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Phylogenetic relationship of six pyrgomorphid species based on alpha amylase and protein page analysis: Depicts non-identical divergence

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Abstract
Proteins and enzymes are the structural units of evolution and serve as a reliable marker to establish phylogenetic relations of grass hoppers, variations in these molecules could be easily resolved on electrophoretic gel. Alpha amylases as well water soluble proteins in this study revealed large variations regard to molecular weight and number. Based on these two features similarity and distance matrix computed and the values obtained are utilized to construct phylogenetic trees. In the phylogenetic trees the species *Atractomorpha crenulata* ramified earlier than other pyrgomorphids. All the six pyrgomorphid grass hoppers showed the monophyletic origin from a common ancestor. Congeneric species *Chrotogonus oxypterus* and *Chrotogonus trachypterus* with common generic morphological features showed the diversification from two different nodes of phylogenetic tree. Six species of pyrgomorphid grasshoppers showed a difference in diversification for Alpha amylases, proteins and combination of these two molecules resulted in phylogenetic trees not identical to each other. The formation of nodes, branch length and position of terminal taxa differed in each one these cases. The knowledge of molecular phylogeny of pyrgomorphid grasshoppers is least compared other orthopterans, in this regard the present analysis has helped to understand the phylogenetic relationship among a few species of pyrgomorphid grass hoppers.

Keywords: Biology, brinjal, leucinodes orbonalis, morphometry

1. Introduction
Evolutionary mode and phylogenetic relationships of pyrgomorphidae are less understood compared to other orthopteran taxa. Even their taxonomic status, after several reviews by different workers of different geographical regions, this family has been considered to be a sister group of Acrididae [1]. Our work on phylogenetic relationship of pyrgomorphidae based on genomic DNA-RAPD analysis and subsequent work on genetic diversity of congeneric grasshoppers has enabled to gain a preliminary knowledge on in between species relationship [2, 3]. These studies revealed variations were due to genetic polymorphism as well duplications. Ohno [4] and Ohta [5] have shown that gene duplication and subsequent diversification have played an important role in progressive changes of organism leading to speciation. Such duplications result in isoforms with unaltered functions as in case of isozymes. Isoforms of amylases and proteins are polymorphic in natural population and the polymorphic state could be easily identified by electrophoresis [6, 7].
Pyrgomorphidae is one of the orthopteran lineages assumed to have later origin compared to other families of acridomorpha, distributed in many parts of the world [1, 8] and has monophyletic origin. Our earlier work on phylogenetic relationship analysis of six species of pyrgomorphidae confirmed the monophyletic origin of the six species analyzed, based on divergence of genomic DNA. In this work we have taken two other factors alpha amylase and proteins to examine the phylogenetic relationships of pyrgomorphidae. These molecules being the structural units of evolution and the variation can be easily resolved by electrophoresis these two components serve as good markers for phylogenetic analysis.
Each gene has its lineage from its ancestor to the present, likewise the proteins and enzymes too represent particular gene product that represents descent or changes with the gene lineage. Variations in the gene product are reflected by changes in the molecular weight, for this reason the gene products have been used as markers to trace genetic diversity and phylogenetic relation of organisms. Till the date many types of markers have been used by several workers but none have claimed one particular molecule or gene to be a perfect marker including the so

called advanced markers. In this context we feel gene products are most ideal marker as these are less expensive and easily reflect variation in terms of molecular weight which could be used to trace the evolutionary lineage and phylogenetic relationships of grass hoppers.

2. Material and Methods

Six grasshopper species of pyrgomorphidae collected from their natural habitat are used for this study, these species include *Atractomorpha crenulata crenulata, chrotogonus oxypterus, Chrotogonus trachypterus), Neorthacris acuticeps acuticeps, Poecilocera picta, Pyrgomorpha bispinosa bispinosa*.

