Short Communication

Molecular phylogenetics in 2D: ITS2 rRNA evolution and sequence-structure barcode from Veneridae to Bivalvia

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1. Introduction

Molecular phylogenetics and molecular taxonomy have traditionally focused on analyzing DNA (or RNA) sequences for phylogenetic and taxonomic assessments. However, in the last few years the exploitation of secondary structure information of ribosomal RNA (rRNA) molecules such as the nuclear ribosomal internal transcribed spacer 2 (ITS2) has revealed a promising approach not only in phylogenetic reconstruction (Coleman, 2003; Schultz et al., 2006; Schultz and Wolf, 2009; Salvi et al., 2010) but also in species diagnosis (Coleman, 2003, 2009; Müller et al., 2007).

The ITS2 is generally organized in four main helix domains (D–IV) of secondary structure and the combined information of both folding and primary sequence has been exploited for phylogenetic inference, taxonomic classification, and species delimitation, providing an increased phylogenetic resolution and reliability than approaches based on primary sequence data alone (Coleman, 2003; Schultz et al., 2006; Bologna et al., 2008; Song et al., 2008; Schultz and Wolf, 2009; Keller et al., 2010; Salvi et al., 2010). Compensatory base changes (CBCs) – mutations in both nucleotides of a paired position in a double-stranded structure of the transcribed RNA – in conserved regions of the eukaryote ITS2 sequence-structure have been shown to correlate with interbreeding incompatibility between species. Several studies on animal, plants, and fungi have demonstrated that the presence of at least one CBC in ITS2 secondary structures is a good classifier (reliability higher than 90%) of two organisms belonging to distinct species (Coleman, 2000, 2003, 2009; Müller et al., 2007). Moreover, some conserved features of the ITS2 secondary structure such as stem-loops domains have shown to be diagnostic of higher taxonomic grouping such as tribes, subfamilies, families, and orders (Oliverio et al., 2002; Bologna et al., 2008; Keller et al., 2008; Salvi et al., 2010). Thus, the information from the ITS2 folding and sequence-structure variation is a useful tool in molecular taxonomy for species and higher-taxa diagnosis.

In this study we analyzed the nuclear ITS2 rRNA primary sequence and secondary structure in Veneridae and comparatively with 20 Bivalvia taxa to test the phylogenetic resolution of this marker and its suitability for molecular diagnosis at different taxonomic levels.

Maximum likelihood and Bayesian trees based on primary sequences were congruent with (profile-) neighbor-joining trees based on a combined model of sequence-structure evolution. ITS2 showed higher resolution below the subfamily level, providing a phylogenetic signal comparable to (mitochondrial/nuclear) gene fragments 2–5 times longer. Structural elements of the ITS2 folding, such as specific mismatch pairing and compensatory base changes, provided further support for the monophyly of some groups and for their phylogenetic relationships. Veneridae ITS2 folding is structured in six domains (DI–VI) and shows five striking sequence-structure features. Two of them, the Basal and Apical STEMs, are common to Bivalvia, while the presence of both the Branched STEM and the Y/R stretches occurs in five superfamilies of the two Heterodonta orders Myoida and Veneroida, thus questioning their reciprocal monophyly.

Our results validated the ITS2 as a suitable marker for venerid phylogenetics and taxonomy, and underlined the significance of including secondary structure information for both applications at several systematic levels within bivalves.

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H3 gene and the ribosomal 18S/28S rRNAs (Chen et al., 2011). To overcome these problems, the use of an extremely variable and easy to amplify nuclear marker such as the ITS2 is highly advisable, but despite a promising test on four species (Cheng et al., 2006), ITS2 has never been used in phylogenetic and taxonomic studies of Veneridae.

The main aim of this study was to integrate the information of individual RNA secondary structures with primary sequences in venerids phylogeny and taxonomy, by employing a combined model of sequence-structure evolution as well as identifying conserved elements of the ITS2 folding. By including several representatives from Bivalvia subclasses we further test the phylogenetic resolution of the ITS2 marker and its suitability as a barcode tool for molecular taxonomy of venerid species to higher taxonomic levels among bivalves.

