Retention of relict satellite DNA sequences in *Anemone* (Ranunculaceae)

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Abstract – Satellite DNA is a genomic component present in virtually all eukaryotic organisms. The turnover of highly repetitive satellite DNA is an important element in genome organization and evolution in plants. Here we study the presence, physical distribution and abundance of the satellite DNA family AhTR1 in *Anemone*. Twenty-two *Anemone* accessions were analyzed by PCR to assess the presence of AhTR1, while fluorescence *in situ* hybridization and Southern hybridization were used to determine the abundance and genomic distribution of AhTR1. The AhTR1 repeat unit was PCR-amplified only in eight phylogenetically related European *Anemone* taxa of the Anemone section. FISH signal with AhTR1 probe was visible only in *A. hortensis* and *A. pavonina*, showing localization of AhTR1 in the regions of interstitial heterochromatin in both species. The absence of a FISH signal in the six other taxa as well as weak signal after Southern hybridization suggest that in these species AhTR1 family appears as relict sequences. Thus, the data presented here support the »library hypothesis« for AhTR1 satellite evolution in *Anemone*. Similar species-specific satellite DNA profiles in *A. hortensis* and *A. pavonina* support the treatment of *A. hortensis* and *A. pavonina* as one species, i.e. *A. hortensis* s.l.

Keywords: *Anemone*, FISH, library hypothesis, satellite DNA

Abbreviations: FISH – fluorescence *in situ* hybridization, **satDNA** – satellite DNA

Introduction

The genus *Anemone* s.str. consists of approximately 150 species (Tamura 1995), mainly distributed in the Northern Hemisphere. The ancestry, phylogenetic differentiation and systematic classification of *Anemone* have been debated for years. To advance the knowledge of the phylogenetic relationships within *Anemone*, considerable efforts based on DNA-analytical approach have been applied successfully and provided new insights into interspecific relationships (Hoot et al. 1994, Ehrendorfer and Samuel 2001, Schuettpelz et al. 2002,

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EHRENDORFER et al. 2009, MEYER et al. 2010). HOOT et al. (1994) and MEYER et al. (2010) suggest that *Hepatica*, *Knowltonia* and *Pulsatilla* as well as the South American genera *Oreithales* and *Barneoudia* should be subsumed within the *Anemone* s. lat and propose a preliminary classification that recognizes two subgenera (*Anemonidium* and *Anemone*), seven sections, and 12 informal subsection groupings. The European *Anemone* taxa belong to the Anemone section, with only one exception, *A. narcissifolia*, which belongs to the Anemonidium section. The Coronaria group is the largest in the Anemone section, comprising all the Mediterranean and American tuberous *Anemone*.

Anemone species were considered favourable plant material in cytogenetic studies because of chromosomal polymorphism, different ploidy levels, as well as variation in DNA content among species (BÖCHNER 1945, HEIMBURGER 1959, ROTHFELS et al. 1966, BAUMBERGER 1970, MLINAREC et al. 2006, MLINAREC et al. 2012a, MLINAREC et al. 2012b). Following the discovery of differential staining methods such as C-banding in the 1970s, *Anemone* again became a subject of interest due to the high quantities of heterochromatin and variation in distribution among species (MARKS 1974, MARKS and SCHWEIZER 1974). The greatest variations in heterochromatin amount and distribution on chromosomes are found among the members of the Coronaria group (MLINAREC et al. 2012b).

Satellite DNA (satDNA) is highly repetitive, non-coding and organized into long arrays composed of thousands to millions of tandemly arranged units. These arrays form constitutive heterochromatin (UGARKOVIĆ and PLOHL 2002). Different satDNA sequences can coexist in genomes, forming what has been defined as a library of satDNAs (FRY and SALSER 1977, MEŠTROVIĆ et al. 1998). Despite well-recognized roles of telomeric and centromeric satDNAs in the stabilization of chromosome ends and in cell division, their overall biological significance remains unclear (CSINK and HENIKOFF 1998).