2.1 Sds-page analysis

50mg of the leg muscle from each grasshopper weighed in a separate vial each and homogenized with 300ul of 1XPBS using tissue grinder and centrifuged at 600rpm for 5mins. Supernatant collected (crude) and stored at 20 ^{0}c for SDS-PAGE analysis. Proteins electrophoresis was carried out and separated by following the methods of Laemmli [9], components used for SDS-PAGE analysis were 1.5M Tris Ph8.8, 1.0MTrispH6.8,30% Acrylamide bisacrylamide, Potassium persulfate, sample loading buffer, crude protein samples TEMED, Protein standard marker,1XTris-Glycine SDS buffer.(4%SDS,20% Glycerol,125Mm Tris-cl,pH6.8 10%B-Mercapthanol,bromophenol blue (dye).Protocol of Sambrook et al Fredrick M Ausubel [10, 11] consisted of 12% resolving gel and 5% stacking gel samples (6ul) boiled with SLB loaded using micropipette(pipette man's from Gilson & Nichipet companies).Electrophoresis carried out at n50V initially for 30mins then increased the voltage to 100V. Gels were stained with Coomassie brilliant blue. The results were photographed and to determine molecular mass four molecular weight markers were used and Molecular weight of each band was determined.

2.2 Zymography

To analyse Amylase Enzyme activity in grasshopper gut samples using 1XPBS extraction buffe,20mg of samples weighed &homogenized with 100ul 1XPBS. Later 20ul of crushed sample was used for zymogram. The method consists of conduction of electrophoresis in polyacrylamide gel polymerized with 0.5% starch and electrophoretic run was done in the adequate P^H. The technique relies on detecting enzymes, so the samples were kept live or frozen to preserve enzyme activity (samples are not boiled during loading) SLB contains glycerol, β-Mercaptoethanol Tris buffer and Bromophenol blue essentially does not contain SDS. Resolving gel of 12% and stacking gel of 5% was prepared. The samples were loaded using micro syringe. Apparatus electrophoresed at 50V at first 30min and then at 100v. Marker lane was stained in Coomassie blue gel was rinsed in distilled water transferred to 50ml renaturation solution (1ml of triton x100 in 49ml of distilled water) and incubated on the rocker for 30mins at room temperature. Gel was rinsed in distilled water and 50ml of developer (10mg of Naphthyl acetate dissolved in sodium phosphate buffer pH 7, with 20mg fast blue) kept in the dark overnight. Electrophoretic gels of zymo washed and stained with Potassium iodide solution so that amylase activity appeared as white bands (due to digestion of starch) on brown back ground.

Distance and similarity matrix computed by co-efficient method to generate evolutionary tree by a Neighbor joining method with Gel Quest software version 1.0 to evaluate the evolutionary distance.

3. Results

PAGE profile of alpha amylase enzymes revealed the presence of more than one type of alpha amylase enzyme in five of the six species screened, one species had one alpha amylase type, in total 16 electrophoretic bands for the six species of pyrgomorphidae (fig 1) representing as many number of alleles. These alleles are unevenly distributed in each of the species *Atractomorpha crenulata crenulata*, *Chrotogonus oxypterus*, *Chrotogonus trachypterus* had three amylase isoforms each but had difference in their molecular weight as represented by band profile single amylase allele was found in *Neorthacris acuticeps acuticeps*, whereas *Poecilocera picta* had two bands and *Pyrgomorpha bispinosa bispinosa*. Four isoforms of amylase. The molecular weights ranged from 87.44 KDa to 48.65 KDa (table 1)

None of the amylase isoforms shared a common molecular weight profile among in these six species. The similarity index and distance matrix is represented in table 3.The similarity matrix for α amylases of six species depicted a range of 100% similarity to 100% dissimilarity (Table.3). SDS-PAGE analysis of water soluble proteins had generated 56 electrophoretic bands for all the six species posed for analysis. The molecular weight of proteins expressed a greater diversity, the range of molecular weights was between 116.5 KDa to 11.06 KDa (fig.2).

The number of bands ranged from least 5 in *A. c. crenulata,* to highest of 14 bands in *N. a. acuticeps* followed by 8 in *C. oxypterus*, 11 bands in *C. trachypterus*, 13 and 12 in *P. picta* and *P. b bispinosa* respectively. The band with least molecular weight 11.06 KDa was in *P. b. bispinosa* and the band with highest molecular weight 166.95 KDa was in *C. oxypterus*. In the remaining species the molecular weight of proteins ranged in between as represented in table (2).The similarity and distance matrix for proteins (table 4) showed a range between 0.82143 to 0.97436.

