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# Assessment of Diversity Indices and DNA Barcoding of Parasitic Fauna Associated with Groundnut Leafminer (GLM), *Aproaerema modicella* Deventer (Lepidoptera: Gelechiidae)

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## ABSTRACT

**Background:** Parasitoid wasps have received a great share of heed in the biological control of pests. However, they are often difficult to identify because of their small body size and profound diversity. In this view, biodiversity indices of parasitic fauna was reckoned and proper identification of those biocontrol agents were done through morphological and molecular technique such as DNA barcoding.

**Methods:** Biodiversity indices of parasitoid of groundnut leafminer (GLM), *Aproaerema modicella* Deventer were reckoned for three agroclimatic zones *viz.,* Irrigated Eastern zone (IE), Rainfed zone (R) and Irrigated Western zone (IW) of Tamil Nadu. Biodiversity indices *viz.,* relative density, Simpson's Index, Shannon-Wiener Index, Pielou's Evenness Index, Margalef Index and Beta diversity were assessed for the parasitic fauna associated with GLM. Apart from this, morphological characterization of prominent parasitoids were done with the help of available literatures and taxonomic experts. The molecular characterization of parasitoids were done using *mitochondrial Cytochrome Oxidase I* (mtCOI).

**Result:** The relative density of parasitoid families and species indicated the dominance of braconidae and *Chelonus blackburni* Cameron. Among the agroclimatic zones, rainfed zone had higher species diversity, richness and evenness. In addition, the morphological and molecular confirmation revealed that the parasitoids associated with *A. modicella* as *Temelucha* spp., *Stenomesius japonicus*, *Bracon hebetor*, *Sympiesis dolichogaster*, *Chelonus blackburni* and *Avga choaspes*. The amplified sequences were submitted to NCBI database and accession numbers were obtained.

**Key words:** *Aproaerema modicella*, Braconidae, *Chelonus blackburni,* DNA barcoding, *mitochondrial Cytochrome Oxidase I* (*mt COI*), Parasitic fauna.

## INTRODUCTION

Groundnut is being ravaged by several insects, of which, Groundnut Leafminer (GLM), *Aproaerema modicella* Deventer is a serious pest of groundnut and soybean in South and South-East Asia (Wightman *et al.,* 1990). The larvae make blister like mines on the dorsal side of the leaf near mid-rib and later, the entire leaf becomes brown, rolled and dried up. In severe infestation, the crop gives burnt up appearance (Ranga Rao and Rameshwar Rao, 2013). The leafminer damage reduces the photosynthetically active leaf area and thereby causes yield losses ranging from 50 to 100 per cent (Namara *et al.,* 2019).

Owing to the concealed nature of the pest, biological control based on entomophages may be an environmentally and economically sound tool for the management of *A. modicella* than the synthetic insecticides. Parasitoids diversity in agro-ecosystems plays a significant role in pest management (Jervis *et al*., 2007). Hence, understanding the distribution and diversity of parasitic fauna may pave way for deploying them in sustainable pest management programme. Among the crops surveyed for the leafminers and its associated parasitic fauna, the GLM was found to harbour diverse parasitoid complex. In view of the above facts, the biodiversity indices were worked out for the parasitoids of GLM.

On the other hand, correct identification of Hymenoptera parasitoids is crucial for assessing their potential in biological control projects (Bigler *et al.,* 2005; Gariepy *et al.,* 2008). Misidentification of parasitoids used in biocontrol may result in serious economic losses (Caltagirone, 1981). Due to their small body size (usually 0.5-2.0 mm), high-quality slide and card-mounted specimens are needed in conventional

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taxonomy. Also, the morphological studies have been the keystone of insect pest identification in the past and continued to be in the present, it offers complementary, faster and more precise options for species identification (Scheffer, 2000). Apart from this, identification system based on DNA barcoding facilitate the identification of known as well as discovery of new species (Hebert *et al.,* 2003). Keeping this in mind, the morphological and molecular identification was done for prominent parasitoids associated with GLM.

## MATERIALS AND METHODS

### Site of collection

The survey was carried out in *A. modicella* infested groundnut fields during 2016-19 in three different agro-climatic zones of Tamil Nadu *viz.,* Irrigated Eastern zone (IE), Rainfed zone (R) and Irrigated Western zone (IW) for working out the diversity indices. For Irrigated Eastern zone (IE), the districts *viz.,* Villupuram, Thiruvannamalai, Cuddalore, Thiruvallur, for Rainfed zone (R), Sivagangai district and for Irrigated Western zone (IW), Coimbatore district were selected as the representatives for assessing the diversity.