Since satDNA evolves through evolutionary processes as predicted by the molecular drive model (DOVER 1986), DNA turnover usually leads to a high interspecific divergence and low intraspecific variation. The balance, persisting between satellite homogenization and persistence of satellite variants, could generate sufficient sequence divergence to cause reproductive isolation between intraspecific lineages, ultimately leading to speciation.

The AhTR1 satDNA monomer is an AT-rich sequence of 560 bp; this satDNA sequence constitutes about 0.14% of the *A. hortensis* genome, roughly about 3.05×10^4 copies (MLINAREC et al. 2009). The same authors only found evidence of AhTR1 in *A. hortensis*; the absence of Southern hybridization signals was found in other relatives tested. Here we characterize the presence, genomic distribution and abundance of the AhTR1 in 22 species of *Anemone* using PCR, fluorescence *in situ* hybridization (FISH) and Southern hybridization. Our goals were to: *i)* evaluate the phylogenetic signal of satDNA family in a genus and *ii)* gain an insight into the karyotype and genome evolution of *Anemone* using satDNA markers.

Materials and methods

Plant material

Information on all plant taxa used in this study is given in table 1. Plants were grown in pots in the Botanical Garden of University of Zagreb. All species were identified by morphological and karyological characteristics. For karyological studies, actively growing

Tab. 1. Species and accessions used in this study. The sectional classification is according to MEYER et al. (2010). Positive (+) and negative (–) PCR amplifications of the AhTR1 of the analysed accessions are also reported.

Taxon	Accession No.	Origin and/or Source	Section	PCR
Anemone apennina L.	1446B	Dizdarica (Montenegro),	Anemone	+
Anemone blanda Schott et Kotschy	12418	BG University of Vienna (Austria)	Anemone	+
Anemone coronaria L.	1725H	Anecor, Esdraelo Plain, Tel Shiron, 25 km SE of Haifa (Israel)	Anemone	+
Anemone hortensis L.	8216R	Island of Hvar (Croatia)	Anemone	+
Anemone palmata L.	12434	Morgion, Marseille (France)	Anemone	+
Anemone pavonina Lam.	12724	Bogdanci, Plajurci (Macedonia)	Anemone	+
Anemone ranunculoides L.	289	Delibatska peščara (Serbia)	Anemone	+
Anemone sylvestris L.	1451B	Čučerje, Medvednica (Croatia)	Anemone	+
Anemone parviflora Michx.	20081630	RBG Edinburgh (UK)	Anemone	–
Anemone obtusiloba D. Don	19861079	RBG Edinburgh (UK)	Homalocarpus	–
Anemone demissa Hook et Thomson	19910632	Upper Mo Chu Dist. (Bhutan), RBG Edinburgh (UK)	Homalocarpus	–
Anemone rivularis Buch.-Ham.	28070	Himalaya (Nepal), RBG Kew (UK)	Rivularidium	–
Anemone hupehensis Lemoine	28069	Sichuan, Huangtuliang Hills (China), RBG Kew (UK)	Rivularidium	–
Anemone tomentosa (Maxim.) C.'Pei	28071	RBG Kew (UK)	Rivularidium	–
Anemone canadensis L.	28068	South Dakota (USA), RBG Kew (UK)	Anemonidium	–
Anemone narcissiflora L.	1864L	Baden-Würtemburg, German Alps near Beuron (Germany), BG University of Zagreb (Croatia)	Homalocarpus	–
Anemone baldensis L.	698G	Vácrátót (Hungary),	Anemone	–
Anemone trifolia L.	562	Botanical Garden of the University of Zagreb	Anemone	–
Anemone cylindrica A. Gray	6559B	Chemnitz (Germany)	Anemone	–
Anemone multifida Poir.	1247	Chemnitz (Germany)	Anemone	–
Anemone nemorosa L.	558	Dubravkin put, Zagreb (Croatia)	Anemone	–
Anemone virginiana L.	11838B	Quebec, Country Deux-Montaignes (Canada)	Anemone	–

root-tip meristems were pretreated with 0.05% colchicine (Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany) for 4 h at RT, fixed in a solution of ethanol and acetic acid (3:1) for 24 h at –20 °C, and stored in 70% ethanol at –20 °C until use. For cloning as well as for Southern hybridization, high quality genomic DNA was isolated from young leaves using the Qiagen mini kit (GmbH, Hilden, Germany) according to manufacturer's instructions.