2. Materials and methods

2.1. Sampling, DNA extraction and sequencing

ITS2 sequences were obtained from live specimens (60%), frozen specimens from trade (4%), or retrieved from Genbank (36%). A total of 45 specimens from 24 venerid species were employed in the molecular analyses. Twenty additional Bivalvia ITS2 sequences from the subclasses Heterodonta, Paleoheterodonta, and Pteriomorpha were also analyzed (see Supplementary Table S1 for taxonomic references, locality data, and Genbank accession numbers). Taxonomic nomenclature in the text, tables and figures follows Mikkelsen et al. (2006). Total DNA purification and PCR amplification were performed according to the standard procedures previously described by Oliverio and Mariottini (2001). Purification and sequencing of Plasmid DNA from positive clones and PCR products were carried out by an external service (Genechron Centre of Sequencing, ENEA, La Casaccia, Rome).

2.2. Secondary structure modeling, sequence alignment, and barcode gap analysis

Preliminary ITS2 sequences alignments were performed by Clustal X 2.0 (Larkin et al., 2007). Afterwards, the alignment was progressively optimized according to secondary structure homology. The best-supported folding models were obtained combining thermodynamic and comparative approaches. On a thermodynamic basis, folding was predicted for each sequence using the software package RNA Structure version 3.71 (available at the Turner Lab Homepage, http://rna.chem.rochester.edu; Mathews et al., 1999). The lowest structure energy alone does not guarantee the best-model prediction (Wolf et al., 2005), thus the folding was optimized by contrasting several candidate low free energy folding models with ITS2 folding models from molluscan taxa (Oliverio et al., 2002; Salvi et al., 2010). The maximized structural homologies were retained and checked for the presence of compensatory base changes (CBCs).

Conserved and variable sites were counted using MEGA 5 (Tamura et al., 2011). We assessed the presence of a barcode gap between intraspecific and interspecific congeneric distance by constructing a histogram based on Kimura 2-parameter distances using the program TaxonDna 1.7.8 (Meier et al., 2006).

2.3. Phylogenetic analysis based on primary and secondary sequence information

Flanking sequence from 5.8S and 28S were trimmed from alignments and the complete ITS2 sequences were used in phylogenetic analyses. According to traditional classification and previous phylogenies, Arctica islandica (Veneroida, Arcticaeida, Arcticidae) was used as the outgroup taxon (see Mikkelsen et al., 2006). In order to test for the effect of the outgroup choice on the Veneridae topology, additional phylogenetic analyses were performed using Corbicula japonica (Veneroida, Corbiculidea, Corbiculidae) or the divergent taxon Potamocorbula amurensis (Myoida, Myoidea, Corbulidae) as alternative outgroups.

Phylogenetic analyses of ITS2 sequences were performed based on primary sequences alone as well as combined analyses including both primary sequences and secondary structure information. Phylogenetic analyses based on primary sequence were carried out using Maximum likelihood (ML) and Bayesian (BA) approaches using the GTR + G model selected by JModeltest (Posada, 2008) according to the AIC criterion. The best ML tree was calculated using heuristic searches (TBR branch swapping, 100 replicates and random stepwise addition) using Paup4.0b (Swofford, 2002) and Treefinder (Jobb, 2008). Nodal support was calculated using 1000 bootstrap replicates (BP) in Treefinder. Bayesian analyses (BA) was conducted in MrBayes 3.1 (Huelsenbeck and Ronquist, 2001). Two independent Markov chain Monte Carlo (MCMC) simulations were run in parallel for 10^7 generations, sampling every 1000 generations. MCMC chains convergence was assessed using the program AWTY (Wilgenbusch et al., 2004) (burn-in = 2500) and the trees sampled after burn-in were used to assess posterior probabilities for nodal support (BPP). Phylogenetic analysis based on primary sequence and secondary structure was performed employing a combined model of sequence-structure evolution with the software ProDistS (Wolf et al., 2008). ProDistS employs a tree construction algorithms suited for RNA sequence-structure alignment including the 4 nucleotides (A, G, C, and U) in three structural states (single strand, stem left, stem right) coded as in Vienna format (“.”, “("), “")”, respectively). The substitution model of sequence and structure evolution employed combines a general time evolution model (GTR) on the sequence level with a substitution model on morphological features of the structure (Wolf et al., 2008). We produced ITS2 sequence-structure alignments by translating the secondary structure information of individual sequences (previously obtained, see above) in a Vienna format. Phylogenetic trees were calculated with neighbor-joining (NJ) and profile neighbor-joining (PNJ) approaches (strNJ and strPNJ, respectively), employing the automatic profile definition procedure implemented in ProDistS. The robustness of the phylogenies produced was tested by 1000 bootstrap replicates.