### Method of collection

*A. modicella* infested groundnut leaves were collected from 25 plants selected at random from each location and infested leaves with live and parasitized larvae were brought to the laboratory and observed for the emergence of parasitoids (Muthiah and Kareem, 2000). The parasitoids collected were preserved in 70% ethyl alcohol. The dried specimens were mounted on pointed triangular cards and studied under a Stemi (Zeiss) 2000-C and photographed under stereo zoom microscope (Leica M 205-A).

### Measurement of diversity

### Relative density

Relative density of the parasitoid families and parasitoid species were calculated separately by using the following formulae:

$$\text{Relative density of parasitoid family (\%)} = \frac{\text{Number of individuals of each parasitoids family}}{\text{Number of individuals of total parasitoids families}} \times 100$$

$$\text{Relative density of parasitoid species (\%)} = \frac{\text{Number of individuals of one parasitoids species}}{\text{Number of individuals of all parasitoids species}} \times 100$$

### Simpson's Index

Simpson's diversity index is a measure of diversity which takes into account, the number of species present, as well as the relative abundance of each species. It is calculated using the formula given by Simpson (1949):

$$D = \frac{\sum n\,(n\text{-}1)}{N\,(n\text{-}1)}$$

Where,
n= Total number of individuals of a particular parasitoid species.
N = Total number of individuals of all parasitoid species.

Subtracting the value of Simpson's diversity index from 1, gives Simpson's index of diversity (SID).

### Shannon-Wiener index

Shannon-Wiener index (H') is an another index calculated by the following formula given by Shannon and Weaver (1949):

$$H' = -\sum Pi \ln (Pi)$$

Where,
Pi = S /N.
S = Number of individuals of one parasitoid species.
N = Total number of all individuals of parasitoids in the sample.
ln = Logarithm to base e.
The higher the value of H', the higher the diversity.

### Pielou's evenness index

Species evenness is calculated by using the Pielou's evenness index (E1) formula given by Pielou (1966):

$$\text{Pielou's evenness index (E1)} = H'/ \ln (S)$$

Where,
H' = Shannon-Wiener diversity index.
S = Total number of parasitoid species in the sample.

As species diversity increases, evenness also increases (Magurran, 1988).

### Margalef index

Species richness was calculated for the three zones using the Margalef index formula given by Margalef (1958) as follows:

$$\alpha = \frac{(S - 1)}{\ln (N)}$$

Where,
S = Total number of parasitoid species.
N = Total number of individuals of parasitoids in the sample.

### Beta diversity

Beta diversity is a measure of how different (or similar) ranges of habitats are, in terms of the variety of species found in them. The most widely used index for assessment of beta diversity is Jaccard index (JI) (Jaccard, 1912).
JI is calculated using the formula:

$$JI \text{ (for two sites)} = \frac{j}{(a + b - j)}$$

Where,
j = The number of parasitoid species common to both sites A and B.
a = The number of parasitoid species in site A.
b = The number of parasitoid species in site B.

### Statistical analysis for diversity indices

The data pertaining to the diversity indices were analysed using online biodiversity indices calculator software (Alyoung studios) and using Minitab software version 17.

### Morphological confirmation of adult leafminers and their parasitoids

The morphological confirmation of parasitoids associated with groundnut leafminer were carried out up to the family level with the help of available literatures (Shanower *et al*.,

1992; Noyes, 2017). In addition, Dr. Santhosh Shreevihar from the Malabar Christian College, Kozhikode, Kerala helped in identifying Bethylidae and Eulophidae. Dr. Sudheer Kalathil from Guruvayurappan College, Kozhikode, Kerala, helped in identifying Ichneumonidae specimen.

**Molecular confirmation of parasitoids associated with groundnut leafminer**

The molecular confirmation of parasitoids associated with GLM were carried out only for the prominent parasitoids found in the respective locations (Murugasridevi *et al.,* 2019).