PCR amplification and cloning

PCR amplifications of the satDNA family AhTR1 were carried out in a 50 µL reaction mixture containing 10 ng template DNA, 0.4 µM of each primer, 200 µM dNTPs, 2.5 U GoTaq® DNA Polymerase and corresponding 1X (1.5 mM MgCl$_2$) Green Reaction Buffer (Promega Cor., Madison, USA), using the primer pairs AhTR1-1 (5'GTGTGAGGTATA-ACACACTGT 3') and AhTR1-2 (5' TAGTGTTGTGGAATACACATC 3'). After an initial denaturing step at 94 °C for 3 min, the amplification was carried out in 30 cycles consisting of denaturation at 94 °C for 1 min, annealing at 54 °C for 10 sec and primer extension at 72 °C for 1 min with final extension at 72 °C for 20 min. Cloning and transformation procedure were carried out using the InsTAclone™ PCR Cloning Kit (Fermentas GmbH, Germany) or pGEM®-T Easy Vector System (Promega, USA) according to the manufacturer's instructions. Sequencing was carried out by Macrogen Inc. (Seoul, Korea).

Sequence alignment and phylogenetic analysis

The number of AhTR1 clones by *Anemone* taxa is indicated in table 2. AhTR1 clones of *A. hortensis* (EU769127-EU769132) were taken from MLINAREC et al. (2009). All sequences obtained in this study are the result of cloning and are deposited in GeneBank under the accession numbers: *A. ranunculoides* AhTR1 (KC148493- KC148496), *A. apennina* AhTR1 (KC148497-KC148500), *A. sylvestris* AhTR1 (KC148501-KC148502), *A. coronaria* AhTR1 (KC148503-KC148505), *A. blanda* AhTR1 (KC148506-KC148509), *A. palmata* AhTR1 (KC148510-KC148514), *A. pavonina* AhTR1 (KC148515-KC148520). Sequences were aligned with Clustal X v1.81 (THOMPSON et al. 1997).

The evolutionary history was inferred using the Neighbor-Joining method (SAITOU and NEI 1987). The evolutionary distances were computed using the Kimura 2-parameter model (KIMURA 1980) and are in the units of the number of base substitutions per site. The analysis involved 34 nucleotide sequences. All positions containing gaps and missing data were eliminated. 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. Evolutionary analyses were conducted in MEGA5 (TAMURA et al. 2011).

Tab. 2. Features of AhTR1 sequences by *Anemone* species.

Taxa	Sequence number	Sequence length	AT %	Polymorphic sites
Anemone apennina	4	490–494	66	104
A. blanda	4	455–458	67	41
A. coronaria	3	457–492	65	106
A. hortensis	6	489–485	68	163
A. palmata	5	385–495	67	100
A. pavonina	6	492–496	69	139
A. ranunculoides	4	488–495	66	84
A. sylvestris	2	496	67	67

Chromosome preparation and fluorescence *in situ* hybridization

Chromosome preparations for FISH are described in MLINAREC et al. (2006). FISH experiments were done according to MLINAREC et al. (2012b). The probes Apav2AhTR1 and Abla3AhTR were directly labelled with Cy3-dCTP (Amersham, GE Healthcare, Little Chalfont, Buckinghamshire, UK) by using a nick-translation kit according to the manufacturer's instructions (Roche Diagnostics GmbH, Mannheim, Germany). After overnight hybridization, slides were given a stringent wash in 1 X SSC. These stringency conditions allowed the target sequences of approx. 56% homology to remain hybridized (SCHWARZACHER and HESLOP-HARRISON 2000). The preparations were mounted in Dako Fluorescent Mounting Medium (Dako North America, Inc., CA93013, USA) and stored at 4 °C. Signals were visualized and photographs captured on an Olympus BX51 microscope, equipped with a highly sensitive Olympus DP70 digital camera. An average of 10 well-spread metaphases was analyzed for each individual. Two individuals per taxa were analyzed.

