Use of the internal transcribed spacer (ITS-1) region to infer *Orius* (Hemiptera: Anthocoridae) species phylogeny

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Abstract

Partial 18S, 5.8S and the intervening internal transcribed spacer region 1 (ITS-1) of nuclear ribosomal DNA from representatives of 10 species of the genus *Orius* and one outgroup species (*Wollastoniella rotunda* Yasunaga et Miyamoto) were analyzed by PCR and DNA sequencing. Within the taxa analyzed, the length of the ITS-1 region varied from 230–663 bp. Phylogenetic trees generated from informative sites within the sequences were highly resolved in the proximal regions of the branches, but more ambiguous deeper within the tree. *Orius* species relationships showed some agreement with anecdotal morphological observations and this study represents a first attempt in establishing *Orius* species relationships.

Key words: *Orius*, phylogeny, ITS-1, nucleotide sequence, molecular systematics

INTRODUCTION

The genus *Orius* contains approximately 67 species found world-wide with 31 described species placed in seven subgenera (*Dimorphella*, *Heterorius*, *Microtrachelia*, *Orius*, *Paraorius*, *Trichorius*, and *Xylorius*) (Yasunaga, 1997c). *Orius* consists of polyphagous predators of many agricultural pests throughout the world. Hence, their value as biological control agents is important in a number of agricultural systems. Although taxonomy and biology have been extensively studied for a number of species, no attempt have been made to determine species relationships of this economically important group of insects. In many instances species differentiation is difficult and the faunas of many regions such as Asia remain unknown (Yasunaga, 1997a).

DNA sequences are now widely used to infer species identification and phylogeny. Ribosomal DNA (rDNA) is an extensively used region for molecular systematics as it offers highly conserved regions for examining animal phyla relationships as well as rapidly evolving regions which are useful in studying closely related

species (Hillis and Dixon, 1991 and references therein). At the species and intraspecific level, the internal transcribed spacer regions (ITS-1 and ITS-2) are used as a taxonomic tool for many groups including fungi (Carbone and Kohn, 1993; Kusaba and Tsuge, 1995), plants (Hsiao et al., 1994; Yuan and Kupfer, 1995) and animals (Bowles and McManus, 1993; Morgan and Blair, 1995), including insects (Wesson et al., 1992; Campbell et al., 1993; Kuperus and Chapco, 1994; Hoy, 1994). Our objectives of this study is to determine whether the use of ITS-1 sequences can be used as a method to infer *Orius* phylogenetic relationships.

MATERIALS AND METHODS

DNA extraction and PCR. A number of *Orius* species and an outgroup species (*Wollastoniella rotunda* Yasunaga et Miyamoto) were obtained from various sources as either live material or preserved material in 100% ETOH (Table 1). Voucher specimens are housed at The Institute of Biological Control, Faculty of Agriculture, Kyushu University, Fukuoka, Japan. DNA was extracted from a single male adult which was crushed with a sterile glass rod in 100 µl of a 5%

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Chelex (Sigma Corp)/sterile water solution with 3 μ l proteinase K (20 mg/ml). The mixture was incubated for at least 4 h at 56°C followed by a 10 min heating period at 95°C. PCR was performed in 50 μ l reaction volumes [10 units Taq polymerase (Perkin Elmer), 1 μ l dNTP's (each in a 10 mM concentration), 3 μ l forward and reverse primer (10 ng), 5 μ l 10 $\times$ reaction buffer, 8 μ l MgCl₂ (100 mM), 25.7 μ l sterile water, and 5 μ l DNA template]. Temperature cycling was carried out in a Perkin-Elmer thermal cycler (Perkin-Elmer Gene Amp PCR System 9600) as follows: 3 min at 94°C, 33 cycles for 40 s at 92°C, 40 s at 51°C, and 40 s at 72°C followed by 3 min at 75°C. One pair of primer sequences was used to amplify ITS-1 regions as follows: forward 5'ACACCGCCCgTCgCTACTACCGA-TT3' and reverse 5'TgCgTTCgACTggTCgAT-gTTCgT3'. These primers were developed from pea aphid (*Acyrtosiphon pisum* [Harris]) conserved 18S and 5.8S rDNA regions, respectively (Kwon and Ishikawa, 1992). Successful amplification was determined by electrophoresing 10 μ l of the PCR reaction on a 1% agarose gel (1 $\times$ TBE) and visualizing with ethidium bromide.

