A Study of Phylogenetic Relationship of Grasshoppers based on Alpha Amylase Diversity

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Abstract: In grasshoppers, phylogenetic studies based on amylase diversity are rare. Insects widely distribute alpha amylases, attracting a few workers to consider these enzymes for phylogenetic analysis and genetic diversity studies. The existence of alpha-amylase isoforms in grasshoppers enabled us to identify molecular diversity and polymorphism between the species. In this study, the isoforms of amylase generated diverse zygomorphic bands ranging from 1 to 4 between species. Twenty-five species of grasshoppers recorded a total of fifty bands, with their molecular weight ranging from 34.08 kDa to 87.44 kDa, reflecting changes in the amino acids in the protein chain and structure of these molecules. The distribution of amylases in these species showed a single band in nine species; three species had four bands; ten species had two isoforms; and in four species, three bands were present. The diversity of isoamylases in these insects has provided new insight into their phylogeny. The phylogenetic analysis of twenty-five species of grasshoppers in this study has not recovered monophyly for the subfamilies Oedipodinae, Acridinae, or Gomphocerinae. The grasshoppers of family pyrgomorphidae appeared as sister taxa to members of different subfamilies of Acrididae. The importance of alpha-amylases as markers in the phylogenetic study of short-horned grasshoppers was proven.

Keywords: Alpha amylase, Isoforms, Paraphyly, Polymorphism, Zymograph

1. INTRODUCTION

Alpha amylases are widely distributed across the taxa, about 3118 alpha Amylases and 280 beta Amylases fully documented in the lineage of organisms, classified into many categories based on their property of breaking starch molecule and biochemically assigned to families 13 and 57 [1,2] of glycoside hydrolases. Only alpha amylase is found in insects [3] as hydrolyzing enzymes of starch and glycogen. A few evolutionary biologists concerned with molecular evolution have chosen alpha amylase and amylase genes as a favorite model among the many nuclear genes over the years to study phylogenetic relationships of organisms. This model has been widely examined in *Drosophila* species as well in many other organisms because amylase enzymes exist in more than one number in a species and give ample of chance for analysis either in terms of duplication or loss of isoforms in different species leading to evolution. Seven types of amylase are found in *D.ananassae* [4] and duplication of amylase producing [amy] genes are found in other taxa also [5]. The Amy genes have been studied in many animals such as insects [6,7,8], in *Aedis aegypti* [9] in honey bees [10] shrimp [11] oyster [12] and human [13], recorded more than one gene for amylase. Alpha amylases have also been studied and characterized in grasshopper *Zonocerous variegate* [14]. Existence of alpha amylase isoforms represent duplicate forms of amylase genes enable the researcher to identify diversity in the organism as well the polymorphism

between the species. Yan and Wu [15] have discussed in depth how the phylogenetic studies of Amylases potentially increase the rate of cloning, industrial application by engineering these enzymes in to new types across the lineages particularly with organisms of industrial or clinical importance. In grass hoppers the study of this enzyme has been done only to little extent. This has given us a chance to understand diversity and evolutionary relationships among grasshoppers. In this study variability in alpha amylase has been assessed through differences in molecular weight of isoforms, variations in molecular weights reflect the changes in amino acids in the proteins or amylase, that can be used with confidence to asses diversity of these molecules. Our work on six species of pyrgomorphidae revealed the interrelationship of taxa within the family limits [16], here these species are combined with other grasshoppers but in indifferent context to understand the phylogenetic relationship with the members of Acrididae and pyrgomorphidae. There are many contradictory views regard to phylogenetic derivation of pyrgomorphids, is considered here to reveal their phyletic status.