Table 1: Amylase polymorphism in six species of grass hoppers

SL NO	MOL WT	Rf VALUE	P20	P21	P22	P23	P24	P25
1	48.65	0.37						1
2	49.04	0.35				1		
3	51.43	0.33						1
4	55.23	0.32			1			
5	56.22	0.31	1					
6	57.58	0.3		1				
7	59.3	0.29		1				
8	63.96	0.26			1			
9	66.59	0.24					1	
10	70.88	0.23	1					
11	75.37	0.22						1
12	75.43	0.22						1
13	76.1	0.21	1					
14	78.88	0.2			1			
15	78.89	0.2		1				
16	87.44	0.15					1	

P20- *Atractomorpha crenulata crenulata*. P21- *Chrotogonus oxypterus* P22- *Chrotogonus trachypterus*. P23- *Neorthacris acuticeps acuticeps*. P24- *Poecilocerapicta* P25- *Pyrgomorpha bispinosa bispinosa*

Table 2: Protein polymorphism in six species of grass hoppers

SL NO	MOL WT	Rf VALUE	P20	P21	P22	P23	P24	P25
1	11.06	0.9						1
2	12.39	0.8			1			
3	13.43	0.79			1			
4	13.65	0.78				1		
5	13.71	0.76					1	
6	14.98	0.75				1		
7	15.1	0.73						1
8	15.3	0.73					1	
9	16.57	0.72					1	
10	17.28	0.7				1		
11	17.35	0.69			1			
12	17.87	0.69						1
13	18.41	0.64				1		
4	18.64	0.64		1				
15	20.8	0.63					1	
16	21.15	0.63				1		
17	24.82	0.62				1		
18	25.56	0.62					1	
19	26.21	0.62			1			
20	26.88	0.6				1		
21	27.11	0.59			1			
22	28.4	0.57						1
23	28.52	0.57					1	
24	29.54	0.57	1					
25	29.68	0.57		1				
26	34.88	0.56				1		
27	35.97	0.55					1	
28	37.25	0.54						1
29	37.91	0.52			1			
30	40.14	0.49				1		1
31	40.27	0.46		1				
32	40.5	0.43			1			
33	40.65	0.42	1					
34	42.88	0.4					1	1
35	43.45	0.39			1			
36	45.33	0.36					1	1
37	47.03	0.36		1				
38	48.44	0.34				1		
39	53.31	0.34						1
40	62.22	0.31				1		
41	63.61	0.29			1			
42	65.52	0.26	1					
43	66.49	0.26		1				
44	66.98	0.26					1	
45	73.17	0.23						1
46	82.32	0.21				1		
47	85.41	0.2	1					
48	86.04	0.16						1
49	87.32	0.15		1				
50	94.68	0.14			1			
51	101.92	0.11				1		
52	104.2	0.1						1
53	107.31	0.09	1					
54	108.91	0.08			1			
55	113.83	0.06		1				
56	166.95	0.01		1				

P20-Atractomorpha crenulata crenulata.,P21-Chrotogonus oxypterus ,P22-Chrotogonus trachypterus., P23-Neorthacris acuticeps acuticeps., P24-Poecilocera picta,P25-Pyrgomorpha bispinosa bispinosa.

Table 3: Similarity and distance matrix for alpha amylases in six species of grasshoppers

'Emzyme_P_P20'	0	1	1	1	0.96825	0.93671
'Emzyme_P_P21'	1	0	0	1	0.95833	1
'Emzyme_P_P22'	1	0	0	1	0.95833	1
'Emzyme_P_P23'	1	1	1	0	1	1
'Emzyme_P_024'	0.96825	0.95833	0.95833	1	0	0.93651
'Emzyme_P_P25'	0.93671	1	1	1	0.93651	0

Table 4: Distance and similarity matrix for proteins in six species of grasshoppers

