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Isoenzyme Profile Of Stingless Bee (Hymenoptera: Apidae: Meliponini) Population Inhabited At Few Distinct Areas Of Southern Karnataka, India

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Abstract
Systematic investigation was conducted to analyze the isoenzyme profile of stingless bee population inhabited at urban, semi-urban, rural areas and rocky hilly areas at Mysore and Chamarajanagar districts of southern Karnataka. Moribund stingless bees were collected and preserved at -20°C and prepared for electrophoresis analysis of α and β-esterase by following standard methods. Results indicated that the stingless bee workers live at urban, semi-urban, rural area and rocky hilly areas revealed alpha and beta-esterase activities in terms of bands formation in varying intensities. Thus, it is evident that α and β esterase activity is not identical and indicated the occurrence of two distinct stingless bee species Tetragonula pagdeni and Tetragonal iridipennis population. Hence, present investigation demonstrated the occurrence of two species of stingless bees namely T. pagdeni and T. iridipennis with clusters at southern Karnataka.

Key words: *Isoenzymes, stingless bees, distinct areas, southern Karnataka*

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I. Introduction

Stingless bees or 'dammer bees' (Hymenoptera; Apidae: Meliponini) are the most abundant and diverse group of social insects, closely related to honeybees, bumble bees and carpenter bees. They are locally called 'Misri jenu' or 'Nasaru jenu'. They live in perennial colonies with specialized division of labour. Their colony composed with single queen (fertile female), few drones (males) and hundred to several thousands of worker bees (sterile females) (Inoue *et al*., 1984 and Michener, 2000). Analysis of isoenzyme profile is considered as one of the best methods in systematic biology (Kumar and Kumar, 2013) that provides an important source of evidences for the presence of genetic variations if any in a bee population (Ruvolo-Takasusuki *et al*., 2006) live at different habitats. Isoenzymes have a variety of physiological functions (e.g. degradation of neurotransmitters, metabolism of specific hormones and pheromones, growth, development and behaviour (Yan *et al*., 2009) in insects. Amongst various isoenzymes, esterase's are the most diverse families of enzymes, their different forms genetically determined by different loci and differently in tissues during various stages of ontogenetic development that help detect their high frequency of genetic variation (Ruvolo-Takasusuki *et al*., 2006 and Ronqui *et al*., 2016). To record such variations, gel electrophoresis has been extensively employed to study the insect systematics (Berlocher, 1984; Yan *et al*., 2009), evolution (Lewontin, 1966 and Loxdale *et al.*,1983), population genetics (Ramesh and Rajasekarasetty, 1980) and supports for phylogenetic relationships among insects (Kumar and Kumar, 2013) due to their high resolving power (Maurer *et al.*, 1972).

Lima and Mestriner (1985) have studied the electrophoretic patterns of esterases and non-specific proteins in 11 species of the Meliponini and recorded the variation in the activity of esterase-6 in *Melipona marginata* species and also the activity variation in esterase-4 of *M. nigra* species. Similarly, Falcao and Contel (1990) have studied the activity of esterase patterns in 10 species of Brazilian stingless bees and recorded the genetic variability in esterase activity expression in four bee species. Contel and Mestriner (1974) have studied the isoenzyme pattern in *M. quadrifasciata* and revealed the monomorphism for the same enzyme. Hashimoto (2003) and Attencia *et al.* (2005) have showed that alterations in the expression of esterase, which were employed to detect the presence of pesticidal and insecticidal residues such as organophosphorus and

