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# Genetic Diversity Analysis of Crab Spider (Araneae: Thomisidae) based on RAPD-PCR

KEYWORDS: Crab spiders, RAPD, diversity, Amravati

**Nandkishor Warghat**
Assistant professor, Department of Zoology, Arts and Science College, Pulgaon, Dist- Wardha, Maharashtra.

**Navin Sharma**
Assistant professor, Department of Zoology, Arts, commerce and Science College , Maregaon, Dist- Yavatmal, Maharashtra.

**Punam Thakur**
Research Scholar, Department of Zoology, Govt. Vidarbha Institute of Science and Humanities, Amravati

***Mumtaz Baig**
Associate professor, Department of Zoology, Govt. Vidarbha Institute of Science and Humanities, Amravati-444604. MH *Corresponding Author

**ABSTRACT** *The current study deals with the genetic diversity of crab spiders using molecular markers. Total scorable bands were produced using six random primers for the 16 species of crab spiders belonging to family thomisidae. Out of all screened primers, OPP 9 produced highest 113 scorable bands, out of which 111 bands were found with 98.28 percent polymorphism. Primer OPA 3 was produced lowest 43 bands with 100 percent polymorphism. Remaining primers (OPA 2, OPA 4, OPN 16 and OPN 18) showed 100 per cent polymorphism with 60, 60, 81 and 51 bands respectively. Along with the molecular markers, a morphometric analysis was also focus on the phylogenetic relationship of crab spiders using UPGMA and NJ approach. The present study is the first report from India to describe the genetic relatedness amongst spider using RAPD-PCR.*

**Introduction**

Crab spiders are small to large size, with two claws and have dark eight eyes on tubercles. Eyes are arranged in two rows, posterior row is usually recurved. Lateral eyes elevated on tubercles which may be jointed or not, median usually larger than others. First and second legs are laterigrade and longer than third and fourth (Tikader, 1982). They are called crab spider because they resemble crabs, with two front pair of legs angled outward having angular flattened bodies. Also, like crabs, Thomisidae can move sideways or backward. Most notably, crab spider do not build web to trap prey, but are hunters and ambushers. These spiders' shows well developed mimicry and generally found on the flowers. Hence, it is called as garden spider. (Lise, 2005).

Order Araneae from phylum Arthropoda contains 110 families, 3849 genera and 42473 species. Thomisidae (crab spider) is one of the families of the order Araneae. It contains 177 genera and 2152 species worldwide (Platnick, 2011). In India 1520 species belonging to 377 genera of 60 families is being identified. As per the checklist of Sebastian and peter (2009) family Thomisidae contains 38 genera and 164 species in India.

The aim of the current study is to produce refined phylogeny of thomisid's spider using DNA markers. Briefly, no study has been attempted in India to infer the genetic diversity of Thomisidae family using DNA markers hence present work is the first attempt to decipher the systematics of Thomisidae spiders using RAPD-PCR.

**Materials and Methods**

**Taxon sampling**

We sampled 16 species of spiders for the RAPD analysis, including 13 Thomisid species belonging to six genera and 3 philodromid species of three genera from the Amravati city, Maharashtra state-India. Amravati city is situated at $20^0$55' and 20.93 North latitude $77^0$45' and 77.75 East longitudes at an elevation of 1125 feet. As Philodromidae have been in the past included as a subfamily of Thomisidae, we include three philodromid taxa (as outgroup) in our study. The population of these selected genera and species are abundantly scattered throughout the area of Amravati city, Maharashtra state- India.

**DNA extraction and PCR Amplification**

Total genomic DNA was extracted from the fresh legs of spiders using the Genetix miniprep kit (genetix biotech). 6 random operon primers i.e. OPA1, OPA2, OPA3, OPA4, OPP9, OPN18 (operon technology, USA) were used for PCR amplification. RAPD-PCR was performed in 25µl reaction using 1.5µl of taq DNA polymerase 7.5 units (Axygen, USA), 1.5µl taq buffer 10x (Axygen, USA), 2µl dNTP's 0.4 mM,(Axygen, USA) 3µl Tris HCl buffer (pH 8.0), 3µl primer (10mM, Operon technology) and 1µl (50 to100 ng) diluted genomic DNA mix with 12µl nuclease free water. The PCR condition for all primers were same for 42 cycles, pre-denaturation at $95^0$C for 3min, denaturation: $95^0$C for 1 min, annealing: $36^0$C for 1 min, extension: $72^0$C for 1.30 min followed by final extension of 8 min. RAPD-PCR product was electrophorsed on 2% Agarose gel with 3ml Ethidium bromide and allow to run 2.30 hours. Band pattern were visualised and photographed with gel-doc imaging system (Kodak MI, US) for further analysis.