'P20_Protein_P'	0	0.82143	0.94595	0.90625	0.96296	0.90625
'P21_Protein_P'	0.82143	0	0.95	0.91429	0.96667	0.84848
'P22_Protein_P'	0.94595	0.95	0	0.9	0.97222	0.92683
'P23_Protein_P'	0.90625	0.91429	0.9	0	0.9	0.97436
'P24_Protein_P'	0.96296	0.96667	0.97222	0.9	0	0.9
'P25_Protein_P'	0.90625	0.84848	0.92683	0.97436	0.9	0

P20- Atractomorpha crenulata crenulata., P21- Chrotogonus oxypterus, P22- Chrotogonus trachypterus., P23- Neorthacris acuticeps acuticeps., P24- Poecilocera picta, P25-Pyrgomorpha bispinosa bispinosa.

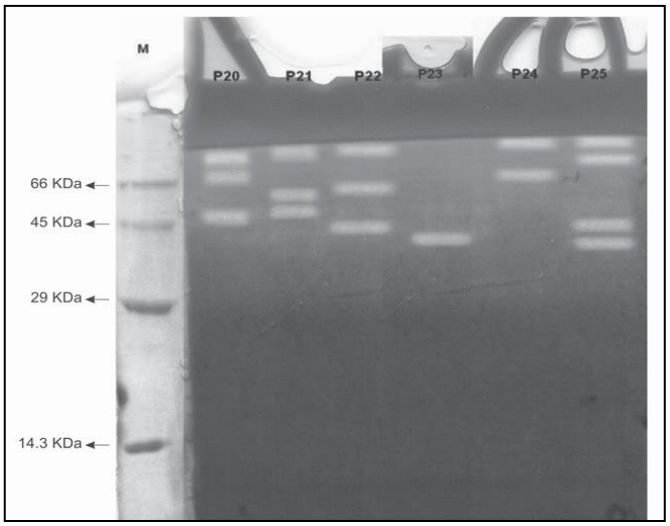

Fig 1: PAGE Profile of alpha amylases of six species of grasshoppers

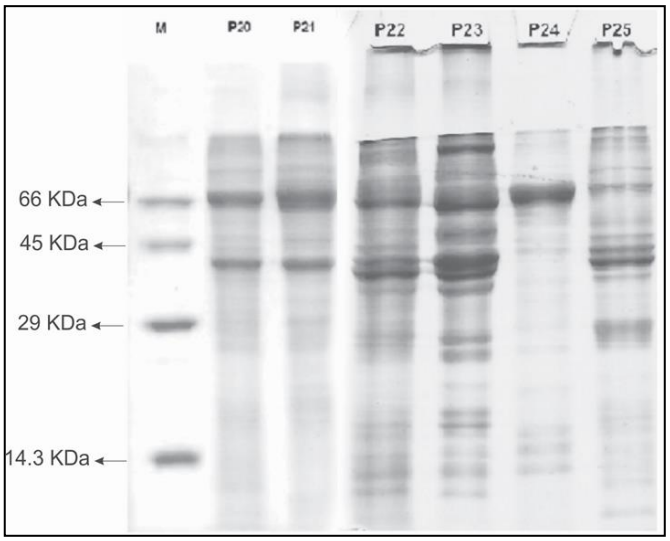

Fig 2: PAGE profile of proteins of six species of grasshoppers

P20-Atractomorpha crenulata crenulata. P21-Chrotogonus oxypterus P22-Chrotogonus trachypterus. P23-Neorthacris acuticeps acuticeps. P24-Poecilocera picta. P25-Pyrgomorpha bispinosa bispinosa.

3.1. Protein based tree

In this tree(Fig. 3) the species *A. c. crenulata* and *C. oxypterus* diverged from the root with two long branches and the node 'A' also resulted from the root further ramifies in to taxon *P. b. bispinosa* and the node 'B', the node 'B' terminates in node 'C' as well in *P. picta*on its other way ramification. The node 'C' ramifies in to two short branches resulting in two terminal taxa species *C. trachypterus* and *N. a. acuticeps* thus remained as outer most taxa in the tree.