Cloning, plasmid purification/recovery, and DNA sequencing. Cloning of ITS-1 PCR products was performed with the use of a prepared cloning kit (TOPO TA Cloning Kit, Invitrogen) following company specifications. DNA purification and recovery were obtained through the use of a prepared miniprep kit (QIAprep Spin Miniprep Kit, Qiagen) using company specifications. Sequencing was performed using an automatic sequencer (373 DNA sequencer Stretch, Applied Biosystems), and a Prism Ready Reaction DyeDeoxy Terminator Cycle Sequence Kit. DNA sequences have been deposited in Gen-Bank with accession numbers given in Table 1.

Phylogenetic analysis. Partial 18S, ITS-1, and partial 5.8S sequences were aligned using Clustal W (Thompson et al., 1994). Phylogenetic analysis and trees were constructed using only informative characters within the sequence alignments and branch and bound parsimony methods as implemented in PAUP 3.1 with *W. rotunda* identified as an outgroup (Swofford, 1993). Equally parsimonious tree sets were

summarized using strict consensus and 50% majority rule consensus for all character matrices. Bootstrap analyses using 1,000 replicates were used to gain confidence limits on individual clades.

RESULTS AND DISCUSSION

The ITS-1 region of *Orius* species appears to be extremely variable in length ranging from 230 to 663 bp (Table 1). In many cases such as *O. minutus*, it appears that there are a number of repeated sections within the sequence, while *O. tantillus* shows some areas where sections may have been deleted. Extreme differences between sequences may also be the effect of differential selection. A single most parsimonious tree (length=540, consistency index=0.73, and retention index=0.67) was produced and the strict and majority rule consensus tree with bootstrap values (50% majority rule and 100% bootstrap) identified a completely resolved tree (Fig. 1). Three of the five Japanese *Orius* species (*O. minutus*, *O. strigicollis*, and *O. sauteri*) formed a monophyletic clade which was recognized in 100% of the trees. Within this clade *O. minutus* and *O. strigicollis* appear to be closely allied. The remainder of the *Orius* species examined formed another group. Although the most proximal branches of the tree are highly resolved, relationships become more ambiguous deeper within the tree.

Some of the relationships described in the tree presented are at least in partial agreement with published taxonomical works. Three of five *Heterorius* species appear to group together (*O. minutus*, *O. strigicollis*, and *O. sauteri*). Yasunaga (1997b) states that *O. strigicollis* and *O. minutus* are closely related and are difficult to distinguish from *O. sauteri*. Although much past confusion has arisen over these three species due to morphological similarity, they do divide into three groups and probably represent good species. The fourth Japanese species, *O. nagaii* is readily distinguishable from the former species and according to Yasunaga (1997b), is most closely allied to the European *O. majusculus* which again agrees with our topology. However, these *Heterorius* representatives group separately which may indicate that they are less closely allied to the former

Table 1. Species, ITS-1 sequence lengths, collection localities and Gen Bank Accession numbers

Species	ITS-1 sequence (length in bp)	Location	Gen Bank Accession numbers
<i>O. tantillus</i>	230	Naha, Okinawa (Okinawa)	AFO61373
<i>O. insidiosus</i>	245	Phoenix, Arizona, United States	AFO61187
<i>O. niger</i>	299	Koppert insectary, The Netherlands	AFO61370
<i>O. tristicolor</i>	321	Phoenix, Arizona, United States	AFO61374
<i>O. nagaii</i>	355	Takarazuka, Hyogo (Honshu)	AFO61369
<i>O. sauteri</i>	404	Amagi, Fukuoka (Kyushu)	AFO61371
<i>O. strigicollis</i>	419	Wakayama, Wakayama (Honshu)	AFO61372
<i>O. majusculus</i>	423	Koppert insectary, The Netherlands	AFO61367
<i>O. laevigatus</i>	437	Koppert insectary, The Netherlands	AFO61366
<i>O. minutus</i>	663	Kawanishi, Hyogo (Honshu)	AFO61368
<i>W. rotunda</i>	534	Kamphaengsaen, Thailand	AFO61375