**Data analysis**

According to the electrophoresis result of RAPD-PCR amplified product, we combined all six primers into single data matrix. According to the band position and mobility on 2% agarose gel, 1, 0 (presence and absence of bands) binary data matrix was prepared in Mesquite 1.12 (Maddison and Maddison, 2007). Nexus file from mesquite was analyzed in PAUP 4.0 (Swofford, 2002). Both UPGMA (Unweighted Pair Group Method with Arithmetic Mean) and neighbor joining clustering methods were adopted as cri-

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teria for reconstructing phylogenetic tree. The RAPD data was processed through branch and bound strategy followed by non-parameric bootstrap with 1000 replicate.

**Character sampling**
We compiled 24 morphological characters scored across 16 taxa (13 thomisids and 3 philodromids). Out of these, 15 were binary and 9 were multistate (Appendix A). All the morphological characters firstly observed, studied, confirmed and then scored in to binary as well as in multistate. In some cases, ambiguous characters sub-state was taken combine as 0&3 (Chart 1).

**Morphology**
All the specimens were observed and studied with stereo zoom DV4 microscope. Morphological characters from male and female genital were excluded in this study to protect further informative complication. Prior to study the morphological characters, preserved specimens were transferred from 70% to 100% ethanol and left overnight. Then specimens were kept in 10% dilute glycerine for 20 minutes.

**Phylogenetic analysis**
Morphological data matrix was prepared in Mesquite 1.12 v. Ambiguous characters were determined with combine state as well as multistate characters were treated as non-additive. Uncorrected distance based UPGMA tree was constructed using Mesquite 1.12 v. Software. Heuristic searches was set to maxtrees 100 and implemented with 500 random additions.

**Result**
A total 408 scorable bands were produced by the 16 crab spider species with 6 random operon primers. The average number of bands per primer was 68. Out of 408 bands 406 bands were polymorphic (99.70%). The average number of polymorphic bands was 67.66 per primer. The primer OPA 2, OPA 3, OPA 4, OPN 16 and OPN 18 produced 100 per cent polymorphism. Only primer OPP 9 (figure 1) produced 98.23 per cent polymorphism (Table.1). Some species were failed to produce any RAPD bands because primers may be failed to recognition complementary sequences of the spider DNA. This data was utilized for further computation.

**RAPD-PCR based phylogenetic analysis of crab spiders**
The phylogenetic analysis was performed to deduce the evolutionary status of crab spider first time from India through the RAPD-PCR study. The present study based on the molecular markers to verify the status of genus and species level phylogenetic position within the family thomisidae. Also, to verify the traditional taxonomical status of genera and species based on the morphological characters of crab spiders. Those characters were selected which have common and found with some variation species to species.

To confirm the family status, three species was taken belonging to family philodromidae. In attempt to improve the resolution of phylogenetic tree, RAPD-PCR data and morphological based data, two methods were adopted for analysis i.e. UPGMA, Neighbor-joining for RAPD-PCR data and UPGMA for mophormetrical data (see appendix A: chart 1)

**Genetic distance**
The genetic distance was used for cluster analysis and phylogenetic tree was constructed for the pooled data. Neighbor-joining and UPGMA trees were produces for the study of 13 species of crab spider and 3 species of spiders from family Philodromidae using RAPD-PCR and morphological characters data.

The RAPD-PCR based distance matrix shows, maximum genetic distance was found (4.753) between the species Thomisus viveki and Tibellus elongatus. The lower genetic distance was found (0.00) between the species Pistius kalimpus and Synaema decorate. The morphological based distance matrix shows, maximum genetic distance was found (0.541) between Thomisus viveki and Tmarus pachpediensis. The lower genetic distance was found (0.166) between Thomisus lobosus and Thomisus andamanensis.

**UPGMA tree**
The tree constructed using UPGMA method show splitting of genera and species in to two major clades designated as I and II. The clade I exclusively includes members from Philodromidae family. The species Philodromus bhagirathai, tibellus elongatus and dieta elongata shows in separate clade I of philodromidae family with high bootstrap value. That's means, the species belongs to philodromidae have separate family status.