2. MATERIAL AND METHODS

Twenty-five grasshopper species of two families - 1. **Acrididae- Sub family-Acridinae**:_*Acrida exaltata* (Walker).*Acrida gigantea* (walker) *Phlaeoba panteli* (I.Bolivar), **sub family -Oedipodinae**: *Dittopternis venusta* (Walker). *Heteropternis respodens* (Walker), *Morphacris citrina* (Kirby). *Oedaleus abruptus* (Thunberg) .*Oedaleus senegalensis* (Karuss), *Trilopidia annulata* (Thunberg), *Acrotylus humbertianus* (Savssure), *Aiolopus thalasinus tamulus* (Fabricius) **sub fam- Calliptaminae:**-*Acorypha glaucopsis* (Walker),**sub fam-Catantopinae**:-*Catantops pinguis innotabilis* (Walker) **sub fam- Coptacanthacridinae** -*Epistaurus sinetyi* (I.Bolivar) ,**subfam-Cyrtacanthacridinae** - *Cyrtacanthocris tatarica tatarica* (Linnaeus), **sub fam – Eyprepoonemidinae:**—*Eyprepocnomis alacris alacris* (Serville). **sub fam-Hemiacridinae**:- *Spathosternum prasiniferum prasiniferum* (Walker) **subfam-Gomphocerinae**: *Leva cruciata* (I.Bolivar), *Gelastorhinus semipictus (*Walker) and ,**2.family-pyrgomorphidae** : *Atractomorpha crenulata crenulata* (Fabricius), *Chrotogonus oxypterus* (Blanchard), *Chrotogonus trachypterus* (Blanchard) *Neorthocris acuticeps acuticeps* (I.Bolivar). *Poekilocera picta* (Fabricius), *Pyrgomorpha bispinosa bispinosa* (Walker). collected from their natural environs are used in this study.

1.1. Extraction of Protein

20mg of Sample was weighed and homogenized with 100µl of 1x PBS using tissue grinder on an ice bath. 20 µl of crushed sample was used for analysis.

1.2. Zymography

The initial protocol of zymography is same as SDS PAGE as followed by Jayashree and Channaveerappa [17]; however the sample loading buffer and sample treatment varies.
Sample loading Buffer for **Zymography** contains glycerol, β-mercaptoethanol, Tris buffer, and Bromophenol Blue. And it essentially **does not contain SDS.** The samples for zymography **are not boiled** during sample preparation for loading

1.3. Protocol: Assembly of glass plates

The glass plates were washed and immersed in 1:20 diluted Nitric acid for half an hour followed by several washes with distilled water and air drying. Dried glass plates were then assembled and sealing was done using molten agarose. It was checked for leakage by filling water between the plates and later discarded.

1.4. Preparation of 12% Resolving gel

The gel mix was prepared as per the following table and poured between the sealed plates till the level reached 3/4th of the capacity. Immediately it was overlaid with water to an additional height of 0.5cm. Gel was allowed to polymerize for nearly 30 minutes. After polymerization the excess water was drained completely. The components required are Distilled water(2.0ml) , 30%Acrylamide-Bisacrylamide(3.6ml) , 10% Starch (970 µl), 1.5 MTris pH 8.8(2.25ml), Ammonium Per sulphate (180µl), TEMED(9 µl), **(**Total volume. 9ml).

1.5. Preparation of 5% stacking gel

Stacking gel mix was prepared as per the table and poured onto to polymerized resolving gel and a clean dry comb was carefully inserted

without trapping air bubbles. Gel was left for additional 30minutes for complete polymerization. Required components include Distilled water (2.085ml), 30%Acrylamide-Bisacrylamide (0.495ml), 1.0 M Tris pH 6.8 (0.375ml), Ammonium Per sulphate (50 µl), TEMED (5 µl), [Total volume 3ml].

