Molecular phylogeny and venom characterization of Indian Scorpions

Thesis submitted in partial fulfillment of the requirements

of Five Year BS-MS Dual Degree Program

Indian Institute of Science Education and Research, Pune

By

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Certificate

This is to certify that this dissertation entitled "**Molecular phylogeny and venom characterization of Indian Scorpions**" towards the partial fulfilment of the BS-MS dual degree programme at the Indian Institute of Science Education and Research, Pune, represents work carried out by Vivek Suranse at IISER Pune under the supervision of Dr. Neelesh Dahanukar, DST INSPIRE Faculty Fellow, Department of Biology, during the academic year 2016-2017.

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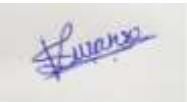
Date: 20 March 2017

Declaration

I hereby declare that the matter embodied in the report entitled "**Molecular phylogeny and venom characterization of Indian Scorpions**" are the results of the work carried out by me at the Department of Biology, IISER Pune, under the supervision of Dr.Neelesh Dahanukar and the same has not been submitted elsewhere for any other degree.



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List of figures –

List of tables –

Acknowledgements

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As this project involved a large number of surveys and field work many people have been instrumental in making this project a success. I want to express a kudos to Nitin Sawant, my lab mate for his sincere and never ending efforts both on field and in lab. I want to thank Arjun C.P. for putting efforts in collecting and transporting the entire collection from Kerela. Pradeep Khumkar and Nikhil Dandekar have had an important share in field trips. Chaitany Risbud and Eshaan Pahade have been of great help in the Goa field trip.

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Most importantly I am thankful to my parents for their support and encouragement in pursuing this project. I want to appreciate all my friends for their constructive inputs at different stages of the project and I urge an encore of the very same in future.

Abstract

Scorpions have always been a neglected group of arthropods from scientific inquiry point of view although they are medically important because of their venom. India has a huge diversity of scorpion fauna yet effective assessment of the available diversity has not been performed. Periodic taxonomic revisions are not undertaken and till date, archaic methods of identification are practiced. Only a handful of studies have looked at the evolutionary relationships of scorpions in India. India is the abode of 'The Indian Red Scorpion (*Hottentotta tamulus*)', a scorpion species regarded as the most venomous scorpion and medicinally very important, yet a thorough venom analysis for this species is not done. In this effect, the current study deals with three aspects, namely (1) A general overview of molecular phylogeny of scorpions in Indian peninsula using the mitochondrial barcoding gene cytochrome oxidase subunit 1 (COI), (2) population genetics and morphometric analysis of *H. tamulus* from regions with high rainfall, moderate rainfall and dry regions and (3) preliminary analysis to characterize venom of *H.tamulus* from different populations to understand population variation. Total 196 samples were collected out of which genetic analysis was successful for 67 samples. I provide first genetic characterization of *Heterometrus phipsoni* of the family Scorpionidae and *Chiromachetes sahyadriensis* of family *Hormuridae* along with several species from family *Buthidae*. Genetic analysis suggests that there are several taxonomic issues with the species of *Lychas*, *Isometrus* and *Hottentotta*, which are likely to harbour undescribed species. Population Genetics study was carried out by studying haplotype diversity in barcoding COI gene. In population genetics study, *H.tamulus* formed three groups corresponding to high rainfall area on the western side of Western Ghats, the moderate rainfall area on eastern side of Western Ghats and dry region. There are various genetic mutations in samples from different *H. tamulus* populations, yet they code for protein with same amino acid sequence except for a few samples. Even though there is variation in populations of H. tamulus, there seems to be presence of conserved peptides in their venom concoctions. Further investigation is required to ascertain the exact nature and composition of venom

Introduction

Scorpions are arthropods belonging to the class of arachnids with their early appearance in aquatic forms during the Silurian Era (444–416 MYA) (Briggs 1987). More than 1500 species of scorpions with 13 extant families have been identified and described (Fet 2000). Scorpion lineage has an evolutionary history of ~400 million years and yet their basic morphology is very conserved and hence they have been aptly regarded as 'living fossils'. However, their presence in diverse habitats and topographies, from arid deserts to mangroves, suggests that they have greatly evolved in order to survive in such a broad range of environments. Scorpions are nocturnal creatures, generally docile and equipped with pincers and venom glands at the end of their tail to subdue their prey. All scorpions are venomous but only about 30 species are known to be fatal to humans.

Scorpion venom is a concoction of diverse peptides, mainly neurotoxins in nature, yet recently Buthids and super-family Chactidae are believed to have cytotoxic properties as well. Scorpion sting has different effects ranging from excruciating pain to as severe as death. Globally, there are around 1.5 million envenomings with around 2600 lethality annually, with more incidences of stinging in adults but severe responses in young children (Chippaux 2012), this might be an underestimation as there are innumerable cases that are not reported. Absence of proper antivenins aggravates the severity of the situation. 'Prozasin' is the only drug available as a treatment to scorpion bite in the country. Studying scorpion venoms is crucial in the present day scenario.

India is a vast nation with myriad natural habitats and harbors a diverse scorpion fauna. The taxon is represented by 18 Genera under 6 Families viz. Buthidae, *Chaerilidae*, *Euscorpiidae*, *Scorpionidae*, *Hormuridae* and *Vaejovidae* (Tikader and Bastawade 1983). Most of the studies undertaken are mainly taxonomy based and lack the understanding of evolutionary relationships in scorpions. Moreover, there have been reports on many new species (Javed et al. 2016; 2010; Kovarik et. al. 2007; Kovarik 2013; Lourenco 2003; Lorenco 2005; Mirza et al. 2016; Mirza and Sanap 2010; Zambre et al. 2011; Zambre and Patil 2011; Mirza and Gowande 2016a,b) suggesting that the current data pool available on Indian scorpions is rather insufficient and an exhaustive

study encompassing taxonomy, evolution, behavioural biology and reproductive biology of these organism is indispensable. Only a handful of phylogenetic studies have been conducted on scorpions of India.