Southern hybridization

Southern hybridization analyses were performed using Gene Images AlkPhos Direct Labelling and Detection System (Amersham, GE Healthcare, UK). Genomic DNA (gDNA) (1.6 µg) was digested over night with *Eco*RV (New England Biolabs, Ipswich, USA). Digested gDNA was loaded per lane on a 1% (w/v) agarose gel and electrophoretically separated for several hours at 100 V. DNA was blotted onto a positively charged nylon membrane (Roche, Basel, Switzerland) for one hour by using the Model 785 Vacuum Blotter (Biorad, Hercules, USA). Crosslinking was performed for 3 min (0.24 J cm^{-2}) on an UVlink CL508M crosslinker (Uvitec, Cambridge, UK). The membrane was prehybridized for half hour and hybridized over night at 55 °C using the probe Apav2AhTR. Subsequently, the membrane was washed at 55 °C and room temperature according to manufacturer's instructions.

Results

PCR amplification of the AhTR1 satellite family in *Anemone*

To detect the presence of AhTR1 satDNA family we performed PCR amplification in *Anemone*. The AhTR1 repeat unit was amplified in 8 of 22 *Anemone* taxa. All eight species in which the AhTR1 was amplified were phylogenetically related, belonging to the Anemone section. Six of the eight taxa were members of the Mediterranean group Coronaria (*A. apennina*, *A. blanda*, *A. pavonina*, *A. palmata*, *A. hortensis* and *A. coronaria*). Surprisingly, AhTR1 was amplified in *A. sylvestris* and *A. ranunculoides*, members of the Multifida and Nemorosa groups, respectively, but not in their closest relatives. As expected, successful PCR amplification usually resulted in a ladder pattern of products (data not shown), suggesting that the AhTR1 family exhibits a tandem repeat organization in the genome.

Sequence analysis

To investigate diversity of AhTR1 satDNA family in *Anemone* we have sequenced a total of 32 monomeric repeats of the AhTR1 family belonging to a total of eight species of the genus *Anemone*. Table 2 details for each species the number of repeat analyses,

monomer length, base composition and polymorphism. The *Anemone* AhTR1 was 385–496 bp and 65–69% in A+T content. The number of polymorphic sites ranged from 41–163, being the lowest in *A. blanda* and the highest in *A. hortensis*. Percentage similarity across all 32 AhTR1 sequences ranges from 53–99%. Previously we found that AhTR1 satellite monomer of *A. hortensis* contains a pentanucleotide sequence (CAAAA) that may have consequence for chromatin packing and sequence homogeneity (MLINAREC et al. 2009). Here we have found this motif in 26 out of 32 cloned AhTR1 sequences.