The major clade II further divided into four smaller subclade designated from Clade-IIA to Clade-IID. Clade IIA contains close similarity between the species of Tmarus pachpediensis and Pistius kalimpus with 100 percent bootstrap value. As well as, the species Runcinia khandari and Xysticus sp.1. also included in the same clade-IIA with high bootstrap value. Clade-IIB includes species Thomisus sp.1. and Thomisus lobosus. Clade-IIC includes species Thomisus viveki and Thomisus pateli 89% bootstrap value. Clade-IIC contains species of Tmarus kotigeharus which shows link with the Thomisus species1 and Thomisus lobosus. Clade-IID contains Thomisus andamanensis and Thomisus danieli with the close similarity. Hence, clade A, B, C and D exibit monoplyly but the species of genus Syneama decorata and Xisticus minutus exhibit paraphyletic status (figure 2).

**Neighbor-joining tree**
Neighbor-joining Tree mainly divided into two major clade, Philodromidae- clade I and Thomisidae- clade II. The species of genera Dieta elongata, Philodromus Bhagirathai and Tibellus elongatus represent to family Philodromidae. Clade II contains genus and species of family thomisidae in separate monophyletic clade. Hence, it is reconfirmed that the philodromidae had independent family status.

Family thomisidae represented by clade II is further divided into four sub-clade i.e. clade IIA to IID. Clade IIA contains spider species Thomisus andamanensis, Thomisus danieli and Thomisus species1 supports with high bootstrap value. Clade II B contains Synaema decorata and Thomisus lobusus as well as Clade II C contains Thomisus viveki and Thomisus pateli with close similarity. Clade IIA, IIB and IIC along with the genus Thomisus exhibit monophyly. Remaining species i. e. Runcinia khandari, Tmarus kotigeharus, Xisticus minutes and Xisticus species1 exhibit paraphyletic status (Figure 3).

**Morphology based phylogenetic analysis of crab spiders**
**UPGMA tree:**
UPGMA tree was constructed on the species identification methodology by recording morphological characters of crab spiders. The tree contains two major clades, philodromidae clade I and thomisidae clade II. Clade I contain three philodromid species i. e. Philodromus bhagirathai, Ti-

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bellus elongatus and Dieta elongata. Again, this clade re-confirmed the separate family status of Philodromidae.

Clade II, divided into four sub-clades, namely IIA to IID. Sub-clade IIA contains the species Thomisus daneili, Thomisus lobosus and Thomisus andamanensis. Thomisus viveki and Runcinia khandari forms sister cluster (IIB in Figure 4). These cluster shows monophyletic status supported with high bootstrap value. Interestingly, remaining two species Thomisus species1 and Thomisus pateli did not cluster with genus Thomisus as shown in cluster IIA and IIB and thus exhibit paraphyly. Tmarus kotegeharus and Tmarus pachpediensis forms sister cluster (IIC in Figure 4). Clade IID contains Xisticus species1 and Xisticus minutus. Similarly, Pistius kalimpus and Synaema decorata cluster together. These clustering shows monophyly with good bootstrap values for some nodes.

**Discussion**

The existing classification either corrects or not is arguable and it is complicated to reconstruct the true phylogeny of spider (Homann, 1975). The Dionycha are a phylogenetic group of spider including 17 families, some of these are Salticidae (jumping spiders), thomisidae (crab spider) and clubionidae. Spiders in this group have better sense of sight and hearing. Dionycha (two tarsal claws) evolved from the web-building Trionycha (three tarsal claws). The monophyly of Dionycha is evenly fragile (Plantnik, 1989, 2000, 2002). It is an old hypothesis in which many families were divided into a group RTA (Retro-lateral Tibial Apophysis) which bears two clawed spiders. The most diverse families of spider like Salticidae, thomisidae, lycosidae and philodromidae considered in RTA clade are entirely webless (Griswold et al., 1998). On the other hand, family thomisidae and philodromidae are placed in RTA clade which is paraphyletic (Benjamin, et al., 2008). Family philodromidae which was previously considered as subfamily of thomisidae, do not group within and hence excluded from thomisidae (Homan, 1975).

In the current study out of 16 spiders, three species i.e. Philodromus bhagirathai, Tibellus elongatus and Dieta elongata belongs to philodromidae and 13 to thomisidae. The phylogenetic tree reconstruction of these 16 spiders reveals that philodromid's do not group with thomisid's in contrast to the earlier studies which suggest that philodromidae is the subfamily of thomisidae (Simon, 1892). NJ and UPGMA trees based on RAPD data suggested the monophyly of thomisid's. Phylogenetic tree construction by Benjamin, et al., (2008) also arrived at same conclusion. Morphological character based UPGMA tree also reconfirms monophyly of thomisidae and also shows paraphyletic relationship between the members of thomisidae and philodromidae.

