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Testing the usage of ITS2 and 28S-D2 markers in identifying some species of *Ammophila, Prionyx,* and *Sphex* genera (Hymenoptera: Sphecidae)

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Abstract - In addition to traditional methods, investigation of rDNA gene regions with molecular techniques is widely used to identify insect taxa. In this study, the usability of the primers (TrITS2, 28S-D2) belonging to second expansion segment (D2) of the 28S subunit (28S-D2) and second internal transcribed spacer (ITS2) rDNA gene regions were tested to identify and differentiate six species of Sphecidae family namely *Ammophila sabulosa, Ammophila heydeni, Prionyx kirbyi, Prionyx nudatus, Sphex flavipennis, Sphex funerarius* for the first time. DNA isolated from samples were amplified by Polymerase Chain Reaction (PCR) and digested by restriction enzymes. PCR products and restriction enzyme analysis of ITS2 obtained from *Prionyx* species displayed species-specific DNA bands indicating that these species could be identified by using TrITS2 primers and restriction enzymes. However, neither DNA bands nor the sequence analysis of 28S-D2rDNA primers provided species-specific data. Consequently, ITS2 marker was suitable for only one of the genera (*Prionyx*), whereas 28S-D2 marker was suitable for none of the taxa examined.

Key words - *Sphecidae, PCR, ITS2, 28S-D2 rDNA, Gene, Sequence, Systematic*

1. Introduction

Sphecidae family (Hymenoptera) includes solitary wasps which are generally distributed in temperate regions all over the world. They are large and conspicuous wasps with bright colors and called as “thread waisted wasps”, “digger wasps”, or “sand wasps”. Adult wasps feeding on nectar of flowering plants but their larvae are carnivorous feeding on other insects and spiders. Female wasps paralyze and carry preys to their nests, therefore contribute to limit their prey’s populations in nature [1].

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As in other insect groups, systematic problems frequently arise in identification and differentiation of Sphecid wasps which based only on morphology. Morphological characters which are used in identification keys such as setation, color of body parts, number and shape of placoids, structure of clypeus etc. may be unclear for some specimens or vary intraspecifically, so they are not wholly reliable [2]. New and reliable methods will provide substantial support to scientists in overcoming this kind of problems. Thus, accuracy of diagnosis based on morphology can be confirmed through some other methods.

DNA based molecular techniques are commonly used in taxonomic and phylogenetic studies as well as they were used to overcome systematic problems recently [3]. Various genes or gene regions are selected as target to differentiate species, to reveal geographical variations among populations, and to determine phylogenetic relationships. One of the most important target regions used in molecular analysis is ribosomal DNA which is relatively less changed. rDNA is composed of 18S, 5.8S, 28S gene regions and internal spaces (ITS1 and ITS2). rDNA genes or nucleotide regions are proved to be useful in taxonomic studies for various taxa [4 - 11**]**. 28S rDNA coding rRNA is accepted to be a highly conserved gene region during evolutionary processes, so it is used to construct phylogenetic trees especially for higher categories. Ribosomal ITS2 is non-coding region located between 5.8S and 28S ribosomal DNA regions. Because this region is thought to change rapidly, it has been used commonly in population genetics, separation of cryptic species, and similar species [12, 13, 14]. Most of these markers have been used in Hymenoptera order, especially in parasitoid wasps.

The aim of this study was to test the usability of the primers belonging to ITS2 and 28S-D2 gene regions in identification and differentiation of six species of Sphecidae family (Hymenoptera).

2. Materials and methods

2.1. Insect samples

Samples were collected from their natural habitats with insect nets in Turkey (Table 1). They were freshly stored at -80°C until used for molecular analysis. Identification of species was carried out in Gaziosmanpasa University, Entomology Research Laboratory, Tokat.