Phylogenetic analysis

To evaluate the phylogenetic signal of satDNA family in a genus we performed analyses with AhTR1 sequences as molecular markers. Un-rooted Neighbor-Joining tree showed
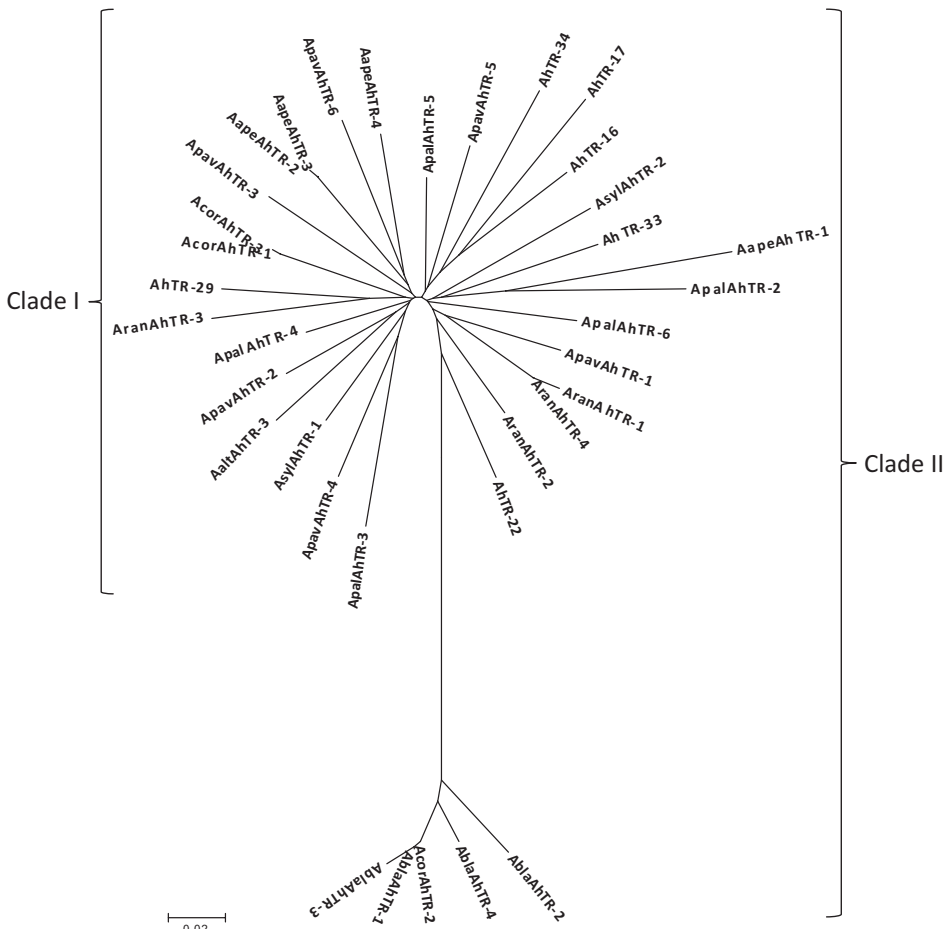

Fig. 1. Phylogenetic relationships among cloned AhTR1 sequences of *A. apennina* (AapeAhTR1), *A. blanda* (AblaAhTR1), *A. coronaria* (AcorAhTR1), *A. hortensis* (AhTR1), *A. palmata* (ApalAhTR1), *A. pavonina* (ApavAhTR1), *A. ranunculoides* (AranAhTR1) and *A. sylvestris* (AsylAhTR1).

separation of sequences into two slightly divergent clades: I and II. The sequences originating from *A. coronaria*, *A. sylvestris*, *A. apennina*, *A. palmata*, *A. hortensis*, *A. ranunculoides* and *A. pavonina* fell within the two distinct clades I and II, while those from *A. blanda* fell only within clade II (Fig. 1). In both clades the sequences are intermingled. The only exception is *A. blanda* which showed a clear separation within clade II. Interestingly, one clone of *A. coronaria* associated with those of *A. blanda*. For more detailed phylogenetic analyses, more clones should be isolated from each individual.

FISH in *Anemone*

Clone Apav2AhTR1 was used as a probe to determine the chromosomal position of AhTR1 satDNA family in the *Anemone* taxa in which AhTR1 was PCR-amplified. The only exception was *A. blanda* in which clone Abla3AhTR was used as a probe as this species proved to have a distinctive AhTR1 family. The AhTR1 sequences were either completely lacking or predominantly localized at the intercalary DAPI-positive heterochromatic regions of chromosomes. Clear FISH signals were visible only in *A. hortensis* and *A. pavonina* (Fig. 2), while negative *in situ* results were obtained in the karyotypes of other six species in which the AhTR1 was PCR-amplified (data not shown).
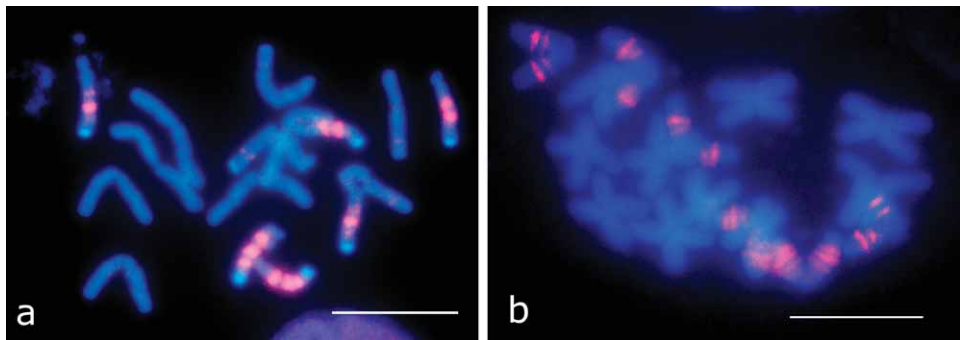

