ssp. *sativa* (Toe8, Toe9, Toe 14, Toe 15 and Toe 19) form a subgroup with more than 87% nucleotide sequence identity. Furthermore, two clones from *O. europaea* ssp. *oleaster* (Too15 and Too16) form another subgroup with more than 98% nucleotide identity. The rest of the clones, although related, do not form distinct subgroups according to species but rather to a supergroup, showing the existing heterogeneity among these sequences (Fig. 2). It is evident that sequences forming the subgroups are descended from ancestral transposons that were distinct from each other in species lineages, while sequences in supergroups spread across the entire *Olea* genus suggesting that their last common ancestral transposon existed before the genus arose.

It has been suggested (Charlesworth 1986) and tested (Pearce et al. 1996) that the heterogeneity of retrotransposons within a genus/species is proportional to their copy number. The copy number of the *RT* sequences in the *Olea* genome has been estimated by dot blotting to be 40,000 in a haploid nucleus (Fig. 4). Taking into account the low homologies between some *RT* sequences within *O. europaea* ssp. *sativa* (the sequence homology between Toe15 – the clone used as a probe – and Toe7 is 63%) and the membrane washing stringency, the estimated copy number is probably an underestimate. According to the Southern-hybridization signal strength, where the total *RT* PCR product from *O. europaea* ssp. *sativa* has been used as a probe, no differences in the copy numbers of retrotransposons between the *Olea* species used were apparent (Fig. 3). From the related genera, only *F. excelsior* seems to have a high copy number of sequences homologous to *Olea* Ty1-*copia* retrotransposons. From the other tested genera some (*P. latifolia* and *J. officinale*) show common bands with *Olea*, revealing structural similarities and possible affinities between retrotransposon families within the Oleaceae. In the genus *Triticum* common bands in Southerns have been used to reconstruct phylogenies (Dvořák and Zhang 1992).

The majority of the isolated *RT* sequences were found most likely not to be active, containing stop codons and frame shifts. Only one clone, Too16, was found to contain a sequence that could be functional. Defective retrotransposons could be the result of mutations and/or mistakes made by the reverse transcriptase during reverse transcription. As a consequence, defective copies and remnants of retrotransposons accumulate within the genomes. Transcripts of plant retrotransposons have been found in roots (*Tnt1* in tobacco; Pouteau et al. 1991), leaves (*BARE*-1 in barley; Suoniemi et al. 1996b) and young microspores (PREM-2 in maize; Turcich et al. 1996). In the present study total RNA was isolated from young leaves and wounded/irradiated young leaves. The wounded leaves were used because some retrotransposons were found to be activated by abiotic stresses, such as wounding, cell culture, protoplast isolation etc. (Wessler 1996; Kumar and Bennetzen 1999). The RNA was probed with the total *O. europaea* ssp. *sativa RT* PCR product. A band of about 3 kb was detected, with some smear along the lanes and an additional band present at about 1.5 kb (Fig. 6). Additional bands in Northern blots have also been reported in barley (*BARE*-1, Suoniemi et al. 1996b) and tobacco (*Tnt*-1, Pouteau et al. 1991). These RNA products could be due to either the variation of the position of transcriptional initiation (Suoniemi et al. 1996b) or could be transcripts of retrotransposon remnants. Based on the molecular weight of the main fragment from the Northern blot and the estimated copy number from the dot blot, it is evident that the Ty1-*copia*-like sequences are abundant and could constitute at least 5.5% of the haploid *Olea* genome. This figure is an underestimate because the full length of the LTR and the transcription initiation site within the 5′ LTR, remain unknown. The size of the LTRs can vary between 138 bp (*Tos*17 element of rice, Hirochika 1997) to more than 1,300 bp (1,829 bp for the *BARE*-1 element of barley, Manninen and Schulman, 1993; 1,307 bp for the PREM-2 element of maize, Turcich et al. 1996). A higher copy number of LTRs was also been observed compared to the reverse transcriptase domain (Waugh et al. 1997; Suoniemi et al. 1996a). A balance to our calculations, regarding the proportion of Ty1-*copia*-like retrotransposons in the *Olea* genome, is provided by the factor that not all elements are of full length and remnants of retrotransposons also exist. In the Northern blot