Venoms have enormous medicinal importance. Their use in many medicinal recipes have been indicated in the folk-lore. They contain a plethora of bioactive compounds that can be harnessed as drugs for many catastrophic diseases. Chemical investigation of peptide fragments suggests that they can be used as scaffolds for drug designing. Many naturally occurring peptides in scorpions have unique structural properties for example a peptide fragment from scorpion has structure similar to T-cell CD4 receptor and thus can be used as a mimetic compound for treatment of AIDS (Huang et al. 2005). Scorpine, an anti-malarial and anti-bacterial compound has been isolated from scorpion venom (Conde et al. 2001). Scorpion toxin-derived potent p53-MDM2/MDMX inhibitor, a mini protein kills dependent tumor cells in p53 dependent manner by competitively inhibiting p53-MDM2/MDMX interaction (Li et al. 2008).

A broad study that addresses scorpion evolution, diversity and distribution and their venom peptide identification and characterization could provide a better understanding about their relationship. This project is in itself a novel approach towards studying ‘Evolutionary Venomics’ and has potential to change the current situation of venom research in India. There are three parts to the project. First part deals with the molecular phylogeny based on mitochondrial cytochrome oxidase subunit 1 (COI) partial gene sequence , which is an extension to our published work (Suranse et al. 2016). Second part deals with population genetics of *H. tamulus* and third part deals with venom characterization of different populations of *H. tamulus*.

Materials and methods

Specimen collection, preservation, identification and museum deposition

Extensive field trips were carried out in eastern and western Maharashtra as well as throughout the Western Ghats and also from other areas in India (Table 1). Specimens were collected from different locations. Some samples were provided by collaborators. The study was carried out with samples collected during the tenure of the project as well as samples collected prior to the project timeline, but processed during the project. From the total of 196 samples, family wise, Buthidae (n=159), Cherilidae (n=3), Euscorpiidae (n=7), Hormuridae (n=6) and Scorpionidae (n=21). The map (Figure 1) shows the location of sampling areas.

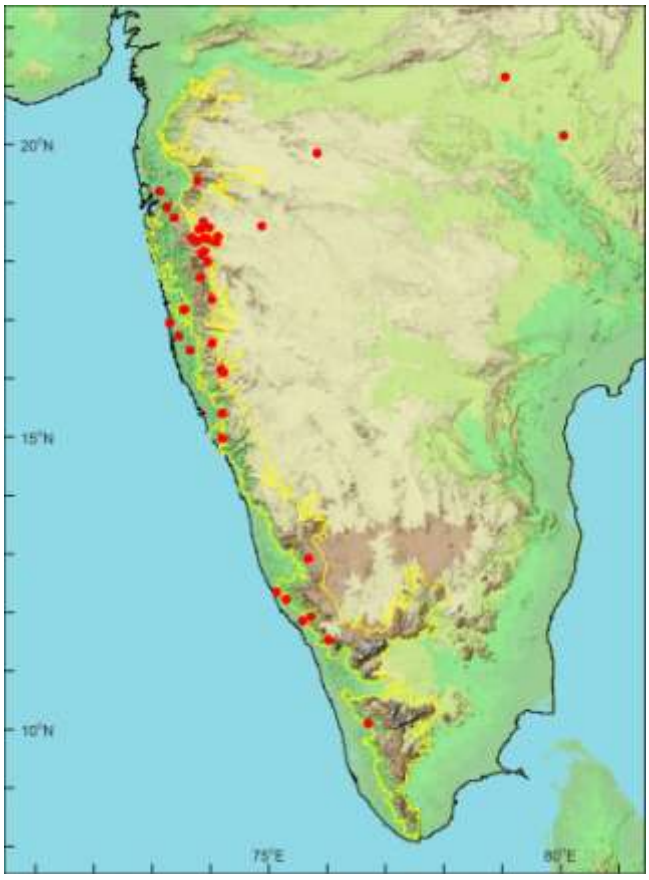


Figure 1: Map of peninsular India with sampling locations. Exact locations of samples from Arunachal Pradesh were not available so those are not shown.

Table 1: Samples collected for the present study. Samples with an asterisk were collected by me with collaborators, while other samples were provided by collaborators.