3. Results

3.1. ITS2 sequence variation

The venerid ITS2 sequences ranged in length from 241 (Saxidomus gigantea) to 417 (Meretrix meretrix) base pairs (bp). For the eight species for which multiple specimens were available no differences in ITS2 sequence length were observed except in Ruditapes philippinarum. In this species, specimens from four different localities showed ITS2 sequences length from 310 to 379 bp with an average length of 363 bp. Intra-individual variation of the ITS2
sequences was observed only in two species, *Ruditapes decussatus* and *Tapes aureus*, with one or two variable sites discriminating the two alleles. Multiple sequence alignment resulted in a total of 625 nucleotide positions including indels, among which 125 positions were constant, 419 variable, and 324 parsimony informative.

The presence of a barcode gap was evident in the comparison of interspecific and intraspecific pairwise distances between ITS2 sequences (Supplementary Fig. S2). Intraspecific genetic distances (K2P) ranged between 0–0.025 (Mean = 0.006; SD = 0.006). Interspecific genetic distances ranged between 0.035 and 0.767 (Mean = 0.514; SD = 0.185) using all the congeneric species comparisons, and 0.035–0.222 (Mean = 0.138, SD = 0.069) excluding comparisons within the genera *Ruditapes* and *Venerupis* as these genera were not monophyletic in our study (see discussion).

3.2. ITS2 secondary structure of Veneridae, Heterodonta and other Bivalvia

The typical venerid ITS2 RNA folding along with conserved secondary structure elements across Veneroida and Myoida superfamilies and Bivalvia subclasses are represented in Fig. 1. The common derived Veneridae ITS2 rRNA structure is generally organized in six stems, defined as DI–VI (see Salvi et al., 2010 for secondary structure nomenclature). The six domains are always identifiable in terms of sequence and position except in three cases: the loss of the DIV was observed in the genera *Protothaca*, *Ruditapes*, and, together with the absence of DV, in the genus *Saxidomus*. Outside Veneridae, either the “six domains” folding or the “four domains” structure standard to most eukaryotes were observed in the ITS2 secondary structure of eight Veneroida superfamilies analyzed, while in the superfamily Tellinoidae both the two folding architectures are present in different families (data not shown).

In venerids, the variable regions of the ITS2 RNA are located in the DI and DIV–VI stems that are poorly conserved in their primary sequences, but still highly conserved in their folding architecture. The basal portion of DII – here defined as Basal STEM – is highly conserved in all venerids and most Bivalvia and consists of two 4 to 6 G/C rich base-pairing stems separated by a segment including a small asymmetric bulge, a 5’-CG/CG-3’ base-pairing, and the mismatching of two pyrimidines (Fig. 1 and Supplementary Fig. S3).

The apical portion of DIII – previously defined as Apical STEM (Salvi et al., 2010) – is confirmed to be present in all the Bivalvia species examined and in venerids is characterized by the fourth adenine invariably exposed, a protruding dinucleotide UU in the opposite base-paired strand, and an apical bulge containing the tetrancleotide 5’-GAAC-3’ (see Fig. 1 and Table 1 for details). Finally, the two conserved pyrimidine and purine sequences (potentially capable to base pairing) located 5’ upstream of the DIII and 3’ downstream of the DIV – here defined Y and R stretch motifs respectively – and the extra stem branching out from the very conserved median bulge of DIII – here defined as Branched STEM – are structural elements of the ITS2 folding remarkably present in all venerids as well as in other Bivalvia subclasses.

![Fig. 1. ITS2 secondary structure model for Veneridae showing the typical six domains folding (exemplified in the type-species Venus verrucosa, Vve1). Conserved sequence-structure elements across Veneroida, Myoida, and Bivalvia subclasses are boxed and named as in the text.](image-url)
representatives of other superfamilies of Veneroida and Myoida (Fig. 1).