1.6. Sample preparation and electrophoresis

Spun down samples in microfuge for 5 minutes. Wells were immediately washed with distilled water to remove non-polymerized acrylamide. Straighten the teeth of the wells using a needle, if necessary. Bottom spacer was removed. Clipped the sandwich to the electrophoresis apparatus filled with 1X Tris-glycine-SDS Buffer in the lower chamber. Care was taken not to introduce any air bubbles between the bottom of the gel and the buffer. Any bubbles caught between the plates at the bottom of the gel can be removed by squirting running buffer through a syringe fitted with a bent needle. The comb was carefully removed from the gel and filled the top of the apparatus with 1X Tris-glycine-SDS Buffer. Checked for leaks. The samples were loaded into the bottom of the wells using microlitre syringe or micropipette fitted with long tip. Carefully recorded the contents in each well. The apparatus was connected to the power supply started electrophoresis at 50 V for the first 30 minutes and then increased the voltage to 100V. When the dye front comes to 0.5 cm above the bottom of the gel, power pack was turned off. Remove the gel plates and gently dry the plates apart. Use a spatula or similar tool to separate the plates. A corner from the bottom of the gel was cut at well no 1 for reference. Wash the gel with distilled water for 30 seconds. Marker Lane transferred in Coomassie staining solution for overnight.

The Gel was rinsed twice with Distilled water for 2minutes each. Gel was transferred to 50ml of Renaturation solution (1ml of triton X100 in 49ml of Distilled water and incubated on rocker for 30minutes at RT. Gel was rinsed twice with distilled water for 2minutes each.50ml of Developer (10mg of Naphthyl acetate in 1ml of acetone, dissolved in 50ml of sodium Phosphate buffer pH 7 containing 20mg of Fast Blue RR) was then added to the gel and kept in dark overnight.

1.7. Distance matrix and Phylogenetic tree

The computation of distance matrix and tree construction carried out by using software GEL QUEST version 1.0. The distance matrix automatically generated by the software, for this Jaccard's similarity was chosen. The phylogenetic trees are constructed, based on UPGMA – Neighbour joining method. Each node is represented using Alpha bets(A,B,C--) manually and any lineage ramified from its ancestral node in to more than one branch then each branch designated with similar alphabet with different numerical (A1,A2,B1,B2----).

3. RESULTS

The Alpha Amylase zygomorphic band profile of twenty-five species of grasshoppers exhibited greater divergence among the species. The bands generated by the isoforms of amylase ranged between 1 to 4 among grasshoppers and in total 50 bands recorded. Among twenty five species, single band was in nine species, three species had four bands, in ten species two isoforms and in four species three bands were present(Fig.1).

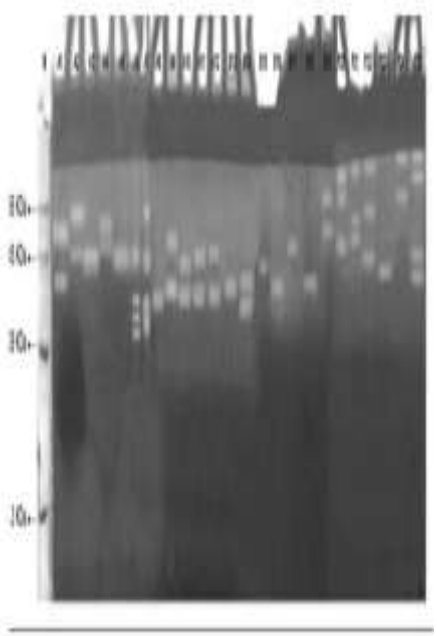

Fig 1 Amylase electrophoresis profile for 25 species of Grasshoppers

A1-A. exaltata, A2-A. gigantea, A3-A. humbertianus, A4-A. t. tamulus, A5-D. venusta, A6-E. sinetyi, A7-H. respondens, A8-M. citrina, A9-O. abruptus, A10-O. senegalensis, A11-P. panteli, A12-T. annulata, A13-A. glaucopsis, B1-C. c. crenulata, B2-E. a. alacris, B3-E. sinetyi, B4-C. p. innotabilis, B5-E. sinetyi, B6-C. tatarica, B7-C. c. crenulata, B8-A. c. crenulata, B9-C. oxypterus, B10-C. trachypterus, B11-A. c. crenulata, B12-C. oxypterus, B13-A. c. crenulata, B14-A. c. crenulata, B15-H. respondens, B16-P. picta, B17-P. picta, B18-P. bispinosa.