FAMILY/Species	Location	Date	Code	Lat (deg)	Lon (deg)	Alt (m)	Genetic analysis
BUTHIDAE							
Buthoscorp sp.	Gadchiroli, Potegaon	02/10/2016	HotGdc1	20.163	80.054	213	Not Worked
*Buthoscorpiops sp. **	Nature's Nest, Tambdi Surla, Goa	12/09/2016	BscNnt1	15.411	74.200	58	Not Worked
*Buthoscorpiops sp. **	Nature's Nest, Tambdi Surla, Goa	12/09/2016	BscNnt2	15.411	74.200	58	Not Worked
*Buthoscorpiops sp. **	Nature's Nest, Tambdi Surla, Goa	12/09/2016	BscNnt3	15.411	74.200	58	Not Worked
*Buthoscorpiops sp. **	Nature's Nest, Tambdi Surla, Goa	12/09/2016	BscNnt4	15.411	74.200	58	Not Worked
*Buthoscorpiops sp. **	Nature's Nest, Tambdi Surla, Goa	12/09/2016	BscNnt5	15.411	74.200	58	Not Worked
Charmus sp.	Matheran	26/06/2016	ChMth1	18.942	73.229	183	Worked
Hottentotta cf pachyurus	Ajara	18/05/2015	HotAj2	16.164	74.155	739	Not Worked
*Hottentotta cf pachyurus **	IISER Pune	01/01/2015	IP1	18.545	73.806	580	Not Worked
*Hottentotta cf pachyurus **	Sinhagad Valley	15/01/2015	Snp1	18.364	73.762	1194	Not Worked
*Hottentotta pachyurus **	Sangrun	08/06/2015	Sng1	18.398	73.678	600	Worked
Hottentotta cf rugiscutis	Kaas Plateau	01/08/2015	KpHr	17.717	73.821	1229	Worked
Hottentotta cf rugiscutis	Kaas Plateau	01/08/2015	KpHra	17.717	73.821	1229	Not Worked
*Hottentotta pachyurus **	IISER, Pune	20/11/2016	HotIp1	18.548	73.806	575	Worked
*Hottentotta pachyurus **	Sinhagad	05/01/2016	HotSin1	18.350	73.740	1105	Not Worked
Hottentotta rugiscutis	Ajara	18/05/2015	HotAj1	16.164	74.155	739	Not Worked
Hottentotta rugiscutis	Ajara	16/09/2015	AjHp	16.111	74.220	669	Worked
Hottentotta rugiscutis	Harpawade Plateau	05/09/2015	PH1	16.615	74.026	814	Not Worked
Hottentotta rugiscutis	Harpawade Plateau	05/09/2015	PH2	16.615	74.026	814	Not Worked
Hottentotta rugiscutis	Harpawade Plateau	05/09/2015	PH3	16.615	74.026	814	Worked
Hottentotta rugiscutis	Tamhini	2016	HotRgTm1	16.111	74.220	669	Not Worked
Hottentotta rugiscutis	Tamhini	05/09/2015	HotRgTm2	18.766	73.360	455	Not Worked
Hottentotta rugiscutis	Tamhini	01/10/2016	HotRgTm3	18.766	73.360	455	Not Worked
Hottentotta sp.	Harishchandra Gadh	01/05/2016	HotHgd2	19.387	73.774	1342	Not Worked
Hottentotta sp.	Harishchandra Gadh	01/05/2016	HotHgd3	19.387	73.774	1342	Not Worked

Hottentotta sp.	Harpawade Ajara	28/03/2016	HotH1	16.111	74.220	669	Worked
Hottentotta sp.	Harpawade Ajara	28/03/2016	HotH2	16.111	74.220	669	Worked
Hottentotta sp.	Tamhini	2016	HoTmL	16.111	74.220	669	Not Worked
*Hottentotta sp. **	Devi Hasole, Laterite Outcrop	13/11/2016	HotDev1	16.736	73.444	170	Not Worked
*Hottentotta sp. **	Devi Hasole, Laterite Outcrop	14/11/2016	HotDev2	16.736	73.444	170	Worked
*Hottentotta sp. **	Nature's Nest, Tambdi Surla, Goa	12/09/2016	HotNnt2	15.411	74.200	58	Worked
*Hottentotta sp. **	Nature's Nest, Tambdi Surla, Goa	12/09/2016	HotNnt3	15.411	74.200	58	Not Worked
*Hottentotta sp. **	Nature's Nest, Tambdi Surla, Goa	12/09/2016	HotNnt4	15.411	74.200	58	Not Worked
*Hottentotta sp. **	Near Talere	12/11/2016	HotTlr1	16.478	73.650	129	Not Worked
*Hottentotta sp. **	Near Talere	12/11/2016	HotTlr2	16.478	73.650	129	Worked
*Hottentotta sp. **	Near Talere	12/11/2016	HotTlr3	16.478	73.650	129	Not Worked
Hottentotta tamulus	Bhor	07/08/2015	HoBho1	18.141	73.836	631	Not Worked
Hottentotta tamulus	Bhor	07/08/2015	HoBho2	18.141	73.836	631	Not Worked
Hottentotta tamulus	Harishchandra Gadh	01/05/2016	HotHgd1	19.387	73.774	1342	Worked
Hottentotta tamulus	Matheran	26/06/2016	HotMth1	18.942	73.229	183	Not Worked
Hottentotta tamulus	Matheran	26/06/2016	HotMth2	18.942	73.229	183	Not Worked
Hottentotta tamulus	Matheran	26/06/2016	HotMth3	18.942	73.229	183	Not Worked
Hottentotta tamulus	Mayureshwar	06/06/2016	HotMyr	18.000	73.950	839	Not Worked
Hottentotta tamulus	Rehekuri	08/06/2016	HotRkr	18.600	74.870	585	Not Worked
Hottentotta tamulus	Sangameshwar, Konkan	10/09/2016	HotSgwr1	17.181	73.549	39	Worked
Hottentotta tamulus	Sangameshwar, Konkan	10/09/2016	HotSgwr2	17.181	73.549	39	Worked
Hottentotta tamulus	Sangameshwar, Konkan	10/09/2016	HotSgwr3	17.181	73.549	39	Worked
Hottentotta tamulus	Sangameshwar, Konkan	10/09/2016	HotSgwr4	17.181	73.549	39	Worked
Hottentotta tamulus	Sangameshwar, Konkan	10/09/2016	HotSgwr5	17.181	73.549	39	Worked
Hottentotta tamulus	Sangameshwar, Konkan	10/09/2016	HotSgwr6	17.181	73.549	39	Worked
Hottentotta tamulus	Sangameshwar, Konkan	10/09/2016	HotSgwr7	17.181	73.549	39	Not Worked
Hottentotta tamulus	Sangameshwar, Konkan	10/09/2016	HotSgwr8	17.181	73.549	39	Not Worked
Hottentotta tamulus	Sangameshwar, Konkan	10/09/2016	HotSgwr9	17.181	73.549	39	Not Worked
Hottentotta tamulus	Sangameshwar, Konkan	10/09/2016	HotSgwr10	17.181	73.549	39	Not Worked
Hottentotta tamulus	Sangameshwar, Konkan	10/09/2016	HotSgwr11	17.181	73.549	39	Not Worked