3.3. ITS2 sequence/structure phylogenetic information from Veneridae to Bivalvia

Phylogenetic analyses based on primary sequence alone (ML and BA) and combined with secondary structure (strNJ and strPNJ) yielded analogous topologies with no supported branch in conflict except for the sister relationship between Ruditapes variegata and R. decussatus (supported in ML and BA analyses, not shown, but not in the sequence-structure strNJ analyses, Fig. 2). Analyses carried out using additional outgroups from Veneroida and Myoida recovered the same overall topology relative to the analyses using the primary outgroup taxa A. islandica (Veneroida), but with lower bootstrap support (data not shown). The profile neighbor-joining procedures (strPNJ) produced virtually the same results both in terms of topologies and node support relative to the initial strNJ. Therefore, only results obtained by strNJ method are shown.

The results from ITS2 sequence/structure analyses based on (i) primary sequence alone, (ii) combined primary sequence-secondary structure and (iii) secondary structure alone were highly congruent at any taxonomic level.

At the species level, all the eight species for which multiple specimens were available consisted monophyletic supported clades (BP > 82; BPP > 99). Moreover, in the three species for which specimens from different geographic localities were analyzed divergent intra-specific phyllogroups were recovered (Fig. 2), suggesting a resolution power of ITS2 sequence data also at the intra-specific level. The ITS2 secondary structure from conspecific individuals of any species was strictly conserved and striking sequence-structure autopomorphies were observed in four species (Supplementary Fig. S4).

At the generic level, in ITS2 sequence/structure analyses Dosinia, Meretrix, and Protothaca formed monophyletic and well supported clades (BP > 84; BPP > 99) with a conserved ITS2 secondary structure (showing six domains in Dosinia and Meretrix, and five domains in Protothaca, see Fig. 2). On the other hand, the genus Venerupis was paraphyletic relative to Tapes and the genus Ruditapes was paraphyletic with respect to Tapes and Venerupis (Fig. 2). The paraphyly of the genera Venerupis and Ruditapes was confirmed by ITS2 structure data: V. lucens shares two striking sequence-structure synapomorphies with T. aureus (Supplementary Fig. S4) and species assigned to the genus Ruditapes show divergent ITS2 secondary structures with six domains in R. decussatus and five domains in R. variegata and R. phylippinorum (Fig. 2).

Multiple species of the subfamilies Chioninae and Tapetinae were analyzed, which provided the opportunity to test whether the combined sequence-structure alignment is also informative at the subfamily ranking. In all the phylogenetic analyses, the species assigned to the subfamily Chioninae were placed in different clades and showed diverged ITS2 secondary structures (with five domains in the genus Protothaca and six domains in the other species), indicating that this taxon is polyphyletic. On the other hand, the monophyly of the subfamily Tapetinae (not including Paphia undulata) was supported by the sequence-structure phylogenetic analyses and by the mismatch in the D11 and two CBCs in the D1, that define even more tightly the relationships between some of them (Fig. 2 and Supplementary Fig. S4).

The resolution of the ITS2 marker in reconstructing the phylogenetic relationships between the nine venerid subfamilies included in our analyses was very low, both as concerns the sequence-structure phylogenetic analyses and the inspection of the secondary structure models (Fig. 2). Indeed, phylogenetic analyses returned a basal polytomy, and failed to disentangle the relationships between venerid subfamilies, while secondary structures were not informative since at deeper nodes most of the taxa showed the plasmogenic “six domains” folding.

Above the family Veneridae, while the phylogenetic resolution of the ITS2 sequence-structure alignments was lower, several conserved features of ITS2 secondary structure can be identified as diagnostic characters of several groups from Veneroida, Myoida, and Bivalvia (see Section 3.2).