neonicotinoids (Klein *et al.*, 2007 and Fermino *et al*., 2011). Ruvolo-Takasusuki *et al.* (2006) have reported the esterase activity regions in *T. angustula* and have identified the presence of two esterase i.e. β-esterase and α-β-esterase regions. Stuchi *et al*. (2012) have reported the inhibition pattern in *T. fiebrigi* and *T.angustula* and classified EST-1 as type I cholinesterase, EST-2 as type II cholinesterase and EST-3 as acetyl esterase. Fermino *et al*. (2011) have observed esterase variations due to insecticides in *T. angustula* and *T. fiebrigi* and recorded that *T. fiebrigi* is more sensitive to the paraquat herbicide than *T. angustula.* Costa *et al.* (2005) have examined the genetic variation in *Melipona rufiventris* population collected from two different geographical regions of Brazil using isoenymes namely: esterase and malate dehydrogenase-1. Ronqui *et al.* (2016) have made the electrophoretic characterization of *T. weyrauchi* esterases to identify esterase types through the use of inhibitors and shown thermo stability tests to check the expression pattern of total proteins. Stuchi *et al*. (2012) have used five types of esterases as molecular markers to identify two stingless bee species (e.g. *Tetragonisca angustula* and *Tetragonisca fiebrigi*) and recorded variations in esterase activity between these species. Moreover, several marked differences have been observed in the electrophoretic patterns of many isoenzymes in stingless bee species (Contel and Mestriner, 1974; Nunamaker and Wilson, 1982; Bitondi and Mestriner, 1983; Del Lama and Mestriner, 1984). Thus, esterases are effective enzymes for catalyzing the hydrolysis of wide ranges of aliphatic and aromatic esters, amides and thioesters, as well as many different processes. Moreover, these enzymes have a broad tissue distribution during insect development (Ruvolo-Takasusuki *et al*., 1997). Hence, isoenzymes are considered as molecular markers to identify different stingless bee species or sub-species levels. However, in India, reports on esterases activity in *Tetragonula* species are sparse and published reports are not available in southern Karnataka region. Hence, the present study was conducted to record the isoenzyme patterns in stingless bee population inhabited at different areas of southern Karnataka.

II. Material and methods

Study area: For the present investigation, urban, semi-urban, rural areas and rocky hilly areas at Mysore (latitude 11º45' to 12º40' N and longitude 75º 57' to 77º15' E) and Chamarajanagar (Latitude 11º40' to 12º06' N and Longitude 76º 46' E) districts of southern Karnataka (Kamath, 2001) were randomly selected. In Mysore district, the urban areas (e.g. Manasagangotri Campus, Devaraj Market, Ontikoppal Flower Market and Ramanuja Road) and rural area (e.g. Hunsur) were selected and in Chamarajanagar district, semi-urban area (e.g. Begur) and rocky-hill area (e.g. Terakanambi) were selected to collect the moribund stingless worker bees (Figure 1).

Sample collection: Maximum 20 moribund stingless bees were collected and preserved at -20°C for further analysis. The head and thorax from the body of worker bees were carefully separated using entomological scissors and homogenized in centrifuged tubes containing 0.1 ml of 40% sucrose solution and added a drop of bromophenol blue indicator. The homogenized mixture was centrifuged at 14000rpm for 15 minutes at -20º C.

Electrophoresis analysis: Native polyacrylamide gel (PAGE) (5%) was prepared and used for the analysis of α and β-esterase. Twenty millilitre of the clear supernatant from the homogenized mixture was taken and layered in the gel slots with the help of micropipettes. The electrophoresis was conducted by placing the gel running unit in refrigerator at 4°C and the unit was connected to the power pack at 40 volt for 20 to 30 minutes. The gels were initially run at 40V for 30 minutes and then at 65V for nearly 5 hours at 4°C employing the setup inside the refrigerator. The electrophoresis process was continued until the tracking dye bromophenol blue migrates to a distance of 7.5 cm in the separating gel. Gels were stained using the substrates α and β naphtyl acetate solution and fast blue RR salt solution which were diluted in phosphate buffer pH 6 and 7.2 for the detection of α and β esterase activity as per Ravolo-Takasusuki *et al*. (2006) with slight modifications. The gels were incubated for 30 minutes under the dark condition and scanned using Gel Doc-2001 Bio- Rad system to record the banding pattern of esterases. Moreover, the gels were fixed in 7% glacial acetic acid solution and scanned again using Gel Doc-2001 Bio- Rad system for the preparation of zymograms to reveal the band profile. Further, esterases were designated numerically according to their electrophoretic mobilities with the help of RF value that has been starting from the cathode with slight modification as required as per Ruvolo-Takasusuki *et al.* (2006). The specificity of the gels were done by staining the mixture with α-naphthyl acetate and β-naphthyl acetate and then results were compared and analyzed the difference in the isoenzyme pattern of esterases in stingless bee population as per Ruvolo-Takasusuki *et al.* (2006).