**Genomic DNA extraction**

Genomic DNA was isolated from single adult parasitoids listed below by following the CTAB (Cetyl Trimethyl Ammonium Bromide) method (Doyle and Doyle, 1987).

| Location | Geographical Co-ordinates (Latitude, Longitude, Altitude) | Parasitoids |
|---|---|---|
| **Irrigated Eastern (IE)** | | |
| Villupuram | 12.147°N 79.102° E, 138 ft | *Bracon hebetor*<br>*Stenomesius japonicus*<br>*Chelonus blackburni* |
| Thiruvannamalai | 12.080° N 79.037° E, 457 ft | *Bracon hebetor*<br>*Stenomesius japonicus*<br>*Chelonus blackburni* |
| Cuddalore | 12.080° N 79.037° E, 471 ft | *Bracon hebetor*<br>*Stenomesius japonicus*<br>*Chelonus blackburnii* |
| Thiruvallur | 13.179° N 79.602° E, 364 ft | *Temelucha* spp.<br>*Sympiesis dolichogaster*<br>*Bracon hebetor*<br>*Stenomesius japonicus*<br>*Chelonus blackburni* |
| **Rainfed** | | |
| Sivagangai | 9.710° N 78.285° E, 299 ft | *Avga chaopses*<br>*Temelucha* spp.<br>*Bracon hebetor*<br>*Stenomesius japonicus*<br>*Chelonus blackburni* |
| **Irrigated Western (IW)** | | |
| Coimbatore | 11.007° N 76.936° E, 1417 ft | *Stenomesius japonicus*<br>*Chelonus blackburni* |

The DNA extraction buffer contained 100 mM Tris-HCl (pH 8), 10 mM EDTA, 1.4 M NaCl, 2.0 per cent CTAB and 5.0 per cent β-mercaptoethanol. Individual insect sample was homogenized with 200 µl of DNA extraction buffer and incubated at 65°C for 1 h. The tubes were removed from the water bath and allowed to cool at room temperature. Equal volume of Chloroform: Isoamyl alcohol mixture (24:1, v/v) (0.8 volume) was added and mixed by inversion for 10 min to form an emulsion. It was centrifuged at 12,000 rpm for 10 min and the clear aqueous phase was transferred to a new sterile tube. Ice-cold isopropanol (0.7 volume) was added and mixed gently by inversion and was stored at -20°C for overnight. It was then centrifuged at 12,000 rpm for 10 min to pellet the DNA and the supernatant was discarded. The DNA pellet was washed with 70 per cent ethanol. After washing, DNA pellet was air dried and dissolved in 20 to 40 µl of Tris-EDTA buffer depending on the size of the pellet and stored at -20°C until use.

**Quality and quantity check of genomic DNA**

Quality of genomic DNA was checked by 0.8 per cent agarose gel. Agarose at 0.8 g was dissolved in 100 ml of 1X TBE (Tris Borate EDTA) buffer. After cooling, 1 to 2 µl ethidium bromide was added from the stock (10 mg ethidium bromide / ml $H_2O$). Then the mixture was poured into a preset template kept with appropriate comb to make wells. 2 µl DNA added with 2 µl loading dye (6X loading dye) were loaded in each well. Electrophoresis was carried out at 65 V for 1 h. Amplified genomic DNA was visualized on UV transilluminator (Bio-Rad, USA) and documented using Gel documentation system (GELSTAN 1312). The quantification of DNA was done using Nanodrop Spectrophotometer (ND-1000). Based on the nanodrop readings, DNA dilutions were made in TE buffer to make a final concentration of 50 ng $\mu l^{-1}$ and stored at 4°C for further use (Sambrook *et al.,* 1989).

**mtDNA (COI) sequencing and phylogenetic analysis**

A fragment of the mitochondrial gene (*Cytochrome Oxidase 1* (CO1) was amplified across the populations of leafminers and parasitoids using Folmer primers LCOI490 (Forward) and HCO2198 (Reverse) (Hebert *et al.,* 2003).