Hottentotta tamulus	Urul Ghat, Satara	30/08/2015	UgHt	17.360	74.028	651	Not Worked
Hottentotta tamulus	Bhatye Plateau, Ratnagiri Konkan	01/10/2015	KnHt	16.963	73.295	62	Worked
Hottentotta tamulus *	Bhatye Plateau, Ratnagiri Konkan	15/11/2016	HotNnt1	16.963	73.295	62	Not Worked
Hottentotta tamulus *	Bhatye Plateau, Ratnagiri Konkan	15/11/2016	HotNnt5	16.963	73.295	62	Not Worked
Hottentotta tamulus *	Bhatye Plateau, Ratnagiri Konkan	15/11/2016	HotBty1	16.963	73.295	62	Not Worked
Hottentotta tamulus *	Bhatye Plateau, Ratnagiri Konkan	16/11/2016	HotBty2	16.963	73.295	62	Not Worked
Hottentotta tamulus *	Bhatye Plateau, Ratnagiri Konkan	17/11/2016	HotBty3	16.963	73.295	62	Not Worked
Hottentotta tamulus *	Bhatye Plateau, Ratnagiri Konkan	18/11/2016	HotBty4	16.963	73.295	62	Worked
Hottentotta tamulus *	Bopdeo Ghat, Kondwa	19/12/2014	Bpd1	18.412	73.903	930	Not Worked
Hottentotta tamulus *	Bopdeo Ghat, Pune	04/10/2016	HotBpd1	18.387	73.944	849	Worked
Hottentotta tamulus *	Bopdeo Ghat, Pune	04/10/2016	HotBpd2	18.387	73.944	849	Worked
Hottentotta tamulus *	Jalna, Aurangabad	30/09/2016	HotJln1	19.853	75.823	539	Worked
Hottentotta tamulus *	Jalna, Aurangabad	30/09/2016	HotJln2	19.853	75.823	539	Worked
Hottentotta tamulus *	Jalna, Aurangabad	30/09/2016	HotJln3	19.853	75.823	539	Worked
Hottentotta tamulus *	Jalna, Aurangabad	30/09/2016	HotJln4	19.853	75.823	539	Worked
Hottentotta tamulus *	Jalna, Aurangabad	30/09/2016	HotJln5	19.853	75.823	539	Not Worked
Hottentotta tamulus *	Jalna, Aurangabad	30/09/2016	HotJln6	19.853	75.823	539	Worked
Hottentotta tamulus *	Jalna, Aurangabad	30/09/2016	HotJln7	19.853	75.823	539	Worked
Hottentotta tamulus *	Kalyan	28/10/2016	HotKyn1	19.202	73.108	11	Worked
Hottentotta tamulus *	Kalyan	28/10/2016	HotKyn2	19.202	73.108	11	Not Worked
Hottentotta tamulus *	Kalyan	28/10/2016	HotKyn3	19.202	73.108	11	Worked
Hottentotta tamulus *	Kalyan	28/10/2016	HotKyn4	19.202	73.108	11	Not Worked
Hottentotta tamulus *	Kayali, Alandi	15/09/2016	HotKyl1	18.685	73.869	570	Worked
Hottentotta tamulus *	Kayali, Alandi	15/09/2016	HotKyl2	18.685	73.869	570	Worked
Hottentotta tamulus *	Kayali, Alandi	15/09/2016	HotKyl3	18.685	73.869	570	Not Worked
Hottentotta tamulus *	Kayali, Alandi	15/09/2016	HotKyl4	18.685	73.869	570	Worked
Hottentotta tamulus *	Kayali, Alandi	15/09/2016	HotKyl5	18.685	73.869	570	Not Worked
Hottentotta tamulus *	Kayali, Alandi	15/09/2016	HotKyl6	18.685	73.869	570	Worked
Hottentotta tamulus *	Kayali, Alandi	15/09/2016	HotKyl7	18.685	73.869	570	Not Worked