Table 1. Number and Collection Places of Examined Specimens

Wasp species	Collection Place (Number of specimens)		
	Tokat	**Amasya**	**Sivas**
Ammophila heydeni	Almus (2), Pınarlı (2), Erbaa (2)	–	–
Ammophila sabulosa	Almus (2), Pınarlı (2), Erbaa(2)	–	–
Prionyx kirbyi	Almus (1), Pınarlı (1), Erbaa (2)	–	–
Prionyx nudatus	Almus (1), Pınarlı (2), Erbaa (1)	–	–
Sphex flavipennis	Tasliciftlik (2), Almus (2), Pınarlı (2), Erbaa (2)	Yesilyenice (1)	Zara (1)
Sphex funerarius	Almus (2), Pınarlı (3), Erbaa (2), Kurtulus (2)	–	Zara (1)

2.2. Extraction of genomic DNA

Total genomic DNA was extracted from thorax of individual wasps with DNeasy Tissue Kit (QIAGEN), in accordance with manufacturer's instructions. The total dilution volume was 100 µl.

2.3. Enzymatic amplification of the ITS-2 and 28S-D2 rDNA

Primers belonging to internal transcribed Spacer-2 (ITS2) and 28S rDNA gene regions, the most widely used primers in Hymenoptera, were preferred for polymerase chain reaction (PCR). The ITS2 genes were amplified using the primers TrITS2F (5'- TGT GAA CTG CAG GAC ACA TG – 3') and TrITS2R (5'- GTC TTG CCT GCT CTG AG – 3') [15]; the 28S-D2 expansion regions were amplified using 28S-D2F (5'- CGT GTT GCT TGA TAG TGC AGC – 3') and 28S-D2R (5'- TTG GTC CGT GTT TCA AGA CGG G-3') primers [16]. Dream Taq Master Mix kit (Thermo Scientific) was used for amplifying DNA samples. The PCR amplification was performed in 25 µl volumes, including 2 µl of each 10 µM primer, 2 µl DNA templates, 13 µl Dream Taq master mix enzyme and sterile distilled water up to 25 µl. The PCR cycles were as follows: 5 min at 94°C; followed by 35 cycles of 1 min at 94°C, 1 min at 55°C, 1.5 min at 72°C; followed by a final extension of 30 min at 72°C.

2.4. Restriction enzyme analysis

PCR products were digested with fast digest restriction enzymes (*Mse*I, *Pvu*I, *Nru*I, *Hind*III) (Thermo Scientific). Each digestion was done in a total volume of 30 µl that consisted of 2 µl PCR product, 2 µl Fast Digest enzyme, 10 µl buffers with loading dye and 16 µl grade water. The reaction was kept at 37° C in dry block heater for 5 minutes. The digested products (10 µl from each) were electrophoresed in 1% (*w/v*) agarose gel and visualized with ethidium bromide staining.

2.5. Sequencing of the 28S-D2 expansion region

Following electrophoresis, the 28S-D2 PCR products of *Sphex* species were purified using the QIA quick purification kit (Qiagen, Valencia, CA). These PCR products were then sent to an automatic sequencer for direct sequencing (Gene Research and Biotechnology, REFGEN).

2.6. Sequence alignment

Sequences were aligned with ClustalW Multiple Alignment in Bioedit v7.0.9 program. The alignments were checked by eyes.

3. Results and Discussion

3.1. PCR amplification

ITS2 fragments were able to be amplified from two *Prionyx* species but not for *Sphex* and *Ammophila* species. The size of ITS2 region of *Prionyx kirbii* and *Prionyx nudatus* was found ~1100 bp and ~1250 bp respectively (Figure 1; Line Uncut).

28S-D2 region was amplified from *Sphex, Prionyx* and *Ammophila* species. The PCR product size of the 28S-D2 region was similar in all of the examined specimens belonging to the species: *Sphex flavipennis - Sphex funerarius, Ammophila heydeni - Ammophila sabulosa,* and *Prionyx kirbii - Prionyx nudatus* (Not shown all. Figure 2; Line Uncut for *Ammophila* species).

3.2. Restriction Analysis and Sequencing

PCR products of ITS2 region from *Prionyx* species and 28S-D2 region from *Ammophila* species were digested by restriction enzymes in which species-specific banding pattern was obtained only for the former genus (Figure 1). NruI cut ITS2 product of *Prionyx kirbii* into three visible bands but it didn't cut the other *Prionyx* species (*P. nudatus*). The other enzyme, Hind III, produced two visible bands for only *Prionyx nudatus* (Figure 1).