4. Discussion

The suitability of the ITS2 as a barcode marker for venerids is highly supported by the present study as assessed against several criteria: (1) the presence of a barcode gap between inter- and intra-specific variation; (2) the clustering of conspecific sequences in distinct groups with high bootstrap supports; and (3) the presence of sequence-structure diagnostic character differences between congeneric species. Based on the ITS2 sequence-structure analyses, it was possible to identify all the venerid species studied

### Table 1: Comparison of Apical STEM sequences among Veneridae

<table>
<thead>
<tr>
<th>Species</th>
<th>Apical STEM sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chamelea gallina (Chioninae)</td>
<td>5’-GGCCGCGGCGCC-3’</td>
</tr>
<tr>
<td>Mercenaria mercenaria (Chioninae)</td>
<td>5’-GGCCGCGGCGCC-3’</td>
</tr>
<tr>
<td>Protothaca staminea (Chioninae)</td>
<td>5’-GGCCGCGGCGCC-3’</td>
</tr>
<tr>
<td>Timocela avata (Chioninae)</td>
<td>5’-GGCCGCGGCGCC-3’</td>
</tr>
<tr>
<td>Cyclina sinensis (Cyclinidae)</td>
<td>5’-GGCCGCGGCGCC-3’</td>
</tr>
<tr>
<td>Dosinia exoleta (Dosinidae)</td>
<td>5’-GGCCGCGGCGCC-3’</td>
</tr>
<tr>
<td>Gouliida minima (Gouldiidae)</td>
<td>5’-GGCCGCGGCGCC-3’</td>
</tr>
<tr>
<td>Meretrix meretrix (Mereticinae)</td>
<td>5’-GGCCGCGGCGCC-3’</td>
</tr>
<tr>
<td>Callista chione (Pitarinidae)</td>
<td>5’-GGCCGCGGCGCC-3’</td>
</tr>
<tr>
<td>Saxidomus giganteus (Pitarinidae)</td>
<td>5’-GGCCGCGGCGCC-3’</td>
</tr>
<tr>
<td>Paphia undulata (Tapetinae)</td>
<td>5’-GGCCGCGGCGCC-3’</td>
</tr>
<tr>
<td>Ruditapes decussatus (Tapetinae)</td>
<td>5’-GGCCGCGGCGCC-3’</td>
</tr>
<tr>
<td>Ruditapes philippinarum (Tapetinae)</td>
<td>5’-GGCCGCGGCGCC-3’</td>
</tr>
<tr>
<td>Ruditapes variegata (Tapetinae)</td>
<td>5’-GGCCGCGGCGCC-3’</td>
</tr>
<tr>
<td>Tapes aureus (Tapetinae)</td>
<td>5’-GGCCGCGGCGCC-3’</td>
</tr>
<tr>
<td>Venerupis lucens (Tapetinae)</td>
<td>5’-GGCCGCGGCGCC-3’</td>
</tr>
<tr>
<td>Venus verrucosa (Venerinae)</td>
<td>5’-GGCCGCGGCGCC-3’</td>
</tr>
</tbody>
</table>

*Note: Superscript letters indicate nucleotides invariantly exposed; in bold Veneridae Apical STEM consensus sequence under 90% minimum identity criterion (see text for details)."
Fig. 2. ITS2 sequence-structure neighbor-joining tree (strNJ) based on evolutionary distances simultaneously calculated on sequences and secondary structures of 24 Veneridae species and rooted with A. islandica (Veneroida, Arcticidea, Arcticidae). Statistical support values from phylogenetic analyses based on primary sequence and secondary structure (strNJ) and primary sequences alone (Maximum likelihood/Bayesian inference) are reported above and below the nodes respectively. Number and uppercase letter after species’ name refer to different individuals; minor case letter after species’ name indicate different alleles from heterozygote individuals. Taxonomic nomenclature follows Mikkelsen et al. (2006).
as reciprocally monophyletic units, which in some cases were further supported by striking apomorphies (CBCs and/or a mismatch pairing) shared by the ITS2 sequence-structure of conspecific individuals. Moreover, ITS2 seems to be a promising marker also for phylogeographic studies as intraspecific monophyletic phylogroups, clustering individuals from distinct localities, were identified in most of the species for which individuals from multiple localities were analyzed.