Hottentotta tamulus *	Kayali, Alandi	15/09/2016	HotKyl8	18.685	73.869	570	Worked
Hottentotta tamulus *	Khalad, Jejuri	20/06/2015	Kld1	18.328	74.079	734	Not Worked
Hottentotta tamulus *	Khalad, Jejuri	20/06/2015	Kld2	18.328	74.079	734	Not Worked
Hottentotta tamulus *	Khalad, Jejuri	20/06/2015	Kld3	18.328	74.079	734	Not Worked
Hottentotta tamulus *	Khalad, Jejuri	20/06/2015	Kld4	18.328	74.079	734	Not Worked
Hottentotta tamulus *	Khalad, Jejuri	20/06/2015	Kld5	18.328	74.079	734	Not Worked
Hottentotta tamulus *	Khalad, Jejuri	20/06/2015	Kld6	18.328	74.079	734	Not Worked
Hottentotta tamulus *	Khalad, Jejuri	20/06/2015	Kld7	18.328	74.079	734	Not Worked
Hottentotta tamulus *	Khalad, Jejuri	20/06/2015	Kld8	18.328	74.079	734	Not Worked
Hottentotta tamulus *	Khalad, Jejuri	20/06/2015	Kld9	18.328	74.079	734	Not Worked
Hottentotta tamulus *	Khalad, Jejuri	20/06/2015	Kld10	18.328	74.079	734	Worked
Hottentotta tamulus *	Khalad, Jejuri	20/06/2015	Kld11	18.328	74.079	734	Worked
Hottentotta tamulus *	Khalad, Jejuri	20/06/2015	Kld12	18.328	74.079	734	Worked
Hottentotta tamulus *	Khalad, Jejuri	04/10/2016	HotKld1	18.328	74.079	734	Worked
Hottentotta tamulus *	Khalad, Jejuri	04/10/2016	HotKld2	18.328	74.079	734	Worked
Hottentotta tamulus *	Khalad, Jejuri	04/10/2016	HotKld3	18.328	74.079	734	Worked
Hottentotta tamulus *	Khalad, Jejuri	04/10/2016	HotKld4	18.328	74.079	734	Worked
Hottentotta tamulus *	Khalad, Jejuri	04/10/2016	HotKld5	18.328	74.079	734	Worked
Hottentotta tamulus *	Khalad, Jejuri	04/10/2016	HotKld6	18.328	74.079	734	Not Worked
Hottentotta tamulus *	Khalad, Jejuri	04/10/2016	HotKld7	18.328	74.079	734	Worked
Hottentotta tamulus *	Khalad, Jejuri	04/10/2016	HotKld8	18.328	74.079	734	Worked
Hottentotta tamulus *	Khalad, Jejuri	04/10/2016	HotKld9	18.328	74.079	734	Worked
Hottentotta tamulus *	Shindavane Gaon	20/06/2015	Sh	18.425	74.116	519	Worked
Isometrus acanthurus *	Kalyan	28/10/2016	IsoKyn1	19.202	73.108	11	Not Worked
Isometrus cf isadensis *	Jalna, Aurangabad	30/09/2016	IsoJl1	19.853	75.823	539	Not Worked
Isometrus cf isadensis *	Jalna, Aurangabad	30/09/2016	IsoJl2	19.853	75.823	539	Not Worked
Isometrus cf isadensis *	Jalna, Aurangabad	30/09/2016	IsoJl3	19.853	75.823	539	Not Worked
Isometrus sp.	Harpawade Ajara	28/03/2016	IHp1	16.111	74.220	669	Worked
Isometrus sp.	Harpawade Ajara	28/03/2016	IHp2	16.111	74.220	669	Not Worked
Isometrus sp.	Harpawade Ajara	28/03/2016	IHp3	16.111	74.220	669	Not Worked

Isometrus sp.	Harpawade Ajara	28/03/2016	IHp4	16.111	74.220	669	Not Worked
Isometrus sp.	Harpawade Ajara	16/09/2015	AjIm	16.111	74.220	669	Worked
Isometrus sp.	Kadumane estate, Manglore, Karnataka	05/11/2016	IsoMng1	12.919	75.656	965	Worked
Isometrus sp.	Kadumane estate, Manglore, Karnataka	05/11/2016	IsoMng2	12.919	75.656	965	Not Worked
Lychas sp.	Kakkayangad, Kannur	17/11/2016	IsoKrl1	11.936	75.710	70	Not Worked
Lychas sp.	Kakkayangad, Kannur	17/11/2016	IsoKrl6	11.936	75.710	70	Not Worked
Lychas sp.	Kakkayangad, Kannur	17/11/2016	IsoKrl7	11.936	75.710	70	Not Worked
Lychas sp.	Kakkayangad, Kannur	17/11/2016	IsoKrl8	11.936	75.710	70	Not Worked
Lychas sp.	Kakkayangad, Kannur	17/11/2016	IsoKrl2	11.936	75.710	70	Worked
Lychas sp.	Kakkayangad, Kannur	17/11/2016	IsoKrl3	11.936	75.710	70	Worked
Lychas sp.	Kakkayangad, Kannur	17/11/2016	IsoKrl4	11.936	75.710	70	Worked
Lychas sp.	Kanganghat	17/11/2016	IsoKrl5	12.348	75.109	17	Not Worked
Lychas sp.	Nirmalagiri	17/11/2016	IsoKrl9	11.855	75.567	55	Not Worked
Isometrus sp.	Sinhagad, Pune	2016	IsoSgd1	18.364	73.762	1194	Not Worked
Isometrus sp.	Tamhini	09/08/2016	TmIm1	18.766	73.360	455	Worked
Isometrus sp.	Tamhini	09/08/2016	TmIm2	18.766	73.360	455	Not Worked
Isometrus sp.	Tamhini	09/08/2016	TmIm3	18.766	73.360	455	Not Worked
Isometrus sp.	Tamhini	1 3 2016	TmIm4	18.766	73.360	455	Not Worked
Isometrus sp.	Tamhini	1 3 2016	TmIm5	18.766	73.360	455	Not Worked
*Isometrus sp. **	Cotigaon WLS	12/10/2016	IsoCoti1	14.970	74.180	100	Not Worked
*Isometrus sp. **	Nature's Nest, Tambdi Surla, Goa	12/09/2016	IsoNnt1	15.411	74.200	58	Not Worked
Lychas sp.	Gadchiroli, Potegaon	02/10/2016	LykGdc1	20.163	80.054	213	Worked
Lychas sp.	Gadchiroli, Potegaon	02/10/2016	LykGdc2	20.163	80.054	213	Not Worked
Lychas sp.	Gadchiroli, Potegaon	02/10/2016	LykGdc3	20.163	80.054	213	Worked
Lychas sp.	Nirmalagiri	16/11/2016	LykKrl1	11.855	75.567	55	Worked
Lychas sp.	Pookode, Kerala	15/11/2016	Lyk1	11.538	76.020	832	Worked
Lychas sp.	Thattekad, Ernakulum	15/11/2016	Lyk2	10.100	76.690	229	Worked
Lychas sp.	Thattekad, Ernakulum	15/11/2016	Lyk3	10.100	76.690	229	Not Worked
Lychas sp.	Thattekad, Ernakulum	16/11/2016	LykKrl2	10.100	76.690	229	Not Worked
*Lychas sp. **	Cotigaon WLS	12/10/2016	LykCoti1	14.970	74.180	100	Not Worked