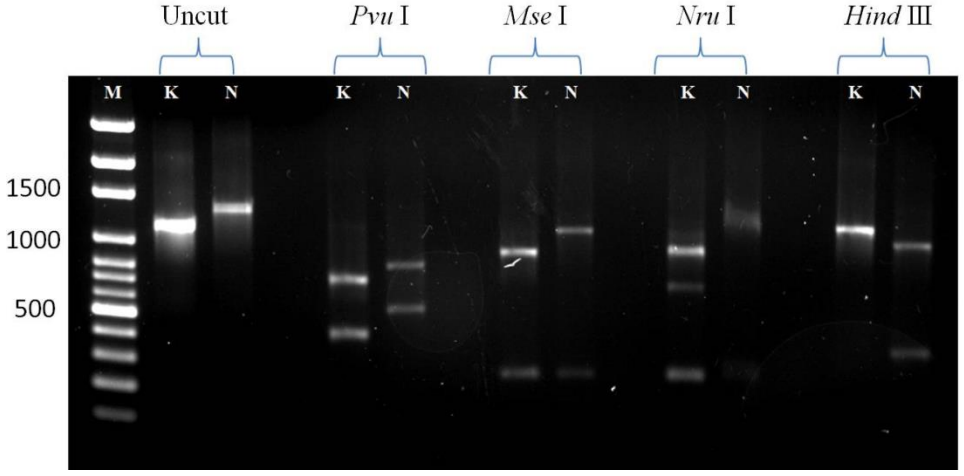

Figure 1. RFLP analysis of ITS2 PCR products, *Prionyx kirbii* (K) and *Prionyx nudatus* (N). *Pvu*I, *Mse*I, *Nru*I, *Hind*III: restriction enzymes, M: DNA size marker.

None of the restriction enzymes were able to digest 28S-D2 PCR product of *Ammophila* species except for *Nru*I, which produced two visible bands, but they are similar in both of the species (Figure 2).

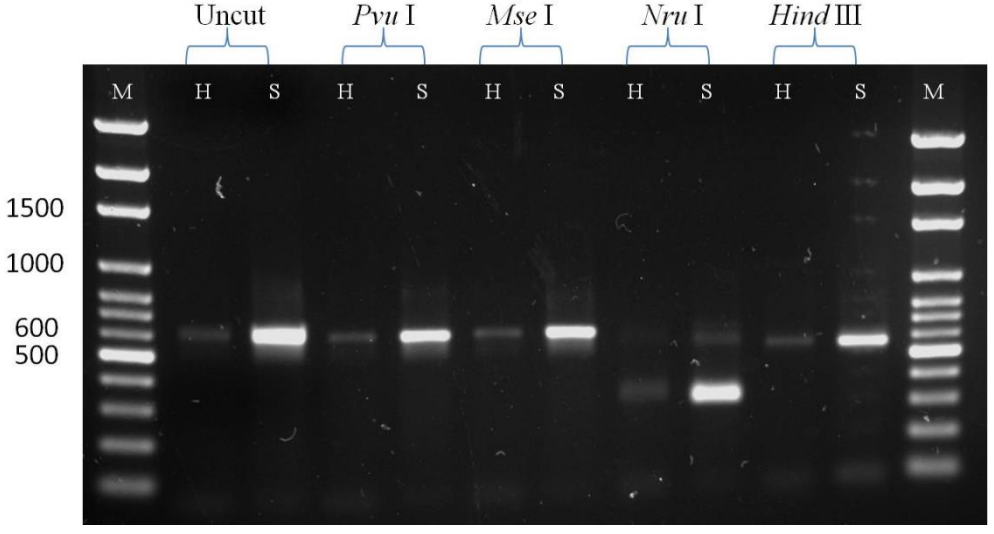

Figure 2. RFLP analysis of 28S-D2 PCR products, *Ammophila heydeni* (H) and *Ammophila sabulosa* (S). *Pvu*I, *Mse*I, *Nru*I, *Hind*III: restriction enzymes, M: DNA size marker.