(Fig. 6), no hybridization intensity differences between the two RNA samples (young leaves vs wounded young leaves) were observed, indicating that either the elements were not activated by the stress conditions within this time range or the response time of the tissues to the stress did not coincide to the RNA extraction time. Apart from activation of plant retrotransposons by stress, an increase in the copy numbers of these elements have been observed when plants are regenerated from cultured cells. In rice, continuing retrotransposition of *Tos*17 (a retrotransposon activated only during cell culture) provided a mechanism for the higher frequency of somaclonal variation observed in plants regenerated from long-term cell cultures compared to short-term cultures (Hirochika et al. 1996). Thus, culture time can play an important role in the amplification of retrotransposons within the genome.

A large number of retrotransposon families were found in the flanking regions of the maize *Adh1*-F gene. Blocks containing different mixtures of these repeats, which make up most of the maize genome (at least 50% of the nuclear DNA maize genome), are hypermethylated in mature plant tissues (SanMiguel et al. 1996). Most highly repetitive sequences, including retrotransposons, can only exist if they target intergenic regions, otherwise lethal mutations can accumulate (SanMiguel et al. 1996). Such intergenic regions are hypermethylated relative to gene sequences and can serve as homing areas of retroelements during their integration (Voytas 1996). From the methylation results (Fig. 5), it is evident that cytosines in GATC and CCGG sites are partially methylated, since extra bands are present in digestions with restriction enzymes insensitive to C methylation. The banding patterns between DNA isolated from young leaves and callus show no differences (Fig. 5), confirming results from previous studies (Katsiotis et al. 1998) about the genetic stability of 9-month-old tissue cultures of *Olea*.

The physical distribution of *RT* sequences in a wide range of genome and chromosome sizes across many taxonomic groups show a dispersed organization across all chromosomes excluded from some regions, such as centromeres, telomeres, NORs and heterochromatic regions (Katsiotis et al. 1995; Brandes et al. 1997; Heslop-Harrison et al. 1997). The *RT* sequences in *Olea* interphase nuclei show a dispersed organization with minor clustering (Fig. 7B). In metaphase chromosomes the smallest pair of homologous chromosomes is weakly labelled (Fig. 7E, double arrows), indicating the presence of Ty1-*copia* sequences in few copies. The low copy number of these sequences in this chromosome pair could be related to the limited number of insertion sites for Ty1-*copia* elements and/or the size of the chromosomes. However, *A. thaliana* chromosomes are smaller and Ty1-*copia* elements are present on all five chromosome pairs. In some of the other *Olea* chromosomes Ty1-*copia*-like sequences are either clustered (Fig. 7E, arrows) or are accumulated in restricted areas (Fig. 7E, arrowheads) of the chromosomes, indicating the absence of insertion sites in the majority of the genome hosted on these chromosomes. Non-uniform insertion and amplification of Ty1-*copia*-like retrotransposons has also been reported in other perennials, such as oil palms (Castilho et al. 2000) and the fern *Pteris cretica* (Brandes et al. 1997). In contrast, a dispersed distribution of retroelements, excluded from loci of *rRNA* genes, has been observed in slash pine, *Pinus elliottii* (Kamm et al. 1996).

The presence of retrotransposon sequences in a perennial plant that is asexually propagated (in order to maintain its characteristics), as well as the absence of structural and copy number differences for Ty1-*copia*-like (same banding pattern in Southern hybridizations) and other tandemly repeated sequences (Katsiotis et al. 1998; Bitonti et al. 1999), between three species belonging to the same section but having different chlorotypes (Lumaret et al. 2000), confirm the conservation of the *Olea* genome and the existence of these sequences prior to their speciation. The cultivated olive tree has 46 chromosomes and is considered a tetraploid. However, since no *Olea* species have been reported with fewer chromosomes, it is impossible to identify if the cultivated olive is an autotetraploid or an allotetraploid. One can only speculate, based on the results from in situ hybridisations with the different probes, that since not all chromosomes are labelled, an allotetraploid origin of the cultivated olive genome is possible. Chromosome morphology and patterns of heterochromatic bands have also led Falistocco and Tosti (1996) to conclude that *O. europaea* evolved from an interspecific hybridization.

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