III. Results

The stingless bee workers collected from urban (e.g. Manasagangotri Campus, Devaraj Market, Ontikoppal Flower Market and Ramanuja Road) and rural area (e.g. Hunsur) in Mysore district and semi-urban area (e.g. Begur) and rocky-hill area (e.g. Terakanambi) in Chamarajanagar district of southern Karnataka revealed alpha and beta-esterase activities in terms of bands formation in varying intensities (Table 1). The polymorphism profile expressed by isoenzymes such as alpha and beta esterases in stingless bee workers population inhabited at distinct areas of southern Karnataka are depicted in Plates 1 to 4.

Alpha (α)-esterase: The lane 'A' represented the stingless bee worker from Manasagangotri Campus (Mysore-urban area) showed six regions of α-esterase activity bands with the retention factor (RF) values 0.552, 0.470, 0.411, 0.364, 0.188 and 0.094 (Plate 1). The lane 'B' represented the stingless bee workers collected from Devaraj Market (Mysore urban area) showed four regions of α-esterase activity bands with RF values 0.552, 0.470, 0.411 and 0.364 (Plate 1). The lane 'C' represented the stingless bee worker collected from Ontikoppal (Mysore-urban area) showed three regions of α-esterase activity bands with RF values 0.552, 0.491 and 0.192 (Plate 1). The lane 'D' represented the stingless bee workers collected from Hunsur (Mysore rural area) showed two regions of α-esterase activity bands with RF values 0.552 and 0.491 (Plate 1). The lane 'E' represented the stingless bee worker collected from Terakanambi (Chamarajanagar - rocky hill area) showed three regions of α-esterase activity bands with RF values 0.552, 0.491 and 0.188 (Plate 1). The lane 'F' represented the stingless bee worker collected from Begur (Chamarajanagar – semi-urban area) showed three regions of α-esterase activity bands with RF values 0.552, 0.411 and 0.188 (Plate 1). Thus, it is evident that α esterase activity is not identical and indicated the occurrence of two distinct stingless bee species population in these distinct areas of southern Karnataka. Further, α-esterase relative flow mobility value was almost nearer to 0.552 with the stingless worker bee population collected from urban, semi-urban, rural and rocky hilly areas in Mysore and Chamarajanagar districts and designated as stingless bee population 'A'. After critical analysis of 61 morphological parameters and molecular genetic analysis (e.g. Mt DNA – COX I gene), it was confirmed that the stingless bee population belong to *Tetragonula pagdeni* (Plate 1). However, results of such observations will be published elsewhere. In contrast to the population 'A', another stingless bee population that showed α-esterase relative flow mobility value less than 0.500 which were collected from the Mysore urban area i.e., Ramanuja Road in Mysore districts and designated as stingless bee population 'B' (Plate 2). Similarly, after critical analysis of 61 morphological parameters and molecular genetic analysis (e.g. Mt DNA – COX I gene), it was confirmed that the stingless bee population belong to *Tetragonula iridipennis* (Plate 1). However, results of such observations will be published elsewhere.

Beta (β)-esterase: The lane 'A' represented the stingless bee workers collected from Manasagangotri Campus (Mysore- urban area) showed four regions of β-esterase activity bands with RF values 0.517, 0.435, 0.376 and 0.317 (Plate 3). The lane 'B' represents the stingless bee workers collected from Devaraj market (Mysore-urban area) showed four regions of β-esterase activity bands with RF values 0.517, 0.435, 0.381 and 0.334 (Plate 3). The lane 'C' represents the stingless bee workers collected from Ontikoppal Flower Market (Mysore urban area) showed three regions of β esterase activity bands with the RF values 0.517, 0.435 and 0.334 (Plate 3). The lane 'D' represents the stingless bee workers collected from Hunsur (Mysore-rural area) showed two regions of β-esterase activity bands with the RF value 0.517, 0.435, 0.376 and 0.317 (Plate 3). The lane 'E' represents the *Tetragonula pagdeni* collected from Terakanambi (Chamarajanagar-rocky-hill area) showed four regions of β-esterase activity bands with the RF values 0.517, 0.388, 0.317 and 0.203 (Plate 2). The lane 'F' represents the stingless bee workers collected from Begur (Chamarajanagar –semi-urban area) showed two regions of β-esterase activity bands with RF value of 0.517 and 0.388 (Plate 3). Moreover, the β-esterase activity relative flow mobility (RF) value was nearer to 0.517 in the stingless bee worker population collected from different distinct areas such as Manasagangotri Campus, Devaraj Market, Ontikoppal Flower market and Ranuja Road (Mysore - urban areas) (Plate 4), Hunsur (Mysore rural area), Terakanambi (Chamarajanagar – semi-urban area) and Begur (Chamarajanagar – rocky hilly area). Interestingly, the β-esterase activity relative flow mobility (RF) value was 0.435 and almost similar in stingless bee worker population at Mysore district i.e., Ontikoppal (Mysore urban area) and Hunsur (Mysore rural area), and Chamarajanagar district i.e., Terakanambi (Chamarajanagar – rocky hill area). Similarly, the β-esterase activity relative flow mobility (RF) value was 0.388 and almost similar in stingless bee worker population collected from Chamrajanagar district i.e., (Begur– semi-urban area and Terakanambi – rocky hill area) (Plate 3). After critical analysis of 61 morphological parameters and molecular genetic analysis (e.g. Mt DNA – COX I gene), it was confirmed that the stingless bee population belong to *Tetragonula pagdeni* (Plate 1). However, results of such observations will be published elsewhere.