The sequence length of 28S-D2 region was 630 bp for both species of *Sphex* genus, in which only four sequences were variable (Table 1). These sequences were deposited in GenBank with the accession numbers KJ680326 and KJ680327.

Table 1. Sequences of D2 expansion region of 28S rDNA, *Sphex funerarius* (fune.) and *Sphex flavipennis* (flav.). Dots (.) indicate identical sequences of *S. funerarius* and *S. flavipennis*. Dashes (-) indicate insertions/deletions. Numbers indicate position in the aligned sequence.

```
fune.  1    ..............................................C..A...........  60
            |||||||||||||||||||||||||||||||||||||||||||||| || ||||||||||||
flav.  1    ..............................................T..T...........  60
fune.  61   ..............................................................  120
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
flav.  61   ..............................................................  120
fune.  121  ..............................................................  180
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
flav.  121  ..............................................................  180
fune.  181  ..............................................................  240
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
flav.  181  ..............................................................  240
fune.  241  ........................................C.....................  300
            |||||||||||||||||||||||||||||||||||||||| ||||||||||||||||||||
flav.  241  ........................................T.....................  300
fune.  301  ..............................................................  360
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
flav.  301  ..............................................................  360
fune.  361  ..............................................................  420
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
flav.  361  ..............................................................  420
fune.  421  ..............................................................  480
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
flav.  421  ..............................................................  480
fune.  481  ..............................................................  540
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
flav.  481  ..............................................................  540
fune.  541  ..............................................................  600
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
flav.  541  ..............................................................  600
fune.  601  ...................A..........  630
            |||||||||||||||||| ||||||||||
flav.  601  ...................-..........  630
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PCR product size and restriction enzyme analysis of ITS2 were found to be different for two species of *Prionyx* (Figure 1). This gene region together with restriction enzyme analysis is thought to be a useful molecular tool in distinguishing species, since it is also compatible with morphological identification. As in other molecular techniques, this method can easily be applicable in many laboratories and provides precise and repeatable results. On the other hand, the same region has not been amplified for other species examined because that the primer is unconvenient for those taxa.

Sizes of PCR products belonging to 28S-D2 region were found to be the same for all species in this study, so this parameter is not a good choice to distinguish them. Additionally restriction enzyme analysis was carried out for *Ammophila* species and 28S-D2 PCR product was sequenced for *Sphex* species but species-specific band or any significant difference were not found (there was only 1% sequence divergence) (Figure 2 and Table1). The sequences obtained in this work are available in Genbank database (Accession numbers: KJ680326, KJ680327). It can be concluded that 28S-D2 region is not suitable to distinguish between *Sphex* species, because both of the data obtained from *Sphex funerarius* and *S. flavipennis* showed 99 % homology with another species (*Sphex ashmeadi*). On the other hand, the region is shown to be applicable in family and higher levels in Hymenoptera order [16 - 18].