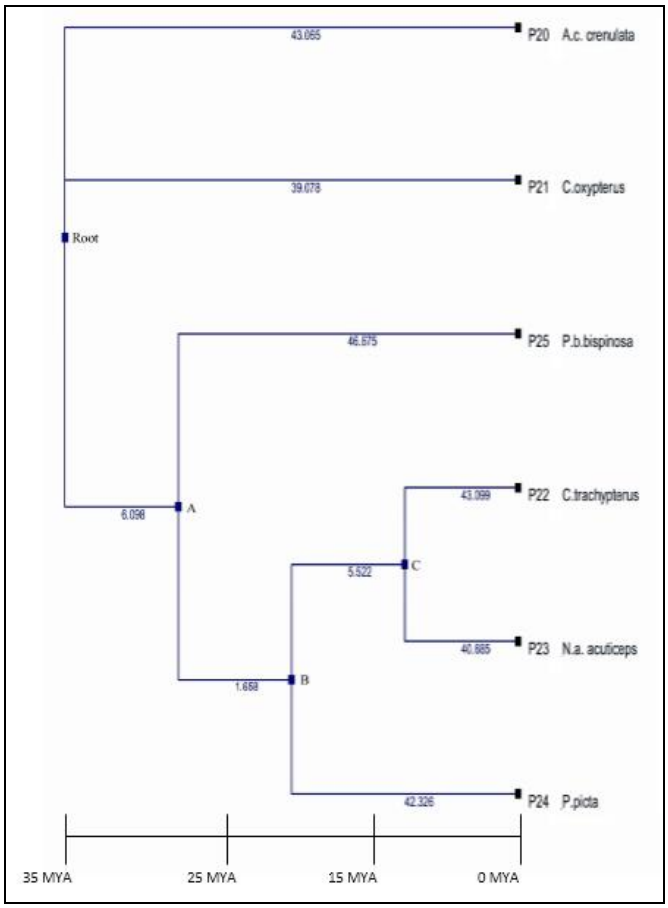

Fig 3: Phylogenetic tree based on Protein profile for 6 species of Pyrgomorphidae.

3.2. Alpha amylase based tree

The tree profile for alpha amylase phylogeny (Fig. 4) of the Pyrgomorphid grasshoppers has revealed that from the root bifurcation has taken place in two directions one carrying the initial lineage of two species *C. oxypterus* and *N. a. acuticeps* and second bifurcation to form the node 'A' that bifurcates in to two nodes 'B_1' and 'B_2' the node 'B_1' diverges to form two terminal taxa *C. oxypterus* and *P. b. bispinosa*. The node 'B_2' divides in to two branches to end up in two terminal species *C. trachypterus* and *P. picta*. This tree is slightly different from the former phylogenetic trees of Pyrgomorphidae.