Fig. 2. FISH on mitotic chromosomes of *Anemone hortensis* (a) and *A. pavonina* (b) with labelled Apav2JME probe (in red). Bar = 10 μm.

Southern blot analysis

To determine the abundance and organization of AhTR1 isolated from A. *hortensis* within different species of *Anemone*, clone Apav2AhTR1 was hybridized to *Eco*RV--digested genomic DNA of *A. apennina, A. blanda*, *A. coronaria*, *A. hortensis, A. palmata*, *A. pavonina, A. ranunculoides* and *A. sylvestris* (Fig. 3). In accordance with the FISH experiments, only *A. hortensis* and *A. pavonina* exhibited a strong ladder-like hybridization signal of similar intensity, suggesting that in these two species satDNA family AhTR1 is highly abundant and represented by a similar copy number. In the lanes of the six other species, the hybridization signal was hardly visible suggesting that in these species AhTR1 is present only as minor repeats.
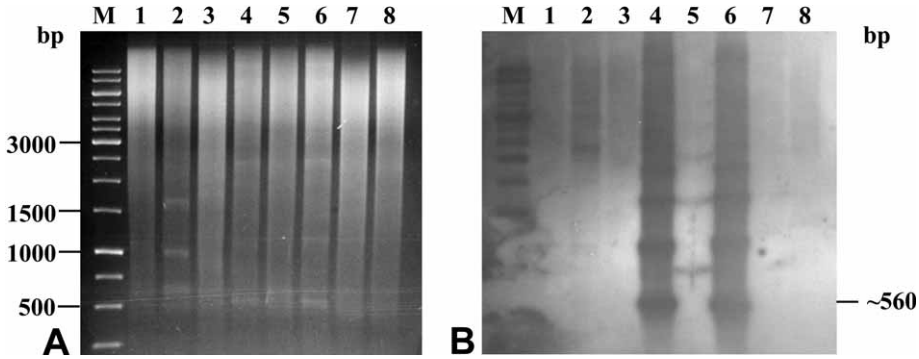
Fig. 3. Genomic restriction digests (A) and Southern blotting analyses (B) of *Anemone apennina* (1), *A. blanda* (2), *A. coronaria* (3), *A. hortensis* (4), *A. palmata* (5), *A. pavonina* (6), *A. ranunculoides* (7) and *A. sylvestris* (8) with restriction endonuclease *Eco*RV and probed with clone Apav2AhTR1. M – marker, bp – base pairs.

Discussion

Distribution of satDNA family AhTR1 is in agreement with phylogeny: the AhTR1 satDNA family is PCR-amplified in the European members of the Anemone section. By contrast, the phylogenetic tree of AhTR1 sequences is not congruent with the phylogeny of these species based on other markers. Using *atpB-rbcL* spacer region and ITS data Meyer et al. (2010) demonstrated that Anemone section contains three major clades: a basal clade composed of the members of the Baldensis group; a second clade composed of the members of the Nemorosa and Multifida groups; and a third clade including all tuberous Mediterranean and American *Anemone* of the Coronaria group. In contrast, the tree based on AhTR1 sequences shows an intermixture of sequences originating from the members of the Coronaria, Nemorosa and Multifida groups with slight divergence of sequences into two clades. The lack of species-specific clustering can be partly explained by divergence of sequences in the ancestors of the species analyzed. However, it is possible that deeper sampling of sequences, within each species, might produce sub-trees that are more representative of species relationships.