4. Conclusions

Molecular markers could be used for identification and/or discrimination of taxa, however choosing a good method for a particular group is still very important for success. For example, in this study, ITS2 marker was suitable for one of the genera (*Prionyx*) but not for the others, and 28S-D2 marker is not suitable for any taxa examined. Mitochondrial DNA regions or other primers to be designed may be successful for some undistinguished taxa (*Ammophila* and *Sphex* species).

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References

[1] R.M. Bohart, and A.S. Menke, Sphecid Wasps of the World. A generic revision. University of California Press, Berkeley, Los Angeles, London. pp. IX+695, 1976.
[2] A. S. Menke, W.J. Pulawski, A Rewiev of the *Sphex flavipennis* Species Group (Hymenoptera: Apoidea: Sphecidae: Sphecini). J Hym Res 9 (2000), 324-346.
[3] V. Lohrmann, M. Ohl, C. Bleidorn, L. Podsialowski, Phylogenie der ‘‘Sphecidae’’ (Hymenoptera: Apoidea) basierend auf molekularen Daten. M D Gesell Allg Ange 16 (2008), 99–102.
[4] M. Dowton, A. D. Austin, Phylogenetic Relationships among the Microgastroid Wasps (Hymenoptera: Braconidae): Combined Analysis of 16S and 28S rDNA Genes and Morphological Data. Mol Phylogenet Evol 10 (1998), 354-366.
[5] J. Schmitz, R. F. A. Moritz, Molecular Phylogeny of Vespidae (Hymenoptera) and the Evolution of Sociality in Wasps. Mol Phylogenet Evol 9 (1998), 183-191.
[6] C. S. Babcock, J. M. Heraty, Molecular Markers Distinguishing *Encarsia formosa* and *Encarsia luteola* (Hymenoptera: Aphelinidae). Ann Entomol Soc Am 93 (2000), 738-744.
[7] Z. X. Li, Molecular Differentiation of the Four Most Commonly Occurring Trichogramma (Hymenoptera: Trichogrammatidae) species in China. Eur J Entomol 104 (2007), 363-367.
[8] H. K. Oh, H. J. Yoon, M. J. Kim, H. U. Jeong, S. R. Kim, J. S. Hwang, C. H. Bae, I. Kim, ITS2 Ribosomal DNA Sequence Variation of the Bumblebee, *Bombus ardens* (Hymenoptera: Apidae). Genes and Genomics 31(2009), 293-303.
[9] O. Doganlar, M. Doganlar, A. Frary, Preliminary phlogeny of the trips parasitoids of Turkey based on some morphological scales and 28S D2 rDNA with description of a new species. Turk J Zool 34 (2010), 279-289.
[10] F. S. Ercan, S. Oztemiz, A. S. Tuncbilek, R. Stouthamer, Sequence Analysis of The Ribosomal DNA ITS2 Region in Two *Trichogramma* Species (Hymenoptera: Trichogrammatidae). Arch Biol Sci Belgrade, 63 (2011), 949-954.
[11] S. M. Sayed, A. M. El-Shehawi, S. A. Al-Otaibi, Molecular and Biological Characterization of *Trichogramma turkestenica* (Hymenoptera: Trichogrammatidae) which Inhabits Taif Governorate at the West of Saudi Arabia, Afr J Biotechnol 10 (2011), 9467-9472.
[12] F. H. Collins, S. Paskewitz, A review of the use of ribosomal DNA (rDNA) to differentiate among cryptic *Anopheles* species. Insect Mol Biol 5 (1996), 1-9.

[13] B. R. Miller, M. B. Crabtree, H. Savage, Phylogeny of fourteen *Culex* mosquito species including the *Culex pipines* complex, inferred from the internal transcribed spacer of ribosomal DNA. Insect Mol Biol 5 (1996), 93-107.
[14] G. Wörheide, J. N. Hooper, M. B. Degnan, Phylogeography of western pacific Leucetta 'chagosensis' (Porifera: Calcarea) from ribosomal DNA sequences: implications for populations history and conservation of the Great Barrier Reef World Heritage Area (Australia). Mol Ecol 11(2002), 1753-1768.
[15] A. I. Ciociola, R. A. Zucchi, R. Stouthamer, Molecular Key to Seven Brazilian Species of *Trichogramma* (Hymenoptera: Trichogrammatidae) Using Sequences of the ITS2 Region and Restriction Analysis. Neotrop Entomol 30 (2001): 259-262.
[16] B. C. Campbell, J. M. Heraty, J. Y. Rasplus, K. Chan, J. D. Steffen-Campbell, C. S. Babcock, Molecular systematics of the Chalcidoidea using 28S-D2 rDNA. In: Hymenoptera, evolution, biodiversity and biological control (ed. Austin AD and Dowton M), CSIRO Publishing, Melbourne, pp. 59-73 (2000).
[17] D. L. J. Quicke, C. Lopez-Vaamonde, R. Belshaw, The basal Ichneumonidae (Insecta, Hymenoptera): 28S D2 rDNA considerations of the Brachycyrtinae, Labeninae, Paxylommatinae and Xoridinae. Zool Scr 28 (1999), 203-210.
[18] M. Dowton, A. D. Austin, Simultaneous analysis of 16S, 28S, COI and morphology in the Hymenoptera: Apocrita: evolutionary transitions among parasitic wasps. Biol J Linn Soc 74 (2001), 87-111.