In contrast to the *Tetragonula iridipennis*, the electrophoretic analysis of beta-esterase activity in *Tetragonula iridipennis* showed only one region of β-esterase activity band with the RF value 0.196 (Plate 4). The beta-esterase activity banding pattern in *Tetragonula iridipennis* didn't show any similarity with the β-esterase activity banding pattern of *Tetragonula pagdeni* populations with reference to RF values (Table 1). These variations further suggested the inter-specific variations existed among the stingless bee population which belong to same species inhabited at different areas of southern Karnataka. In contrast to the population 'A', another stingless bee population that showed only one region of β-esterase relative flow mobility value 0.196 (Plate 4). The β-esterase activity banding pattern in stingless bee workers collected from urban, semi-urban, rural and rocky hilly areas in Mysore and Chamarajanagar districts didn't show any similarity with the β-esterase activity and designated as stingless bee population 'B'. Similarly, after critical analysis of 61

morphological parameters and molecular genetic analysis (e.g. Mt DNA – COX I gene), it was confirmed that the stingless bee population belong to *Tetragonula iridipennis* (Plates 1 to 4 and Tables 1 and 2). However, results of such observations will be published elsewhere.

IV. Discussion

The differences of esterase activity in terms of their different banding patterns in the body of stingless bee, *Tetragona* species indicated considerable variations. The activity of esterase's in ten different bands utilizing the α-naphthyl acetate substrates, where eight α-esterase bands were obtained in stingless bee population 'A' (e.g. *Teragonula pagdeni*) worker bees and only two α-esterase bands were observed in stingless bee population 'B' (e.g. *Teragonula iridipennis*). Moreover, nine different bands β-naphthyl acetate substrates, where eight β-esterase bands were observed in stingless bee worker population 'A' (e.g. *Teragonula pagdeni*) and only one β-esterase band was observed in stingless bee worker population 'B' (e.g. *Tetragonula iridipennis*). In contrast to the present investigation, Ruvolo-Takasasusuki *et al.* (2006) have reported the presence of two esterase banding activity in *Tetragonisca angustula* and seven banding activity in *Tetragonisca clavipes.* Ronqui *et al.* (2016) have reported the presence of six esterase banding activity in *Tetragonisca weyrauchi.* Moreover, in *Melipona ruventris* the activity of esterases varied from single band to two bands within the species suggesting the difference in esterase banding activity due to different geographical regions. Thus, present study corroborate the observations of Ruvolo-Takasusuki *et al.* (2006) who have recorded the genetic variation in two *Trigona* species collected from two cities in the southern Brazilian state of Parana and detected two bands of esterase in *T.angustula* and seven bands of esterase in *T. clavipes*. The esterase-6 activity found in abdomen and esterase-7 was found in head and thorax region. The involvement of these enzymes in ontogenetic development is not clear and need to be further investigated. Fermino *et al.* (2011) have also evaluated the changes in the expression of isoenymes (e.g. esterases (EST), malate dehydrogenase (MDH) and superoxide dismutase (SDM) in *T. angustula* and *T. fibrigi* which were collected from natural nests located at the State University of Maringa, Brazil, after herbicide (e.g. Nicosulfuron and Paraquat) contamination and recorded the influence on the expression of esterases and superoxide dismutase. The results showed that the *T. fiebrigi* was more sensitive to herbicides compared to *T. angustula*. Perhaps, at distinct areas of Mysore and Chamarajanagar districts, locally existed different physiographic features and ecological factors might have interfered with the activity of α and β esterase's and that could have expressed different banding patterns which is evidenced during the present investigation. To support the different banding patterns of stingless bee population inhabited at different areas such as urban, semi-urban, rural area and rocky hill areas of Mysore and Chamarajanagar districts indicated survival of two species namely: *Teragonula pagdeni* and *Tetragonula iridipennis* in this part of the state. However, to conclude for the existence of two different species, in depth molecular genetic analysis is necessitated. Such type of investigations are made to arrive the existence of *Teragonula pagdeni* and *Tetragonula iridipennis* population at urban, semi-urban, rural and rocky hilly areas of Mysore and Chamarajanagar districts. Results of such observations will be published elsewhere.