*Lychas sp. **	Nature's Nest, Tambdi Surla, Goa	12/09/2016	LykNnt1	15.411	74.200	58	Worked
*Lychas sp. **	Nature's Nest, Tambdi Surla, Goa	12/09/2016	LykNnt2	15.411	74.200	58	Not Worked
*Lychas sp. **	Nature's Nest, Tambdi Surla, Goa	12/09/2016	LykNnt3	15.411	74.200	58	Not Worked
*Lychas sp. **	Nature's Nest, Tambdi Surla, Goa	12/09/2016	LykNnt4	15.411	74.200	58	Worked
*Lychas sp. **	Nature's Nest, Tambdi Surla, Goa	12/09/2016	LykNnt5	15.411	74.200	58	Worked
*Lychas sp. **	Nature's Nest, Tambdi Surla, Goa	12/09/2016	LykNnt6	15.411	74.200	58	Worked
*Lychas sp. **	Nature's Nest, Tambdi Surla, Goa	12/09/2016	LykNnt7	15.411	74.200	58	Not Worked
*Orthochirus bastawadei **	Jalna, Aurangabad	30/09/2016	OcBdJl1	19.853	75.823	539	Not Worked
*Orthochirus bastawadei **	Jalna, Aurangabad	30/09/2016	OcBdJl2	19.853	75.823	539	Not Worked
*Orthochirus bastawadei **	Jalna, Aurangabad	30/09/2016	OcBdJl3	19.853	75.823	539	Not Worked
*Orthochirus bastawadei **	Kayali, Alandi	15/09/2016	OcKyl1	18.685	73.869	570	Not Worked
*Orthochirus bastawadei **	Kayali, Alandi	15/09/2016	OcKyl2	18.685	73.869	570	Not Worked
Orthochirus bicolour	Bhor	07/08/2015	OcBho	18.185	73.895	616	Not Worked
*Orthochirus bicolour **	Baner Hills	14/06/2015	OcBh	18.554	73.790	658	Worked
CHAERILIDAE							
Chaerilus pictus	Arunachal Pradesh	2015	Cp	--	--	--	Not Worked
Chaerilus pictus	Arunachal Pradesh	2015	SS	--	--	--	Not Worked
Chaerilus pictus	Arunachal Pradesh	2015	ES	--	--	--	Not Worked
EUSCORPIIDAE							
Neoscorpiops sp.	Harishchandra Gadh	01/05/2016	NeoHgd1	19.387	73.774	1342	Not Worked
Neoscorpiops sp.	Matheran	26/06/2016	NeoMth1	18.942	73.229	183	Not Worked
Neoscorpiops sp.	Matheran	26/06/2016	NeoMth2	18.942	73.229	183	Not Worked
Neoscorpiops sp.	Matheran	26/06/2016	NeoMth3	18.942	73.229	183	Not Worked
Neoscorpiops sp.	Matheran	26/06/2016	NeoMth4	18.942	73.229	183	Not Worked
Neoscorpiops sp.	Matheran	26/06/2016	NeoMth5	18.942	73.229	183	Not Worked
Neoscorpiops deccanensis	Sinhagad Uphill	10/06/2015	NeoSgd1	18.364	73.762	1194	Not Worked
HORMURIDAE							

Chiromachetes sahyadriensis	Tamhini	09/08 2015	TCS1	18.766	73.360	455	Worked
Chiromachetes sahyadriensis	Tamhini	09/08 2015	TCS2	18.766	73.360	455	Worked
Chiromachetes sahyadriensis	Tamhini	09/08 2015	TCS3	18.766	73.360	455	Not Worked
Iomachus sp.	Arunachal Pradesh	2016	I1M	--	--	--	Not Worked
Iomachus sp.	Arunachal Pradesh	2016	I2M	--	--	--	Not Worked
Iomachus sp.	Arunachal Pradesh	2016	I3M	--	--	--	Not Worked
SCORPIONIDAE							
*Heterometrus sp. **	Cotigaon WLS	12/10/2016	HetCoti1	14.970	74.180	100	Not Worked
Heterometrus sp.	Gadchiroli, Potegaon	02/10/2016	HetGdc1	20.163	80.054	213	Not Worked
Heterometrus sp.	Gadchiroli, Potegaon	02/10/2016	HetGdc2	20.163	80.054	213	Not Worked
Heterometrus sp.	Gadchiroli, Potegaon	02/10/2016	HetGdc3	20.163	80.054	213	Not Worked
Heterometrus sp.	Harpawade Ajara	15/08/2015	HH1	16.111	74.220	669	Worked
Heterometrus sp.	Harpawade Ajara	15/08/2015	HH2	16.111	74.220	669	Not Worked
Heterometrus sp.	Harpawade Ajara	28/03/2016	HetH1	16.111	74.220	669	Not Worked
Heterometrus sp.	Kadumane estate, Manglore, Karnataka	05/11/2016	HetMng1	12.919	75.656	965	Not Worked
*Heterometrus sp. **	Kalyan	28/10/2016	HetKyn1	19.202	73.108	11	Not Worked
Heterometrus sp.	Kanhangad, Kasargod	17/11/2016	HetKrl2	12.348	75.109	17	Not Worked
Heterometrus sp.	Kannur, Peringome	17/11/2016	HetKrl1	12.240	75.300	132	Not Worked
Heterometrus sp.	Matheran	26/06/2016	HetMth1	18.942	73.229	183	Not Worked
Heterometrus granulomanus	Nagpur Outskirt	01/01/2016	HgN1	21.155	79.039	330	Not Worked
Heterometrus granulomanus	Nagpur Outskirt	01/01/2016	HgN2	21.155	79.039	330	Not Worked
*Heterometrus sp. **	Nature's Nest, Tambdi Surla, Goa	12/09/2016	HetNnt1	15.411	74.200	58	Not Worked
*Heterometrus sp. **	Nature's Nest, Tambdi Surla, Goa	12/09/2016	HetNnt2	15.411	74.200	58	Not Worked
*Heterometrus sp. **	Sangrun	08/06/2015	HetSng	18.398	73.678	600	Not Worked
Heterometrus xanthopus	Wagholi Pune	26/12/2015	HxW2	18.585	73.978	588	Not Worked
Heterometrus xanthopus	Wagholi Pune	26/12/2015	HxW3	18.585	73.978	588	Not Worked
Heterometrus xanthopus	Wagholi Pune	26/12/2015	HxW4	18.585	73.978	588	Not Worked
Heterometrus xanthopus	Wagholi, Pune	26/12/2015	HxW1	18.585	73.978	588	Not Worked