Forward primer (5′-3′): GGTCAACAAATCATAAAGATATTGG
Reverse primer (3′-5′): TAAACTTCAGGGTAACCAAAAAATCA

Polymerase chain reactions were performed with 25 µl volumes in PCR machine (Sure cycler 8800, Agilent Technologies). The composition of cocktail mixture (for 25 µl reaction mix) is narrated below:

| Contents | Volume | Stock concentration |
|---|---|---|
| Nuclease free water | 16.5 µl | As required to make up to 25 µl |
| Reaction buffer 10X | 2.5 µl | 1X |
| $MgCl_2$ | 0.5 µl | 25 mM |
| dNTPs | 1.0 µl | 250 µM of each dNTP |
| Forward primer | 1.0 µl | 10 µM |
| Reverse primer | 1.0 µl | 10 µM |
| Taq polymerase | 0.5 µl | 1.5 unit |
| Template DNA | 2.0 µl | 50 ng/µl |

The following programme (thermo cyclic conditions) was used for COI amplification:

| Activity | Temperature | Time | No. of cycles |
|---|---|---|---|
| Initial denaturing | 95°C | 3.00 min | |
| Denaturing | 95°C | 0.20 sec | |
| Annealing | 52°C | 0.30 sec | 35 cycles |
| Extension | 72°C | 0.40 sec | |
| Final extension | 72°C | 10.00 min | |
| Storage | 4°C | Storage | |

Amplified products of COI gene were screened using agarose gel electrophoresis (1.5%), 5 µl of PCR product along with 2 µl of loading dye loaded on the agarose gel, electrophoresed at 65 V for 1 hour. The products were then visualized on UV transilluminator and the gel was documented using gel documentation system (GELSTAN, 1312).

PCR products (20 µl) and their respective forward and reverse primers (10 µl each per sample) were labelled appropriately and sent to Agrigenome Labs Pvt. Ltd., Cochin, Kerala for sequencing as per the following procedure. The PCR products of leafminers and parasitoids were sequenced by double pass method in both forward and reverse direction. The PCR products were purified using Pure Link PCR purification Kit and the sequencing PCR were set up by using Big Dye Terminator V3.1 Cycle Sequencing Kit. The resulting sequencing information were retrieved from the client database of Agrigenomelabs online portal. Then the sequences were aligned, edited and trimmed using the programme Geneious and outgroups obtained from GenBank using the *blastn* algorithm to search for nucleotide (nr/nt) data base.

### Similarity analysis

The nucleotide sequences were compared to identify the similarity between each host by Basic Local Alignment Search Tool (BLAST) and Barcode of Life Database. The gene sequences were aligned using the ClustalW algorithm (Thompson *et al.,* 1994). The phylogenetic tree was constructed using MEGA version 6.06 and the tree was drawn using neighbourhood joining method.

## RESULTS AND DISCUSSION

The studies on the biodiversity in agroecosystems aims to understand the relationship between plants, insects and natural enemies (Ananthakrishnan, 2004). The diversity of parasitoid faunal complex varies from place to place with the variation in the agroclimatic conditions of the locality. Hence, an understanding of the species richness, diversity and interaction of parasitoids in an ecosystem are necessary for implementing effective insect pest management strategies. With this view, the biodiversity indices of the parasitoids of GLM were reckoned.

A total of 2,829 of parasitic hymenopterans associated with GLM were obtained through the survey conducted in three agroclimatic zones *viz.,* Irrigated Eastern zone (IE) (Villupuram, Thiruvannamalai, Cuddalore and Thiruvallur), Rainfed zone (R) (Sivagangai district) and Irrigated Western zone (IW) (Coimbatore district) of Tamil Nadu. This constituted 13 species of hymenopteran parasitoids belonging to 8 families under 3 super families *viz.,* Ichneumonoidea (Braconidae and Ichneumonidae), Chalcidoidea (Eulopidae, Eupelmidae, Pteromalidae and Chalcididae) and Chrysidoidea (Bethylidae). Among the 13 species, four species of braconids *viz., Chelonus blackburni* Cameron, *Avga choaspes* Nixon, *Apanteles* spp. and *Bracon hebetor* Say, three eulophids *viz., Stenomesius* spp., *Aprostocetes* spp. and *Sympiesis* spp., one each in Ichneumonid, (*Temelucha* spp.), Eurytomid, (*Eurytoma* spp.), Pteromalid, (*Pteromalus* spp.), Eupelmid, (*Eupelmus* spp.), Bethylid, (*Goniozus* spp.) and Chalcid (*Brachymeria* spp.) were recorded (Fig 1). The parasitoids were mostly larval parasitoids except *C. blackburni*, which is an egg-larval parasitoid.

Among the parasitoid families, braconidae accounted for 40.19 per cent and was the highest in the collection followed by eulophidae (20.96%) and Ichneumonidae (14.95%). These three families together constituted 76.10 per cent of total collection (Fig 2). Besides, the families *viz.,* Eulopidae, Eupelmidae, Pteromalidae, Chalcididae and Bethylidae together contributed only 23.90 per cent of the parasitic fauna.