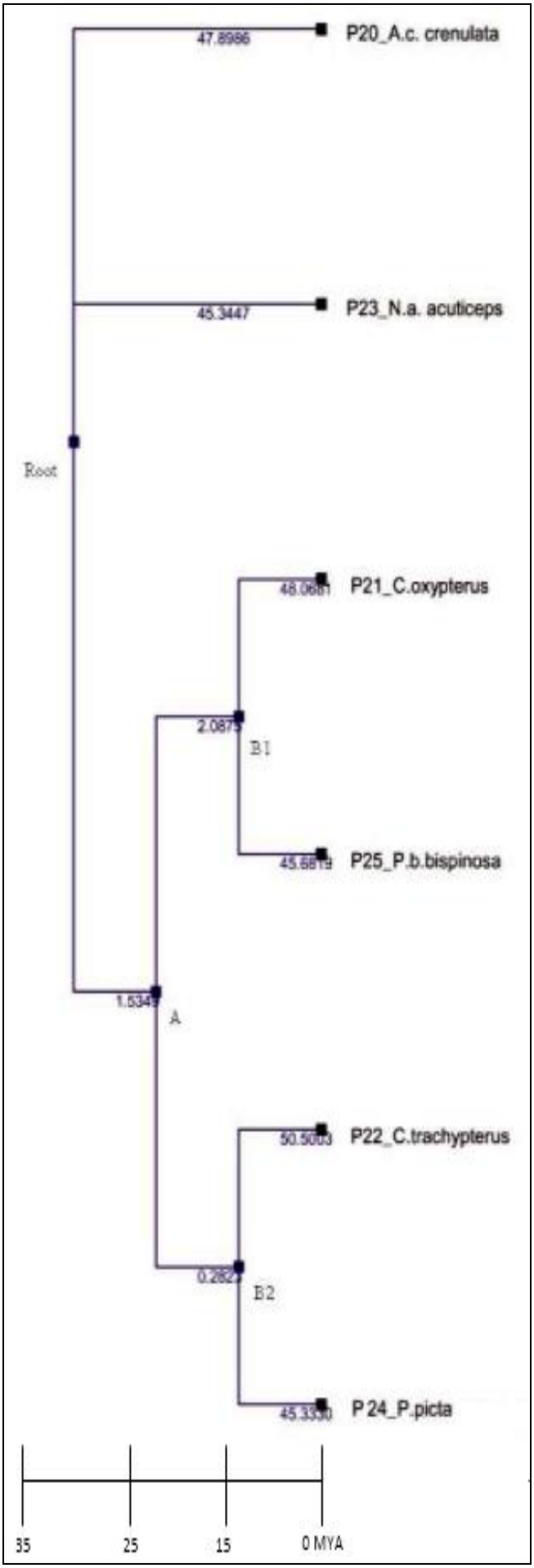

Fig 4: Phylogenetic tree based on Alpha Amylase profile for 6 species of Pyrgomorphidae.

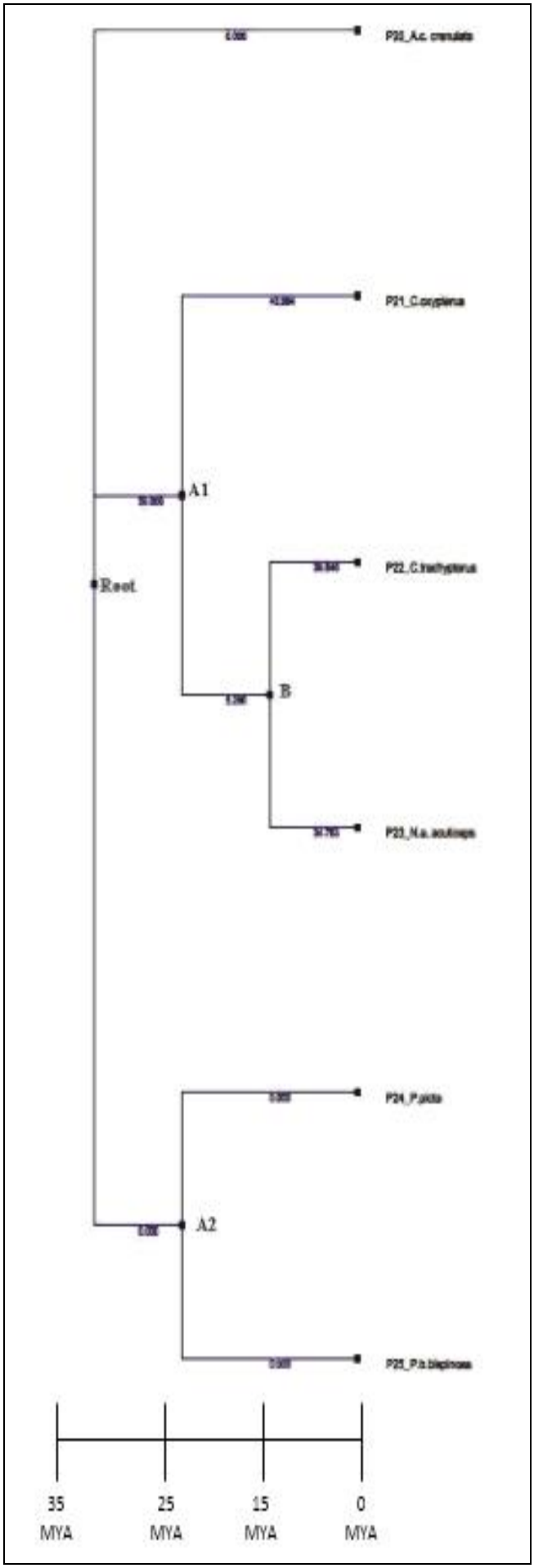

Fig 5: Phylogenetic tree based on Protein and Alpha amylase profile combined for species of pyrgomorphidae.