From the collection, *Hottentotta tamulus* genus was selected as the group for venom analysis and the specimens were subjected to venom extractions. All specimens were preserved in molecular grade ethanol. Taxonomic identification was done as per various taxonomic revisions available for all the genera used in the study (Fet et al., 2013; Kovarik 1997; Kovarik 2000; Kovarik 2004; Kovarik and Affilastro 2009; Kovarik 2015 a, b, c; Kovarik and Ahmed 2016; Lourenco and Duhem 2010; Tikader and Bastawade 1983). Specimens collected during the study are in the process of deposition in the museum collection of Bombay Natural History Society (BNHS), Mumbai; Wildlife Information Liaison Development (WILD) Society, Coimbatore and Institute of Natural History Education and Research (INHER), Pune.

Morphometry and Morphometric Analysis

Morphometric measurements were made for 66 specimens of the *Hottentotta tamulus* from eight populations used for population genetics study (Table 2). A total of 15 characters were identified and measured. Cephalothorax length (ct), Carapace length (cl), Carapace Anterior Width (ca), Carapace Posterior Width (cp), Mesosoma length (ml), Pedipalp length (pp), Femur length (fl), Patella length (pl), Manus length (mn), Fixed finger length (ff), Movable finger length (mf), Pectin Length (pc), Pectin Count left, Pectin Count right and Pectinal teeth length (pt) were the parameters selected for morphometric study. Length Measurements were made using digital Vernier calipers (Mitutoyo Digimatic Caliper CD-8” CSX. Other characters were extracted by observing the specimens under microscope.

Morphometric data was analyzed with PAleontological STatistics (PAST) version 3.0 (Hammer et. al., 2001), a tool that enables users to analyze data and perform various statistical operations on raw data. This tool has been used to process morphometric data. A multivariate analysis was carried out for 66 specimens of *Hottentotta tamulus*. A total of 15 characters were used for the analysis. The analysis was performed with Non-metric multidimensional scaling (NMDS) approach. A Shephard’s plot was used to determine the relationship between the actual dissimilarities between objects and the ordination distances so as to understand the reliability of NMDS in depicting the distances between individuals. Well correlated values will lead to low stress values and thereby confirm that the visualization is a good representation of the data in a lower number of axes.