On this line, several researchers (Contel and Mestriner, 1974; Ramesh and Rajasekarasetty, 1980; Nunamaker and Wilson, 1982; Loxdale *et al*., 1983; Bitondi and Mestriner, 1983; Del Lama and Mestriner, 1984; Lima and Mestriner, 1985; Falcao and Contel, 1990; Costa *et al*., 2005; Kumar and Kumar, 2013; Ronqui *et al*., 2016 and Ruvolo-Takasusuki *et al*., 2017) have reported the esterase enzymes activity in social bee species such as *Apis mellifera, Melipona rufiventris, Tetragonisca weyrauchi T. angustula* and *T. clavipes* and non-social insects such as *Drosophila* and Aphids. Bitondi and Mestriner (1983) have recorded in esterase isozymes variability in *Apis mellifera*. Costa *et al*. (2005) have recorded the isoenzyme variation in *Melipona rufiventris* collected from Minas Gerais State, Brazil. Similarly, Del Lama and Mestriner (1984) have analyzed exopeptidase activity and identified 14 different species of bees. Moreover, Falcao and Contel (1990) have recorded the isozyme patterns and polymorphism for esterases and recorded the variability in natural populations of Brazilian social bees. Further, Nunamaker and Wilson (1982) have studied the isozyme changes in the honeybee, *Apis mellifera* during larval morphogenesis. Ronqui *et al*. (2016) have studied the esterases using electrophoretic methods to distinguish stingless bee, *Tetragonisca weyrauchi* at different regions. Ruvolo-Takasusuki *et al.* (2017) have identified the *Tetragonisca angustula* and *Tetragona clavipes* using esterases enzymes. However, Ramesh and Rajasekarasetty (1980) have studied the isozyme variations in *Drosophila nasuta* subgroup. Thus, during the present investigations, esterase's help distinguish the occurrence of different species of stingless bee population at distinct areas of Mysore and Chamarajanagar districts of southern Karnataka. Hence, activity of esterases varied from species to species and within the species collected from different distinct areas within Mysore and Chamarajanagar districts. Thus, isoenzyme analysis help identify the variation within the stingless bee population and between the species of stingless bees. This analysis has demonstrated the occurrence of two species of stingless bees namely *T. pagdeni* and *T. iridipennis* with clusters at southern Karnataka. Our observations are similar to the observations of Contel and Mestriner (1974), Ramesh and Rajasekarasetty (1980), Nunamaker and Wilson (1982), Loxdale *et al*. (1983), Bitondi and Mestriner

(1983), Del Lama and Mestriner (1984), Lima and Mestriner (1985), Falcao and Contel (1990), Costa *et al.* (2005), Kumar and Kumar (2013), Ronqui *et al*. (2016) and Ruvolo-Takasusuki *et al*. (2017).