Among the parasitoids collected, *C. blackburni* was the highest and relatively abundant parasitoid followed by *Stenomesius* spp., *Temelucha* spp. and *A. choaspes* (Fig 3). The parasitoids *viz.*, *Aprostocetes* spp., *Apanteles* spp. and



**Fig 1 a:** *C. blackburni.*

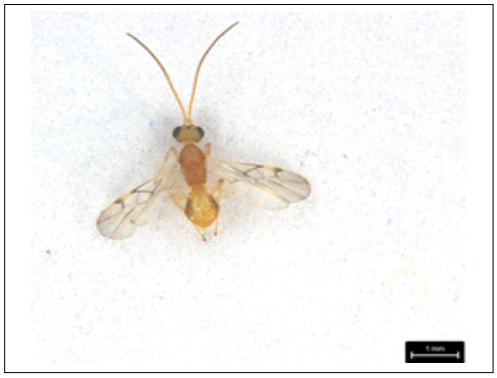

**Fig 1 b:** *A. choaspes*



**Fig 1 f:** *Aprostocetes* spp.



**Fig 1 c:** *Apanteles* spp.



**Fig 1 g:** *Sympiesis* spp.



**Fig 1 d:** *B. hebetor*

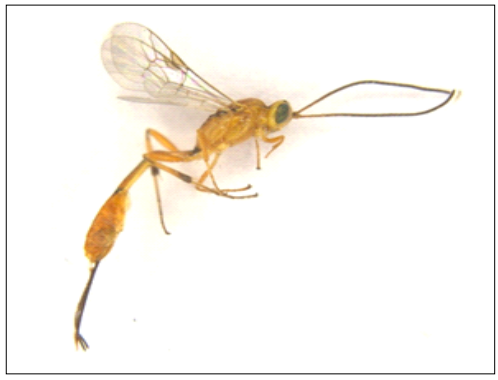

**Fig 1 h:** *Temelucha* spp.



**Fig 1 e:** *Stenomesius spp.*



**Fig 1 i:** *Eurytoma* spp.



**Fig 1 j:** *Pteromalus* spp.



**Fig 1k:** *Eupelmus* spp.



**Fig 1l:** *Gonrozus* spp.



**Fig 1m:** *Brachymeria* spp.

**Fig 1:** Parasitoids of GLM on groundnut.

*Pteromalus* spp. were found to be relatively least abundant in all the zones surveyed.

The studies on relative density of parasitoid families and species indicated the dominance of braconidae family and *C. blackburni.* This dominance may be brought about by the nil (or) no anthropogenic activity in the ecosystem. These results corroborates with Barbieri and Dias (2012) who suggested that braconids are the biological indicators of ecosystem disturbance. In addition, Muthiah and Kareem (2000) recorded maximum activity of egg larval parasitoid, *Chelonus* spp. (26.00%) on GLM at different regions of Tamil Nadu. Similarly, Praveena (2010) also recorded highest parasitism of *Chelonus* spp., on GLM at Bagalkot. In addition, the dominance of braconidae as well as *C. blackburni* may be due to the specific relationship that these parasitic wasps have with their host and the host plants (Pérez-Rodríguez *et al.,* 2013).

Apart from relative density, other indices *viz.,* Simpson index of Diversity (SID), Shannon-Wiener (H'), Pielou's Evenness Index (E1), Margalef Index (a) and Beta diversity (b) were also worked out with data obtained from the surveys conducted during 2016 to 2018 and the results are presented in Table 1.

From the results, it is observed that SID ranged between 0.84 to 0.88. Though the values were found to be much closer in all the three zones, SID was found to be high in Rainfed (R) zone (0.88) followed by Irrigated Eastern (0.87) and Irrigated Western (IW) zone (0.84).

Similar trend was also observed for Shannon and Wiener diversity index (H') and Margalef index (a). It was also observed that the species diversity and species richness were found to be higher in Rainfed (R) zone as indicated by Shannon and Wiener diversity (H') and Margalef indices (a).

Pielou's evenness value (E1) clearly showed that the Rainfed (R) zone had maximum evenness pattern with evenness index value of 0.87 followed by Irrigated Eastern (IE) (0.86) and Irrigated Western (IW) zone (0.55).