Heterorius group. The fact that they cluster with both *O. insidiosus* and *O. tristicolor* indicates a possible relationship with these two unplaced and closely related species although morphological examination and more samples need to verify this result. The two *Orius* species (*O. laevigatus* and *O. niger*) within the nominate *Orius* subgenus also group together and may have an older association with *Orius* (*Paraorius*) *tantillus*; an unusual species which possesses a number of distinct morphological

and genitalic differences (Yasunaga, 1997c). Again, detailed morphological study will probably define these relationships.

In the past, rDNA sequences have been used to infer phylogenetic relationships due to their versatility. Some regions such 18s evolve slowly and are ideal for examining ancient evolutionary events. Other regions such as ITS-1 and ITS-2 evolve at much quicker rates and may be used to infer relationships among closely related taxa. Another supposed advantage in using rDNA is that it can easily be sequenced as it is a multicopy gene. However, this fact has recently generated controversy as highly evolving regions (i.e. ITS-1 and ITS-2) may demonstrate a high level of variation within individuals. Rich et al. (1997) found that within individuals, ITS-2 nucleotides varied by 4% which was nearly half of what was reported between ticks from separate geographic locations and they have cautioned using this region in inferring closely related tick relationships. On the other hand, processes of concerted evolution often may homogenize ribosomal arrays within lineages and ITS-2 sequences have been used reliably to determine relatedness for certain species of mosquitoes because they appear relatively invariable within individuals (Fritz et al., 1994; Wesson et al., 1992). Within individuals, variation of ITS-1 sequences does not appear to pose a problem for *Orius* phylogenetic studies either as there is very little variation among individuals from distant geographic locations. For example, *O. laevigatus* specimens tested from The Netherlands and France were

Fig. 1. Strict consensus tree of *Orius* species and *W. rotunda* outgroup using a branch and bound search (length=540, consistency index=0.73, and retention index=0.67). Bootstrap values are found on tree branches (1,000 replicates).

sequenced and found to vary in only 2% of their nucleotides. Moreover, *O. sauteri* from Kyushu and Honshu were sequenced and found to vary in only 3% of their nucleotides. Hence, we can conclude that in general, *Orius* ITS-1 sequences are fairly conserved among geographically distant individuals and it follows that there is even less within individual variation as well.

The decision in identifying a particular gene region that is most useful for solving systematic questions is probably the most critical step in any phylogenetic study. For example, highly conserved sequences among taxa are relatively useless as the data will be too invariant. In contrast, regions may be too variable so that the number of homoplasies and equally good possible alignments increase to the point such that a phylogeny will lack robustness. The latter case appears to limit the utility of ITS-1 sequences with respect to *Orius* species phylogeny. Our high retention index (0.67) indicates a high degree of homoplasy and Hillis and Dixon (1991) state that alignments become increasingly ambiguous when sequences differ more than 30%. If this criterion is utilized, we must hesitate in concluding that ITS-1 is a good sequence region to use for *Orius* phylogenetic studies, as some of our sequences differ by over 50% (i.e. *O. minutus* and *O. tantillus*). Such differences made alignments difficult and greatly reduced the number of useful character states for analysis.

This is not to say the results in this study have no merit. We have shown that there is little ITS-1 variation within *Orius* species and there is a high degree of variation among species which indicates that this region may be useful for species identification and we are currently investigating the use ITS-1 RFLP's to discriminate between *Orius* species. In addition, no study exists in utilizing *Orius* morphological characters to define species relationships, and this study represents a first attempt at defining *Orius* species relationships. As such, it is difficult to fully evaluate our results. Although a resolvable tree was obtained and there is at least partial agreement with morphological studies, it will no doubt vary from others as more character states and species are studied. It is advised that more conservative gene regions (i.e.

28s or mitochondrial DNA) be examined and compared with morphological data. Only after a combining such character states (including those reported herein) will we be able to produce a total evidence analysis which may help resolve *Orius* species relationships.

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