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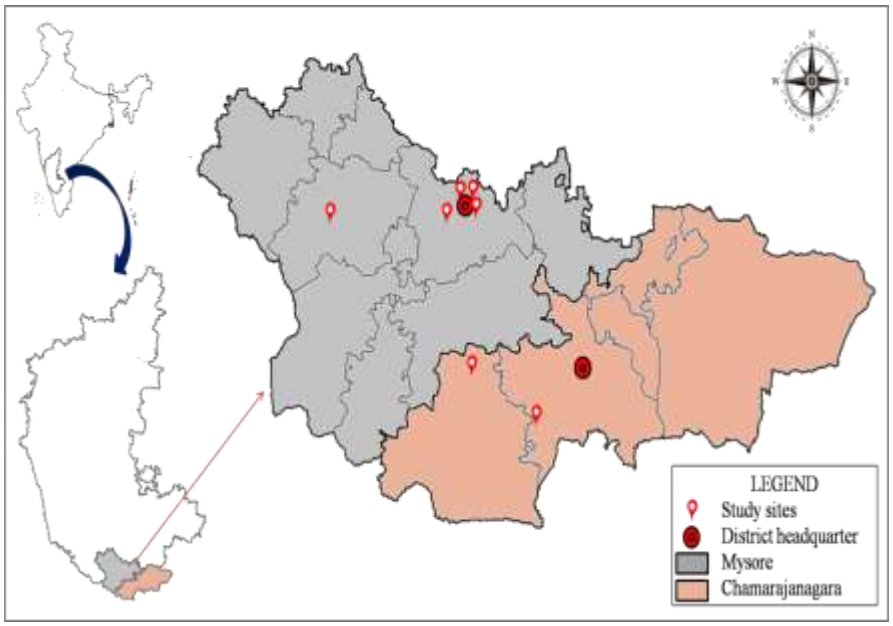

Figure 1. Map showing the study areas selected at Mysore and Chamarajanagar districts of southern Karnataka

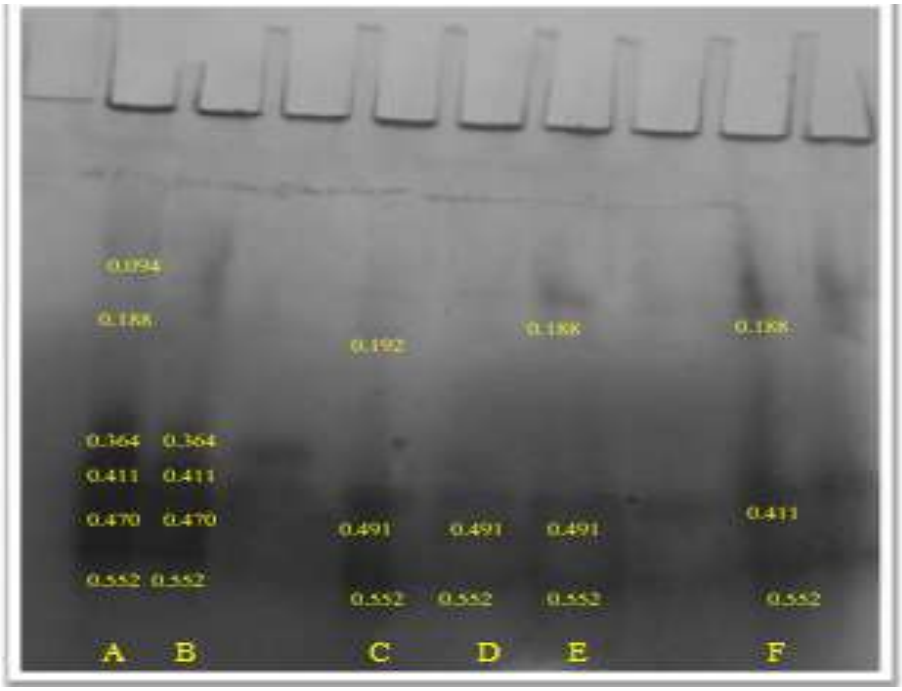

Plate 1. Banding pattern and activity of alpha-esterase in *Tetragonula pagdeni* workers inhabited at different geographical areas of southern Karnataka

'A': Manasagangotri Campus (Mysore - urban area); 'B': Devaraj Market (Mysore - urban area); 'C': Ontikopal Flower Market (Mysore - urban area); 'D': Hunsur (Mysore - rural area); 'E': Terakanambi (Chamarajanagar – rocky hill area) and 'F': Begur (Chamarajanagar – semi-urban area).