Comparison of species similarities using the Beta diversity (b %) index between the zones, taken in pairs showed 92 per cent similarity between the Irrigated Eastern (IE) and Rainfed (R), 61 per cent similarity between the Rainfed (R) and Irrigated Western (IW) and 53 per cent similarity between the Irrigated Western (IW) and Irrigated Eastern (IE) zones.

The results also revealed that the rainfed zone had higher species diversity, richness and evenness. This may be due to minimal or no plant protection measures against groundnut leafminer in rainfed zone, which might have favoured more parasitoid populations *via* increased pest populations. Another possible reason behind the increased parasitoid density in rainfed zone is due to the higher temperature present in these locations which may induce the parasitoids to invest their effort into egg production to produce higher-quality offspring. Additionally, Romo and Tylianakis (2013) also reported increased parasitization of

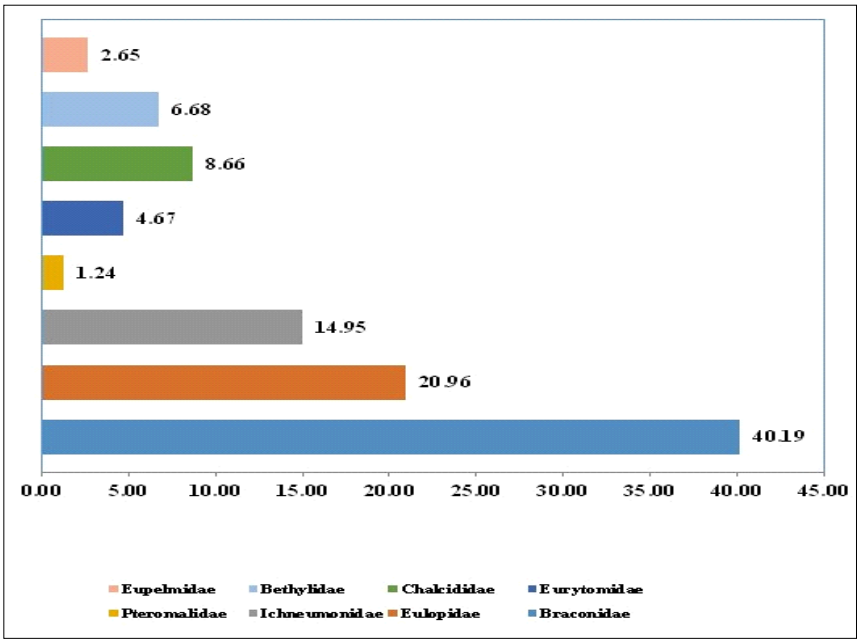


**Fig 2:** Relative density of parasitoid families.

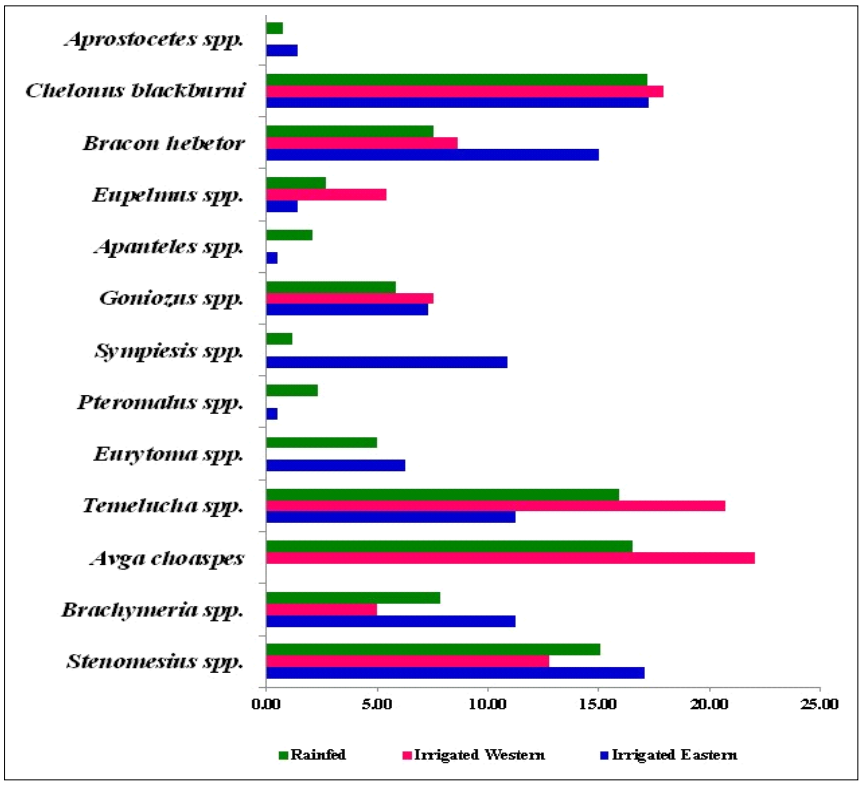


**Fig 3:** Relative density of parasitoid species.

*Diaeretiella rapae* on *Brevicoryne brassicae* at higher temperatures.

Morphological and molecular characterization of prominent parasitoids of *A. modicella* at groundnut growing regions of Tamil Nadu revealed as *Temelucha* spp., *S. japonicus*, *B. hebetor*, *S. dolichogaster*, *C. blackburni* and *A. choaspes*. In addition, the amplified sequences were submitted to NCBI database and accession numbers were obtained (Table 2). The present results are in concurrence with the findings of Shanower *et al.* (1992) and Praveena (2010) who have also reported the above parasitoids on *A. modicella*.

**Table 1:** Biodiversity indices of parasitoid fauna associated with groundnut leafminer (GLM) in groundnut growing regions of Tamil Nadu.

| Location | SID | H' | E1 | a | b (%) |
|---|---|---|---|---|---|
| **Irrigated Eastern (IE)** | | | | | |
| Villupuram | | | | | |
| Cuddalore | 0.87 | 2.15 | 0.86 | 1.57 | 92 (IE-R) |
| Thiruvannamalai | | | | | |