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A common representative amylase band for all the twenty-five species was not found. Sixteen species of grasshoppers had two or more duplicates of the amylase. The molecular weight of these bands ranged from34.08 K.da to 87.44 K.da (Fig 1).The computed similarity index for these Alpha Amylases showed a range 0 to 1.0 indicating 100% similarity to 100% dis similarity in between different species of grasshoppers (Table 1).

Phylogenetic tree

While presenting the results of tree topology the term derived ancestor is used to represent the nodes of the phylogenetic tree extended from the root towards the terminus of the tree. The basal bifurcation represented by two species *A. exaltata* and *P. b. bispinosa* - a Pyrgomorphid. second ramification from the root resulted in node 'A' the innermost derived ancestral representative, that further bifurcated into 'two branches ended up in nodes 'B_1' and 'B_2' from these two nodes lineages 'C_1','C_2','C_3' originate, further derivation of these are 'D_1' and 'D_6' nodes. The D_1 and D_2 had two moderate length branches each terminating in *G. semipictus* and *P. picta* and *M. citrina* and *L. cruciata*. other species *H. respondens* directly derived as terminal taxa

from the node B1. The branch 'D_3' ramified in to 'E_1' and 'E_2' nodes each of these branched twice in to medium branches ending in *A. gigantea* plus *A. t. tamulus* and *A. humbertianus* plus *E. a. alacris*. The node 'D_4' ramified in to two nodes 'E_3' and 'E_4' each bifurcating in to a branch and a node, branch of 'E_3' terminated in *O. abruptus* and branch 'E_4' terminates in *N. a. acuticeps*. The node 'D_5' ramified in to two medium branches bearing *P. panteli* and *A. glaucopsis*, the node 'D_6' ramifies in to a branch with taxa *E. sinetyi* and a node 'E_5'. The node 'F_1' originates from 'E_3' forming in to a node 'G' and a branch having *A. c. crenulata.* The node 'F_2' branched to terminate in *O. senegalensis* and *T. annulata* and the nodes 'F_3' and 'F_4' as basal nodes derived in to two short branches each bearing *D. venusta* and *S. p. prasiniferum* and *C. tatarica* and *C. oxypterus,* the species *C. oxypterus* appeared as sub terminal node derivatives. The last node 'G' derived from 'F_1' terminated into two shortest branches bearing *C. p. innotabilis* and *C. trachypterus*. (Figure 2).