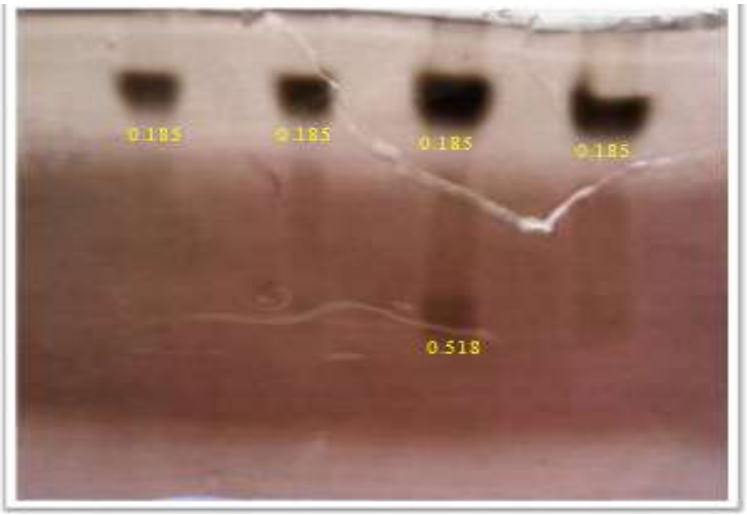

Plate 2. Banding pattern and activity of alpha-esterase in *Tetragonula iridipennis* worker inhabited in Ramanuja Road (Mysore - urban area).

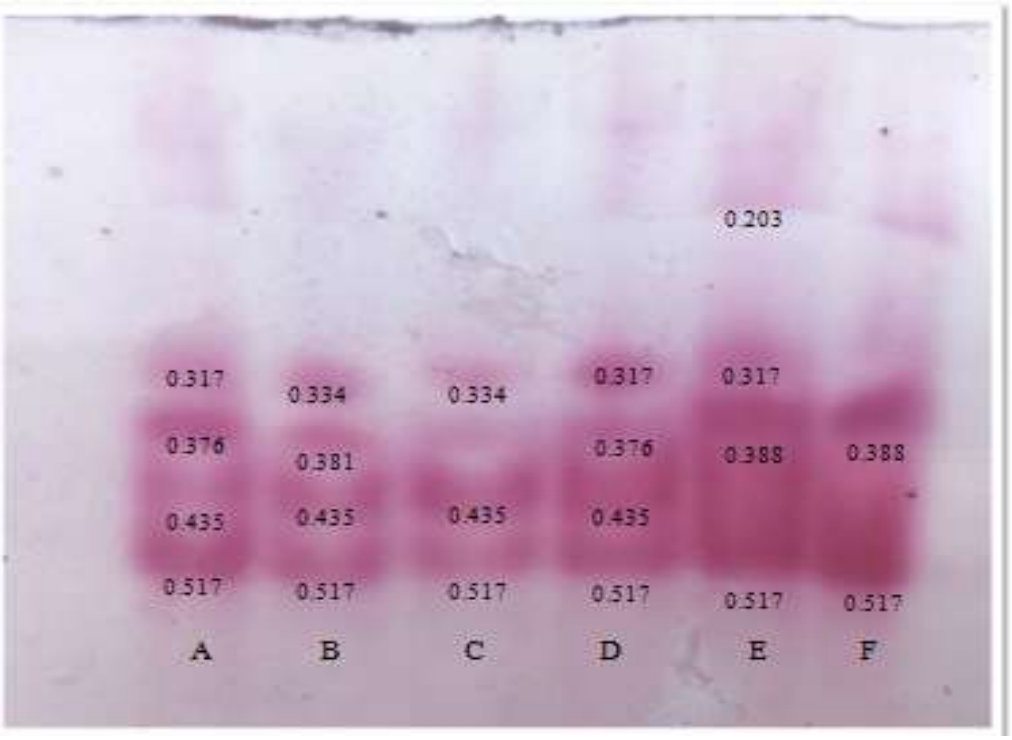

Plate 3. Banding pattern and activity of beta-esterase in *Tetragonula pagdeni* workers inhabited at different geographical areas of southern Karnataka

'A': Manasagangotri Campus (Mysore - urban area); 'B': Devaraj Market (Mysore - urban area); 'C': Ontikopal Flower Market (Mysore - urban area); 'D': Hunsur (Mysore - rural area); 'E': Terakanambi (Chamarajanagar – rocky hill area) and 'F': Begur (Chamarajanagar – semi-urban area).