**The following morphological differences are proposed to define thomisidae and philodromidae:**

1. Legs I and II longer and stronger than legs III and IV (Ono, 1988; Wunderlich, 2004; Jocque´ and Dippenaar-Schoeman, 2006). While philodromids have leg II slightly longer than leg I (Homann, 1975).
2. Lateral eyes on tubercles, larger and much more developed than the median eyes. In philodromids congruent eye tubercles absent (Ono, 1988).
3. Presence of a group of setae instead of colulus. While philodromids have colulus (Homann, 1975).

RAPD-PCR based phylogenetic analyses by UPGMA, NJ as well as morphology based UPGMA analyses reveal that 13 species of crab spider grouped into a single monophyletic clade designated as clade II, but within this clade the generic relationship in inferred trees is not well established. All trees (RAPD based- UPGMA, NJ and morphology based- UPGMA) branched into four sub-clades viz. IIA, IIB, IIC and IID with unexpected grouping. Genus Thomisus is morphologically homogenous and supposed to be monophyletic (Benjamin, 2011) but in the present study Thomisus species separate out into discrete sub-clade IIA and IIB and formed monophyletic groups. In contrast to this, Thomisus species1 and Thomisus pateli exhibit paraphyly. RAPD based UPGMA trees, placed Thomisus species into sub-clade IIB, IIC, IID, while in NJ tree it grouped into sub clade IIA, IIB, IIC. Morphologically character based phylogenetic relationship of crab spider has proved to be reciprocally monophyletic in UPGMA tree. Clade IIC and clade IID contains those species which are morphologically similar and cluster as per the established systematics i. e. species Tmarus kotigeharus with Tmarus pachpediensis, Xisticus species1 with Xisticus minutus and Pistius kalimpus with closely related Synaema decorata. On the other hand, RAPD based UPGMA tree contains genus Runcinia, Tmarus and Xisticus thereby exhibiting monophyly. Additionally, NJ tree contains genus Runcinia, Tmarus and Xisticus exhibiting paraphyly. This dramatic behavior of several genera of thomisidae is not surprising and was anticipated in the recent study by Benjamin (2008, 2001). In consequence, NJ and UPGMA illustrate more or less similar genetic relatedness. Morphology based UPGMA tree also pointed towards monophyly but some genera exhibit paraphyletic status for unknown reason. This might be due to inadequate taxon sampling or less informative morphological characters such as male genital morphology, relative size of eyes, presence of cheliceral teeth (Coddington, 2005).

Finally, to establish more clear and vivid evolutionary relationship within the thomisidae family; there is a need to carry out extensive sampling of the relevant spider family followed by distinctive morphological studies in conjunction with recent molecular techniques.

**Table 1: Scorable RAPD bands generated by six random primers (operon).**

| Sr. No. | Primers | Total no. of bands produced | No. of polymorphic Bands | Per cent produced polymorphism |
|---|---|---|---|---|
| 1 | OPA 2 | 60 | 60 | 100 |
| 2 | OPA 3 | 43 | 43 | 100 |
| 3 | OPA 4 | 60 | 60 | 100 |
| 4 | OPP 9 | 113 | 111 | 98.23 |
| 5 | OPN 16 | 81 | 81 | 100 |
| 6 | OPN 18 | 51 | 51 | 100 |
| Pooled | | 408 | 406 | 598.23 |
| Average | | 68 | 67.66 | 99.70 |

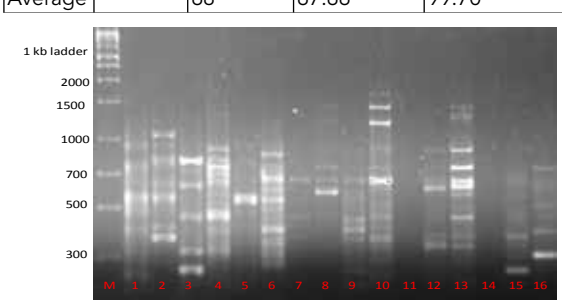


**Figure 1: RAPD-PCR Band patterns of primer OPN 16.**

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M- marker, 1-Thomisus viveki, 2-Thomisus pateli, 3-Tmarus pachpediensis, 4-Pistius calimpus, 5-Thomisus andamanensis, 6-Thomisus danieli, 7-Thomisus species1, 8-Synaema decorata, 9-Dieta elongata, 10-Thomisus lobosus, 11-Runcinia khandari, 12-Xisticus minutus, 13-Philodromus bhagirathai, 14-Tmarus kotigeharus, 15-Xisticus species1 and 16-Tibellu elongatus.