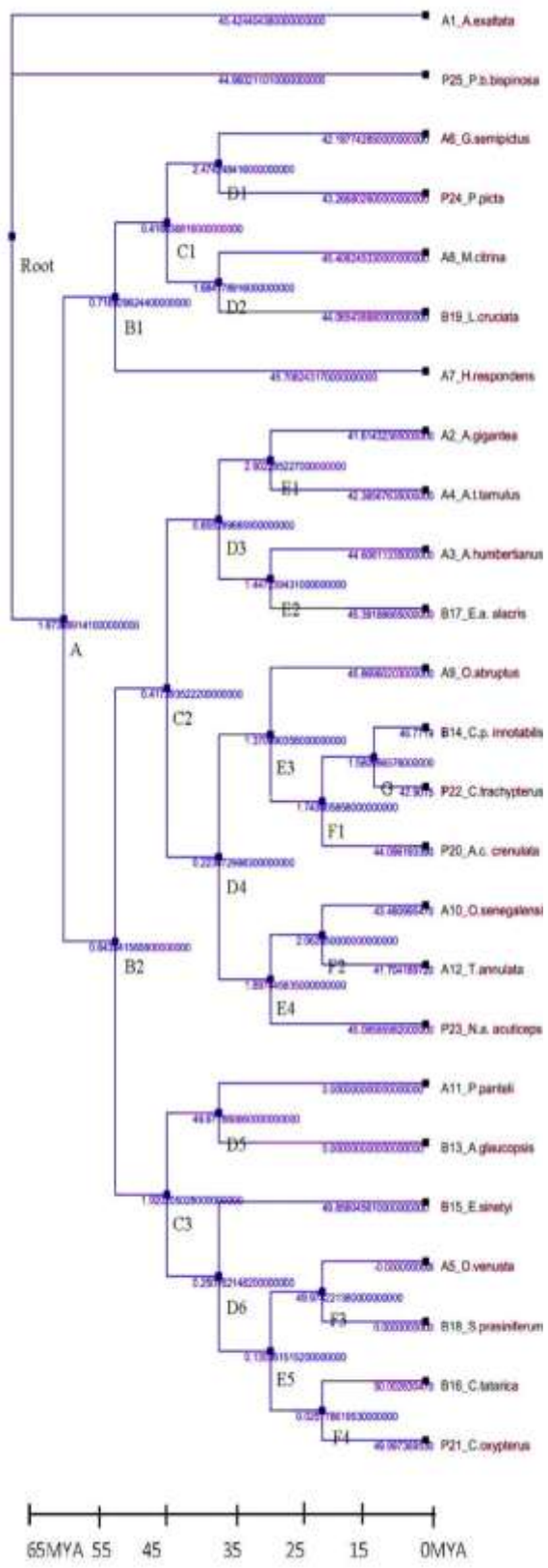

Fig.2 Phylogenetic tree based on Amylase profile for 25 species of Grasshoppers.

4. DISCUSSION

Existence of more than one amylase with different molecular weights, in sixteen of the twenty species indicated the possible existence of more than one amylase genes in grasshoppers. Gene duplicate conditions exist in many insects as we have visualized in introductory part. Ohno [18] has explained the mechanism of gene duplication as a major source of evolution and the possible fate of duplicated genes. Standing variations and further changes in alpha amylases are crucial to grasshopper as these insects depend on starch-based food source for their survival and reproduction. In grasshoppers' different isoforms of amylases are recorded implied that all the duplicates are functional and if the functions were to be lost there could have been no electrophoretic band formation. Formation of a new enzyme seems to occur by gene duplication and divergence of its function which is favored by innovation, amplification and divergence; to evolve in to a new type requires a new activity to contribute to the fitness of the organism and in some cases to enhance the fitness towards environmental changes such gene duplication often lead to speciation [19] but amy gene duplication leading to speciation is still to be understood in grasshoppers. Alpha amylases are functional units in an organism and have been considered to be units of evolution. The variation in number and molecular weight of these enzymes in each of the species may be due to specific adaptation to its food sources and for their survival. In our study more than 30% of the grasshoppers had single amylase yet these survive that showed the other evolutionary facet of non-duplication of gene for amylases. Possibly these insects might have different mode of adaptation by existing amylase enzyme but it needs a confirmative study.

Explaining the phylogeny of grasshoppers in this geographical region will provide an insight in to the evolution among Indian grasshoppers because the phylogenetic studies in this region are too less compared to grasshoppers of other regions and it is relevant to mention acridids of only two families exist in Indian subcontinent. In the phylogentic analysis of grasshoppers there are a few hypotheses based on morphological and molecular data other than alpha amylases [20-29] but there is lack of consensus on phylogenetic tree derivation based on morphology. Many workers opted to consider molecular data for phylogenetic

analysis and a few selected mitochondrial genes for this purpose [30] but use of mitochondrial genome has not been fully appreciated by a few (cross findings) [31]. All these works majorly concentrated on deriving monophyly for families of Acridoidea and their subfamilies.