3.4. Protein+ Alpha amylase tree

Combined analysis of protein and alpha amylase variation has produced a rooted tree (Fig. 5) with a difference from the previous tree in its taxa profile and slight variation in branching pattern. From the root the ultimate ancestor of Pyrgomorphidae three simultaneous branching had taken place to produce an initial ancestral taxon *A.C. crenulata* two nodes 'A_1' and 'A_2'. The node 'A_1' ramifies in two directions to give rise to *C. oxypterus* and node 'B' that bifurcates to produce two taxa derivative *C. trachypterus* and *N. a. acuticeps* with shortest branches in the tree. The node 'A_2' forms the basal node in the analysis continue into two taxa *P. picta* and *P. b. bispinosa* with moderate branch length.

4. Discussion

Many evolutionary biologists concerned with molecular evolution have chosen alpha amylase genes as a favorite model among the many nuclear genes over the years. This model has been widely examined in *Drosophila* species as well in many other organisms. As many as seven types amylase are found in *D. ananassa* [13] and about 12 different types in other species of *Drosophila* [14]. This kind of duplication of amylase producing [amy] genes are generally found in other taxa also [15].

Phytophagous insects too have evolved different types of alpha amylase without loss of function and each isoform represent an allele [16], isoforms of amylase are important to insects as these provide an increased capability to adapt to different kinds of food and to resist activity of amylase inhibitors Grasshoppers are a diverse group in orthopterans and the evolution and radiation of these insects related with origin and radiation of grasslands [8, 9]. Diversity in diet may result in several morphological changes as progressive modifications due to different diets [10]. Pyrgomorphids are mainly graminiferous insects and many are phytophagous insects, a few feed on toxic plants thus exhibit greater diversity in their food habit. Such adaptations to feed on different starchy food source depends on adaptation of these insects by means of enzyme or protein changes. This divergence of habit might have led to production of different types of Amylases and proteins. In millions of years of diversification the pyrgomorphids might have developed many molecular strategies to combat innovative variations. Such variations taken origin from a common ancestor and its lineages provide an opportunity to trace the evolutionary relationship between the existing pyrgomorphids in relation to diversification of Amylases among these species. This diversification may be independent of several other factors.

Work on the role of protein polymorphism in speciation and evolutionary mechanisms in *Drosophila melanogaster* by Hubby and Levinton[12]through simple SDS-PAGE analysis generated a new line of thinking on molecular evolution through changes in protein. The importance of protein and enzyme polymorphism added a new dimension in phylogenetic analysis of plants [13, 14] and animals [15] Proteins being structural units of evolution these have a role to play in evolutionary process. Considering the importance of proteins and enzymes polymorphism in evolution, we have taken up the phylogenetic analysis of this family based on PAGE protein and alpha amylase analysis to identify the diversity within pyrgomorphidae to establish interspecies relationships. PAGE analysis of proteins and enzymes is still a powerful tool to understand genetic variation by separation of these molecules based on their molecular weight that reflects variation in structure of enzymes and proteins.

The emergence of new enzymes occur by gene duplication and divergence of its function which is favored by innovation-amplification-divergence but 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 [16, 17].Assuming that during in millions years of diversification of orthopterans, already innovation has taken place [8] and this has resulted in gene duplication as witnessed through electrophoresis result, by this the diversification could be analyzed. This diversification resulted by food type and related enzyme might have compelled a species to isolate from congenera as to become a separate species as evident in Darwin's Finches [18].