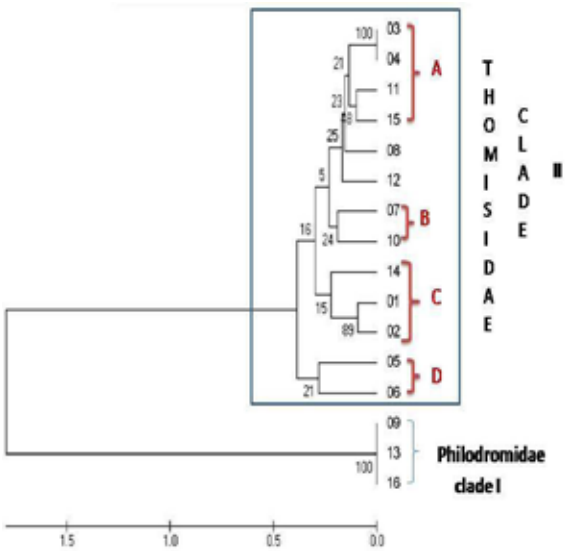


Figure 2: UPGMA tree of crab spider and philodromid spiders. 1-Thomisus viveki, 2-Thomisus pateli, 3-Tmarus pachpediensis, 4-Pistius calimpus, 5-Thomisus andamanensis, 6-Thomisus danieli, 7-Thomisus species1, 8-Synaema decorata, 9-Dieta elongata, 10-Thomisus lobosus, 11-Runcinia khandari, 12-Xisticus minutus, 13-Philodromus bhagirathai, 14-Tmarus kotigeharus, 15-Xisticus species1 and 16-Tibellu elongatus.

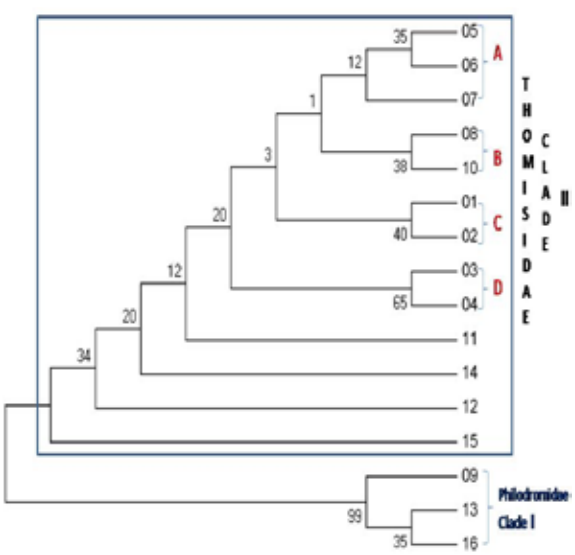


Figure 3: Neighbor-joining tree of crab spider and philodromid spiders. 1-Thomisus viveki, 2-Thomisus pateli, 3-Tmarus pachpediensis, 4-Pistius calimpus, 5-Thomisus andamanensis, 6-Thomisus danieli, 7-Thomisus species1, 8-Synaema decorata, 9-Dieta elongata, 10-Thomisus lobosus, 11-Runcinia khandari, 12-Xisticus minutus, 13-Philodromus bhagirathai, 14-Tmarus kotigeharus, 15-Xisticus species1 and 16-Tibellu elongatus.

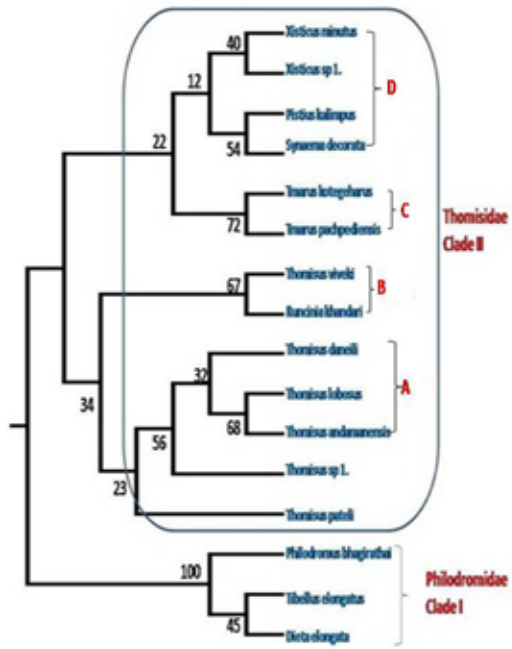


Figure 4: UPGMA tree of crab spider based on recorded morphological characters.