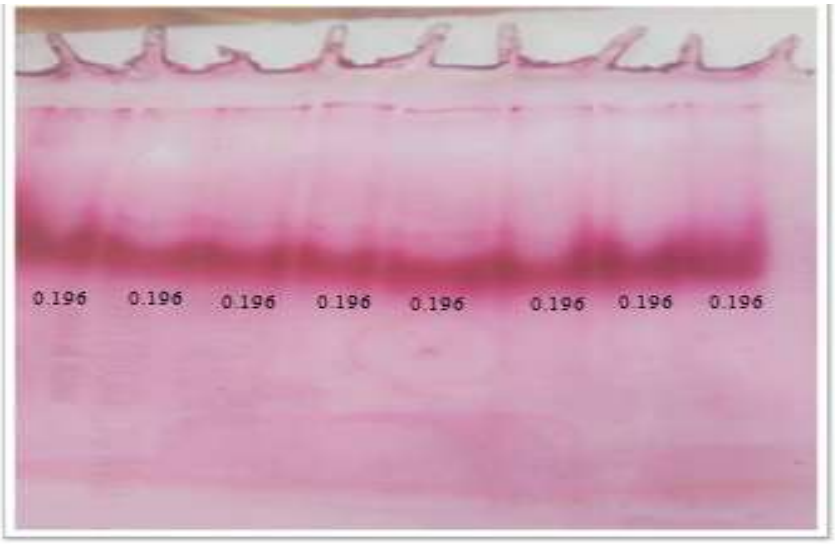

Plate 4. Banding pattern and activity of beta-esterase in *Tetragonula iridipennis* workers inhabited at Ramanuja Road (Mysore - urban area)

Table 1. Electrophoretic variations recorded in alpha esterase banding pattern in *Tetragonula* species inhabited at different geographical areas of southern Karnataka

Habitat	Area Region	Species identified	Banding pattern with retention factor (RF) value										No. of Bands recorded
			0.552	0.518	0.491	0.47	0.411	0.364	0.192	0.188	0.185	0.094	
Urban Area	MGC	*T. pagdeni*	+	-	-	+	+	+	-	+	-	+	6
	DM		+	-	-	+	+	+	-	-	-	-	4
	OFM		+	-	+	-	-	-	-	-	-	-	2
	RR	*T. iridipennis*	-	+	-	-	-	-	-	-	+	-	2
Rural Area	H	*T. pagdeni*	+	-	+	-	-	-	+	-	-	-	3
Rocky-hill Area	TKN		+	-	+	-	-	-	-	+	-	-	3
Semi-urban Area	B		+	-	-	-	+	-	-	+	-	-	3

Note: +: Present; -: Absent; MGC: Manasagangotri Campus; DM: Devaraj Market; OFM: Ontikopal Flower Market; RR: Ramanuja Road; H: Hunsur; TKN: Terakanambi and B: Begur.

Table 2. Electrophoretic variations recorded in beta esterase banding pattern in *Tetragonula* species inhabited at different geographical areas of southern Karnataka

Habitat	Area Region	Species identified	Banding pattern with retention factor (RF) value									No. of Bands recorded
			0.517	0.435	0.388	0.381	0.376	0.334	0.317	0.203	0.196	
Urban Area	MGC	*T. pagdeni*	+	+	-	-	+	-	+	-	-	4
	DM		+	+	-	+	-	+	-	-	-	4
	OFM		+	+	-	-	+	-	+	-	-	4
	RR	*T. iridipennis*	-	-	-	-	-	-	-	-	+	1
Rural Area	H	*T. pagdeni*	+	+	-	-	-	+	-	-	-	3
Rocky-hill Area	TKN		+	-	+	-	-	-	+	+	-	4
Semi-urban Area	B		+	-	+	-	-	-	-	-	-	2

Note: +: Present; -: Absent; MGC: Manasagangotri Campus; DM: Devaraj Market; OFM: Ontikopal Flower Market; RR: Ramanuja Road; H: Hunsur; TKN: Terakanambi and B: Begur