In the AhTR1 tree, the only exception is *A. blanda,* which shows low intra-individual nucleotide diversity and clear separation from the other Anemone taxa in which AhTR1 was amplified. The phylogenetic position of *A. blanda* has been debated for years. In the tree based on *atpB-rbcL* spacer region and ITS data *A. blanda* has a basal position to all anemones of the Anemone section. Thus, the position of *A. blanda* in the AhTR1 tree agrees with the position based on other molecular markers suggesting that although similar morphological characteristics and geographic distribution unite *A. blanda* with other Mediterranean anemones in the Coronaria group, this species underwent different evolutionary pathways than the other Mediterranean tuberous *Anemone* from the Coronaria group.

The DNA library model proposed by Fry and Salser (1977) hypothesized that closely related species share a set of satellite DNA families (satDNA library) differing in copy number and sequence divergence (Ugarković and Plohl 2002). As a consequence of the

amplification of a particular satDNA family in a species, this satDNA becomes highly abundant, while the others are present only as minor repeats. This leads to species-specific profiles (UGARKOVIĆ and PLOHL 2002). In *Anemone* we have found a support for this hypothesis. The presence of AhTR1 in all Mediterranean anemones as well as in the representatives of the other two groups of the Anemone section (the Multifida and Nemorosa groups) suggests that this family was present in the common ancestor of the Anemone section. However, the highly dynamic nature of satDNA turnover resulted in considerable fluctuation in satellite copy number. In *A. hortensis* and *A. pavonina* it became highly abundant, while in other members it stayed only as minor repeats, even in the close relative *A. coronaria*. Furthermore, the AbS1 satDNA family constitutes the major fraction of *A. blanda* interstitial AT-rich heterochromatin, about 2 % of its genome, while in sister species *A. apennina* the family is present as minor repeats (HAGEMANN et al. 1993). These two satellite DNA families, AhTR1 and AbS1, are highly divergent (<35% similarity) suggesting that in *A. hortensis* and *A. blanda* different satDNA families were amplified and became abundant. This further supports the »satDNA library« hypothesis. Besides, it is worth mentioning that in the six species where AhTR1 was present in lower numbers, a ladder PCR pattern was still recovered. The tandem arrangement might be important for maintaining the identity of these repeats via homogenization mechanisms. There are, to our knowledge, few empirical examples for the »satDNA library« hypothesis in plants (KOUKALOVA et al. 2010, QUESADA DEL BOSQUE et al. 2011).

The Mediterranean anemones are mostly diploids (except for *A. palmata* in which the autotetraploid 2n=4x=32 populations are reported, MÉDAIL et al. 2002). The experimental hybrids between *A. coronaria*, *A. palmata* and *A. hortensis* exhibit meiotic asyndesis and are completely sterile (MAÏA and VENARD 1976). As repetitive sequences constitute the major fraction of the genome, it is reasonable to suggest that these are responsive to maintaining reproductive isolation between the Mediterranean *Anemone* taxa. On the other hand, experimental hybrids between *A. hortensis* and *A. pavonina* exhibit relatively normal meiosis and fertility (MAÏA and VENARD 1976). Considering this and the existence of molecular and morphological similarities between *A. hortensis* and *A. pavonina*, EHRENDORFER et al. (2009) proposed to treat *A. hortensis* and *A. pavonina* as one polymorphic species, i.e. *A. hortensis* s.l. The results of this study showed that *A. hortensis* and *A. pavonina* share similar species-specific satDNA profile and thus clearly support the treatment of *A. hortensis* and *A. pavonina* as one species, i.e. *A. hortensis* s.l.

In summary, the study of AhTR1 satellite DNA in *Anemone* reveals a complex evolutionary history where differential levels of satellite DNA amplification in different lineages, at different evolutionary times, and in different chromosomal places gave rise to sequence variants persisting as »library« (MEŠTROVIĆ et al. 1998).

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