| Thiruvallur | | | | | |
| **Rainfed (R)** | 0.88 | 2.24 | 0.87 | 1.67 | 61 (R-IW) |
| Sivagangai | | | | | |
| **Irrigated Western (IW)** | 0.84 | 1.94 | 0.55 | 0.55 | 53 (IW-IE) |
| Coimbatore | | | | | |

SID- Simpson's Index of Diversity
H'- Shannon-Weiner Index
E1- Pielou's evenness index
a- Margalef index
b - Beta diversity (Jaccard index)

**Table 2:** Accession number for parasitoid fauna associated with groundnut leafminer (GLM) in groundnut growing regions of Tamil Nadu.

| Location | Parasitoids | Accession numbers |
|---|---|---|
| **Irrigated Eastern (IE)** | | |
| Villupuram | *Bracon hebetor* | MN525197 |
| | *Stenomesius japonicus* | MN525203 |
| | *Chelonus blackburni* | MN525200 |
| Thiruvannamalai | *Bracon hebetor* | MN525199 |
| | *Stenomesius japonicus* | MN525190 |
| | *Chelonus blackburni* | MN525201 |
| Cuddalore | *Bracon hebetor* | MN525195 |
| | *Stenomesius japonicus* | MN525204 |
| | *Chelonus blackburni* | MN525192 |
| Thiruvallur | *Temelucha* spp. | MN525187 |
| | *Sympiesis dolichogaster* | MN525188 |
| | *Bracon hebetor* | MN525196 |
| | *Stenomesius japonicus* | MN525189 |
| | *Chelonus blackburni* | MN525193 |
| **Rainfed (R)** | | |
| Sivagangai | *Avga chaopses* | MN525180 |
| | *Temelucha* spp. | MN525186 |
| | *Bracon hebetor* | MN525194 |
| | *Stenomesius japonicus* | MN525184 |
| | *Chelonus blackburni* | MN525191 |
| **Irrigated Western (IW)** | | |
| Coimbatore | *Stenomesius japonicus* | MN525202 |
| | *Chelonus blackburni* | MN969921 |

## CONCLUSION

Holistically, the parasitic fauna associated with GLM was abundant and diverse in rainfed zone. Habitat manipulation strategies using hedgerows and strips of non-crop plants in a spatial and temporal way may benefit parasitoids and their role in natural control of insect pests. Additionally, the present results suggest that combination of traditional taxonomy and molecular methods, enhance the accuracy and reliability of identification. Overall, this study contributes for the reliable identity of parasitoids based on morphological and molecular characterization.

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