The tree derived on the basis of alpha amylase divergence is distinct from other two trees generated based on DNA and protein divergence for the 6 species of Pyrgomorphids. This tree resulted in a profile dominated by short branches. Manifestation of four taxa out of 6 species, as short branched terminal taxa is the unique feature of this tree. From the node basal bifurcation has taken by *A. c. crenulata* and *N. a. acuticeps* similar to that of DNA tree representing these more closer. The second line of deviation resulted in node 'A' that bifurcates in two directions to result in ancestors 'B_1' and 'B_2' lineages. From 'B_1' *C. oxypterus* and *P. b. bispinosa* are derived as the terminal taxa, likewise from node 'B_2' *C. trachypterus* and *P. picta* are derived as terminal taxa. Thus four taxa deviated simultaneously as terminal taxa but there is greater branch value difference in the 'B_2' diverged taxa. Even here the two congeneric forms remained as paraphyletic taxa in the monophyletic tree. The species relationship shared between the three trees generated differed for these 6 species of Pyrgomorphids.

Like amylase model, several workers have chosen protein diversity from muscles as a model to analyze genetic diversity. Phylogenetic relationship and evolutionary process in animals and plant have been studied using variations in protein electromorphs [19, 20, 21]. Evolutionary trees are constructed using many related factors like muscle tissue [22] and muscle proteins Actin, myosin and MRF families have been reviewed mentioning different types of trees as well changes in molecules and phylogenetic relationships between the molecular changes have been recorded. In some cases like Geckos [23] and Fishes [24] phylogenetic relationship is drawn based on the SDS PAGE profile of proteins and also using motor proteins of arthropods [25] The phylogenetic analysis of muscle related genes and proteins has revealed the grasshoppers and other orthopterans muscle related protein genes have evolved late among Neopteran insects, further diversified among these protein isoforms and genetic mechanism for producing them. This rich diversity suggests that studying of invertebrates muscle related proteins and genes can be usefully applied to resolve phylogenetic relationships and to understand protein assembly evolution [26]. Phylogeny of the family pyrgomorphidae has been analyzed based on the morphological features applying cladistic method has confirmed monophyletic origin of pyrgomorphids [27] that also coincides with our study, despite of dissimilar branching pattern of phylogenetic trees with respect to the molecules concerned in this study.

The tree generated based on protein profile is slightly modified from that of DNA based tree. [2] The initial ramification from the root leads to diversification of two species *A. c. crenulata* and *C. oxypterus* both of these have

long branches with different branch values. On the other way of ramification node 'A' is derived the sub ultimate ancestral lineage for Pyrgomorphids, from this a clade deviates to result in *P. b. bispinosa* and other clade ends in an ancestral lineage 'B' that further diverges to result in a branch with sub terminal taxa *P. picta* and ancestral lineage 'C' that ramifies to derive two species *C. trachypterus* and *N. a. acuticeps*. The *N. a. acuticeps* represented as one of the basal taxa in DNA based tree, here represented as terminal and youngest taxa. The *C. trachypterus* derived from second lineage in DNA tree has also represented as terminal taxa. Even the tribal level organization of the species in this tree profile is distinct from that of DNA tree.

Combined analysis of protein and alpha amylase variation has produced a rooted tree with a difference from the previous tree in its taxa profile and slight variation in branching pattern from the root the ultimate ancestor of Pyrgomorphidae three simultaneous branching had taken place to produce an initial ancestral taxon *A.C. crenulata* two nodes 'A_1' and 'A_2'. The node 'A_1' ramifies in two directions to give rise to *C. oxypterus* and node 'B' that bifurcates to produce two taxa derivative *C. trachypterus* and *N. a. acuticeps* with shortest branches in the tree. The node 'A_2' forms the basal node in the analysis continue into two taxa *P. picta* and *P. b. bispinosa* with moderate branch length. The hypothetical timeline [8]for each of the trees drawn showed the time and diversification of each species of grass hoppers with reference to either Alpha amylases or proteins or combination of both was different for each of the criteria as well did not show similarity in branch length and terminal taxa.

5. Conclusion

Evolutionary mode in six species of pyrgomorphids, as indicated by variations in alpha amylases has followed increase in number of isoforms for amylase and more number of muscle proteins. Polymorphism for these functional molecules revealed a wide range of variation in between the species. The phylogenetic trees represented monophyletic origin in these species but tree profile was not similar and had shuffled combination of individual species when compared with each other. Presence of greater polymorphism with least monomorphism among these indicated greater rate of evolution through variations independent of each other in each of the species that may be the reason to generate different trees for proteins and alpha amylase and combination of both these in this phylogenetic analysis.

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