In Venus clams both the ITS2 primary sequences and the secondary structure have proven valuable for reconstructing the phylogenetic relationships providing the highest phylogenetic resolution and robustness at the genus level. The genera Dosinia, Meretrix, and Protophatka formed monophyletic units in the ITS2 (sequence and structure) phylogenetic reconstructions, in accordance with previous molecular studies, although the taxonomic composition of the genus Dosinia needs to be re-assessed (Kapper and Bieler, 2006; Mikkelsen et al., 2006; Chen et al., 2011). The ITS2 secondary structures of Protophatka species showed a “five-domains” folding architecture, unique within Veneridae, which emerges as a synapomorphy of this genus. On the other hand, the monophyly of Vennerus and Ruditapes is not supported either by ITS2 data or by previous molecular studies based on mitochondrial and nuclear markers (Canapa et al., 2003; Kapper and Bieler, 2006; Mikkelsen et al., 2006; Chen et al., 2011). Indeed, the closer phylogenetic relationship of V. lucens to T. aureus rather than to V. senegalensis is further supported by a double CBC in the D1 shared by the first two species. Likewise, the paraphyly of the genus Ruditapes was supported by phylogenetic and structural ITS2 data, suggesting that these three genera need revision as proposed by previous studies (references above). The monophyly of the subfamilies Venerinae, and Tapetinae and the inclusion of Chononida gallinae in the subfamily Venerinae (rather than in the Chionidae) were strongly supported in agreement with previous molecular phylogenies (Canapa et al., 2003; Kapper and Bieler, 2006; Mikkelsen et al., 2006; Chen et al., 2011).

Contrary to previous studies (Coleman, 2003; Song et al., 2008; Salvi et al., 2010), in venerids the inclusion of the ITS2 secondary structure information did not extend at higher taxonomic levels the phylogenetic applicability of this marker attained by comparisons of primary sequences. However, this might be due to the fact that most of the Veneridae subfamilies may not represent natural groups (see Mikkelsen et al., 2006 and Chen et al., 2011), rather than to a lack of phylogenetic signal in the ITS2 sequence-structure, although phylogenetic signal of the venerid ITS2 rRNA is strongest below the family level, the ITS2 secondary structure showed several conserved elements which also occur outside the Veneridae, thus providing clues on ITS2 molecular evolution in venerids as well as at higher taxonomic levels. The most common derived Veneridae ITS2 rRNA shows five structural-sequence features: (a) six domains folding architecture with highly conserved basal D1 and apical DII regions; (b) two Y and R stretches; (c) the Branched STEM sequence; and (d) the Apical STEM sequence. Conserved sequence-structure motif in the ITS2 secondary structure are rare or unique features likely to be of single (monophyletic) evolutionary origin and thus representing invaluable tool for molecular diagnoses of higher-level taxa. The presence of the Branched STEM/Y stretch/R stretch marks the ITS2 rRNA folding of heterodent superfamilies of the orders Veneroida and Myoida (Fig. 1), thus supporting their close phylogenetic relationships, albeit questioning the reciprocal monophyly of the orders Myoida and Veneroida, in agreement with previous morphological and molecular studies (Morton, 1990; Adamkiewicz et al., 1997; Canapa et al., 2001). Moreover, while the Basal and the Apical STEMs emerged as a common features of the Bivalvia (with the exception of three Pteriomorpha orders which lack the Basal STEM), the specificity of their consensus sequences to lower taxonomic groups such as Veneridae and Pectinidae (this study; Salvi et al., 2010) could provide a molecular (sequence-structure) barcode tool for their diagnosis.

Interestingly, when comparing the phylogenetic reconstruction of Veneridae attained in this study with those recovered in previous molecular studies, we found that the ITS2 provides the same phylogenetic resolution as much longer mitochondrial and nuclear fragments including the 16S rRNA (Canapa et al., 1996, 2003) and the COI (Mikkelsen et al., 2006; Kapper and Bieler, 2006; Chen et al., 2011) mitochondrial fragments, as well as the combined 16S + COI + 28S + H3 (Mikkelsen et al., 2006) and 16S + COI + H3 (Kapper and Bieler, 2006; Chen et al., 2011) mitochondrial-nuclear datasets. Taking into account that the use of mitochondrial markers can be problematic due to double uniparental inheritance of mitochondrial genomes in some venerids (Mikkelsen et al., 2006), and given the very low resolution of nuclear markers such as the H3 gene and the ribosomal 18S/28S rRNAs (Chen et al., 2011), the ITS2 gene emerges as a candidate optimal marker for accurate and wide-range taxonomic and phylogenetic studies in venerids.

Acknowledgments

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ympev.2012.07.017.

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