**Appendix A**

Characters and their character states are means which describe to the features of an organism. Mesquite program supports morphological characters whose states are categorical. Categorical characters other than molecular characters can have 55 states. Standard Categorical Matrix states are discrete and not necessarily ordered. Characters can have as many as 55 states, whose symbols by default are (0 – 9), (A – H), (K – N), (P – Z), (a – h), (k – n), (p – z). Polymorphisms (e.g., state 0 and 2) are indicated by (0&2); uncertainty (e.g., state 0 or 2) is indicated by 0/2. A completely unknown state is indicated by (?) by default. If the character is inapplicable to that taxon, the symbol is (-) by default.

**The recorded morphological characters are as follows.**

**1.Cephalothorax:**

0-High 1- Depressed, 2.Cephalothorax shape: 0- As long as wide 1- Broader than long 2- Wider than long 3- Longer than wide, 3.Cephalothorax> Antero-lateral side with marking: 0- Absent 1- present, 4.Cephalothorax> Mid-dorsal portion with marking: 0- Absent 1- present, 5.Ocular area coloration: 0- absent 1- colored or chalk white, 6.Clypeus: 0- long 1- very high 2- Median 3.- Narrow 4- moderately high 5- broad, 7.Clypeus margin provided with spine-like hairs: 0- absent 1- present 2- seven 3- six 4- two 5- eight, 8.Clypeus: 0- Obtuse 1- Sub-rectangular, 9.Sternum: 0- Oval shape 1- Heart shape 10.The chelicerae: 0- Fused at the base 1- Free at the base, 11.Tarsal claw pairs: 0- one 1- two 2. Three, 12.Chelicerae teeth: 0- Absent 1- Present, 13.Abdomen shape: 0- Rounded 1- Pentagonal 2- Slender and long 3- Oval 4- Longer than wide 5- Elliptical, 14. Abdomen overlapping on Cephalothorax: 0- Overlapping 1- Not overlapping, 15. Legs> the adult spiders with the longest legs represented by: 0- I and II 1- II 2- I and II sub-equal, 16. First two pairs of legs: 0- Laterigrade 1- Not laterigrade, 17. Tibiae and Metatarsi of legs with spines: 0- Absent 1- Present, 18. Whether present: 0- Six 1- Four 2- Five 3- Two 4- Three, 19. Tibiae and Metatarsi with

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marking or spot: 0- Absent 1- Present, 20. Position of eyes when in 8 pattern: 0- AME slightly larger than ALE 1- AME slightly smaller than ALE 3- ALE situated antero-lateral edge 4- PME smaller than PLE5. PLE eyes larger, 21. Eyes: 0- Anterior four eyes and PME forms hexagonal area 1- Not hexagonal, 22. Eyes with two rows: 0- Anterior row recurved 1- Posterior row recurved 2- Both rows recurved, 23. Eyes coloration: 0- All clear and glassy 1- All dark and round, 24. Eyes when on tubercles: 0- Provided with tubercles 1.- Not provided with tubercles

**Chart 1-Numerical morphometric data for the 16 crab spiders.**

Thomisus_daneili- 0310153110111110012?11211
Thomisus_pateli- 011?11011011112012131211
Thomisus_viveki- 0301101110111110012001211
Thomisus_lobosus- 00101001101111011101211
Thomisus_andamanesis- 02101001101111001(1 3)011211
Thomisus_Species1- 0000050110111110000?011211
Runcinia_khandari- 0300131110114100140(2 4)1211
Pistius_kalimpus- 00000000001141001(0 1)0-1210
Xysticus_minutus- 0010022?1011300013151210
Xysticus_Species_1- 001002??1011300013151210
Synaema_decoreta- 1000031?1011500011021210
Tmarus_kotegeharus- 1410112?1011400114021210
Tmarus_pachpediensis- 1410143?1011400111051110
Philodromus_bhagirathai- 1310031?1011(2 4)11014050210
Tibellus_elongatus- 1400025?10112010141?0211
Dieta_elangata- 0411034?101120101111?0210

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