Phylogenetic tree based on alpha amylases is unique , the initial ramification is shared by *A.exaltata* an Acrididae member and *P.b.bispinosa* a Pyrgomorphid these two species had variations in number of amylases, the first outer most lineage 'A' ramifies to generate the 'B_1' and 'B_2' nodes and the node 'C_1' that further ramifies to generate *G.semipictus* and *P.picta* and .The node 'C_2' through its ramification derives species *A.gigantea* and *A.t.tamulus* ,*A.humbertianus* and *E.a.alacris* ,whereas *O.abruptus* ,*A.c.crenulata* and *C.p.innotabilis.* The other ancestor 5 th order lineage directly results in *N. a.acuticeps* on one of its branches,and *O.senegalensis* and *T.annulata* on the other. These branches sharing between members of different sub families of Acrididae as well between the members of the family Pyrgomorphidae bear no clarity in relationship and correlation with taxonomic grouping of these insects.

The 'C_3' ancestral cluster has five extended ancestral lineage 'D_5' to 'F_4' nodes include species *P.panteli, A.glaucopsis* ,s *E.sinetyi* , *D.venusta* plus *S.p.prasiniferum* and *C.tatarica* and *C.oxypterus*; other congeneric grasshopper *C.trachypterus* and a Catantopine *C.p.innotabilis* are represented as youngest in the lineage . All the three sets of congeneric species *A.exaltata, A.gigantea, O.abruptus, O.senegalensis, C.oxypterus* and *C.trachypterus* found to originate from different ancestor as paraphyletic taxa. Branch sharing between different species of Acrididae and pyrgomorphidae proves these families are a sister group derived through the lineages of common Acridomorpha ancestors. Shuffling of species position in phylogenetic tree may be due to changes in Alpha amylases at different times of (compare ancestral nodes with hypothetical time line) evolutionary period. So derived isoforms have retained the function of starch digestion, served here as good markers to trace evolution among twenty-five species of grasshoppers and Such retention of isofunction or failure to retain the original molecular function by a gene or its product in course of species evolution is well discussed by Ohta [32]. The branch sharing between the species either of Acrididae or of pyrgomorphidae is not influenced by number of amylase isoforms. Three species with four isoforms shared branching with other species having two or three bands, this also seen in species having three and two electromorphic bands indicated that the branch sharing might have been influenced by molecular weight of first identical or nearly indentical molecular weight band of a species with the corresponding band of other branch shared species.

Monophyly of Acrididan sub families are widely examined regard to relationship of subfamilies of Acrididae, different topologies are obtained from different data sets while determining the relationship by zhang *et al* [25] showed that Cyrtocanthocridinae, Catantopinae, Calloptominae, Oxyinae and Melanoplinae to be closely related. The sub family Acridinae were found to be a split lineage and placed closer to Oedipodinae and these authors found Gomphocerinae were monophyletic. Using the cyto-B and COI genes as markers subfamilies Catantopinae and Gomphocerinae were found to be monophyletic [29,27], using 18s rDNA and mitochondrial genes have resolved monophyly for the grasshoppers of Oedipodinae [26] but sub families Acridinae including Truxalinae, Gomphocerinae and Oedipodinae are not monophyletic but a fuzzy set. A few have also worked on phylogentic relationship of single species of grasshoppers [30, 31] but such works can justify inclusion of a species in a targeted sub family. The phylogenetic analysis on twenty-five species of grasshoppers in this study has not recovered monophyly of the sub family Oedipodinae or Acridinae or Gomphocerinae which is similar to the findings of Chapco and Contreras [26]. The grasshoppers of family pyrgomorphidae appeared as sister taxa to members of different sub families of Acrididae, has to be considered that pyrgomorphidae are derivatives of Acrididae ancestral lineage.

5. CONCLUSION

Amylase diversity in grasshoppers may have adaptive value and helpful to adjust to new food sources. The migratory events during past ecological innovations might have forced evolution of isoforms of amylase enzymes. If

two closely related species or different species of a sub family move to new food sources by natural migration, one could assume changes in the amylase of each species to adjust to specific food resources. Such changes on comparison between members of subfamilies will never be similar regard to amylase contents developed in those species. Such changes may be the cause for paraphyletic relationships of species from different sub families of Acrididae, in the phylogeny based on alpha amylases.

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