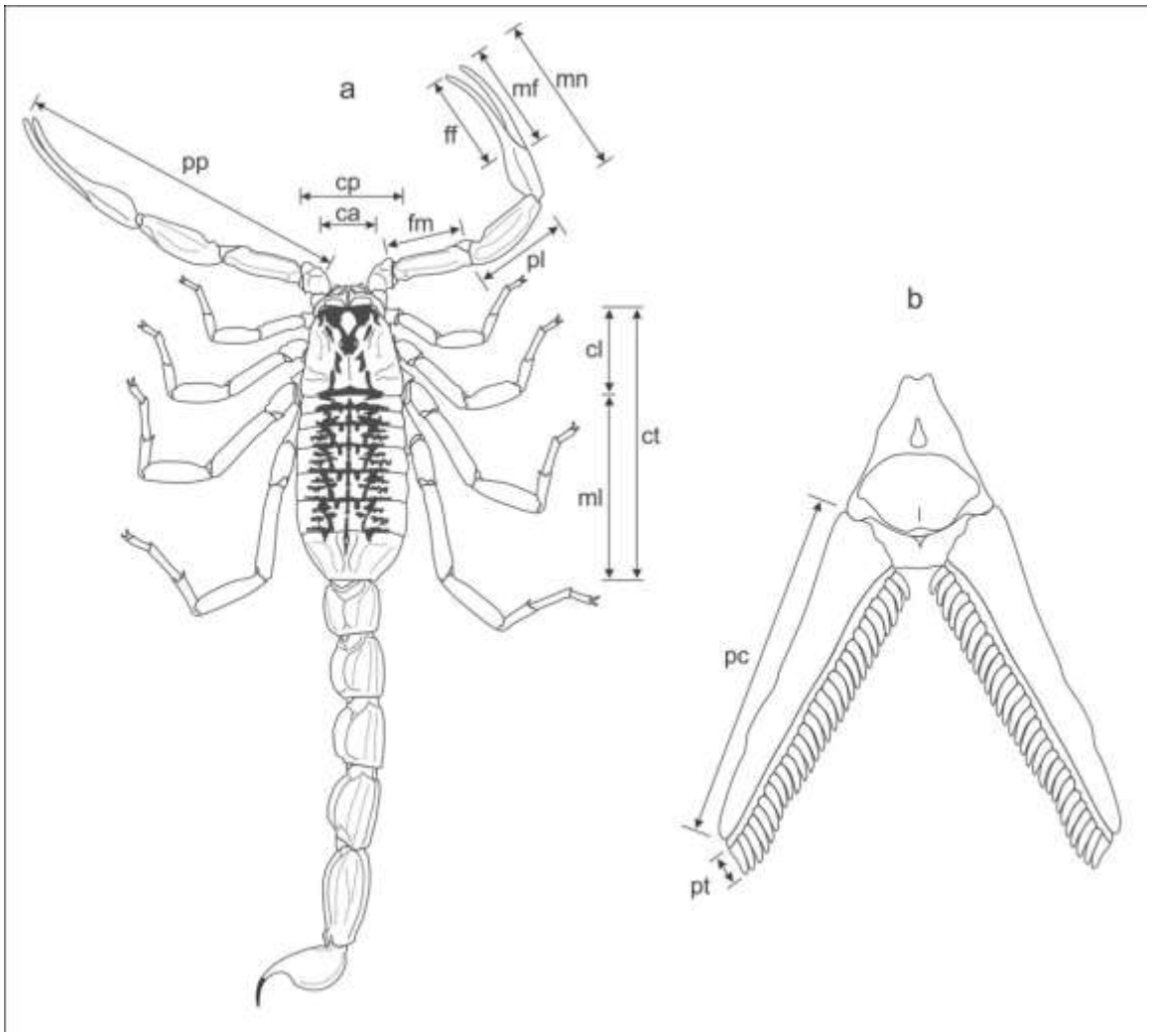


Figure 2: Characters used for morphometric analysis. (a) complete specimen and (b) pectin teeth.

Table 2: Specimens of *H. tamulus* used for population genetics and morphometric studies.

Location	Number of specimens used	
	Genetics	Morphometry
Bhatye Plateau	2	6
Sangameshwar	6	11
Jejuri	12	20
Shindavane	6	7
Pashan	3	3
Alandi	5	8
Kalyan	2	4
Jalna	6	7

Molecular Work

DNA Extraction

Muscle tissues were harvested from mannus, metasoma and telson of freshly preserved specimens and were subjected to DNA extraction. The extraction was carried out with QIAamp DNA Mini Kit (Qiagen, Hilden, Germany, Catalog No. 51306) with modifications. Tissue sample was taken in 1.5ml microfuge tube and 180µl of STE buffer (0.1M NaCl, 0.05 M Tris-HCl, 0.01M EDTA, 1%SDS) was added with 5µl Proteinase K (20mg/ml). Tube was incubated at 56ºC till tissue dissolved. Samples were spin at 12000rpm for 5 minutes and transferred to a new tube using cut tips avoiding any debris. To this 5µl of 20mg/ml RNAse was added and tube was incubated at 37ºC for 1 hour. After incubation, 200µl AL buffer was added and tube was incubated at 70ºC for 10min. After incubation, 200µl of absolute ethanol was added and tubes were vortexed. Full volume was transferred to DNA mini kit column and was spun at room temperature for 2 min at 8000rpm. Solution in collection tube was discarded and 500µl of AW1 buffer was added. Tube was spun at room temperature for 2 min at 8000rpm. Solution in collection tube was discarded and 500µl of AW2 buffer was added. Tube was spun at room temperature for 3 min at 14000rpm. Solution in collection tube was discarded a dry spin was given for 1 min at room temperature at 15000rpm. Columns were placed in fresh collection tubes and 50µl of AE buffer was added to the columns. Column was incubated at room temperature for 5 mins. Column was spun at 13400 rpm for 1.5 min to collect DNA. Collected DNA was stored at -20ºC.

Gene amplification

Mitochondrial COI subunit I gene being a barcoding gene, was selected as the potential target for evolutionary study. For amplification of gene of interest polymerase chain reaction was performed. Mastercycler Personal 5332 eppendorf ver 2.22.32 was used for amplification. Table 3 below show the list of primers used for PCR, their sequence and melting temperature (Tm) and Table 4 provides the combinations tried with their annealing temperatures (Ta). Five sets of primers were used for PCR. The reaction was performed in a 25µl reaction volume containing 13µl of template DNA (~250 ng), 5µl of 10X reaction buffer (100 mM Tris pH9.0,

500 mM KCl, 15 mM MgCl2, 0.1% Gelatin), 3µl of 25 mM MgCl2, 1µl of 10 mM dNTPs, 1µl of each primer and 0.5µl Taq polymerase (2.5 units). The thermal profile was 10 min at 95ºC, and 35 cycles of 1 min at 94ºC, 1 minute at Ta and 2 min at 72ºC, followed by extension of 10 min at 72ºC following Suranse et al. (2016). Agarose gel (1%) was made and PCR products were verified for presence of the amplicon. The gel electrophoresis was carried out in 1x TAE solvent environment at 100V.

Table 3: List of primers used for PCR with sequence and melting temperature.

Name	Direction	Sequence	Tm (˚C)
SPCOI F	Forward	GGTCAACAAATCATAAAGATATTGG	61.2
SPCOI R	Reverse	TAAACTTCAGGGTGACCAAAAAATCA	66.9
LCO1490	Forward	GGTCAACAAATCATCATAAAGTTGG	52.9
NANCY	Reverse	CCHGGTAAAATTAAAATATAAACTTC	47.9
U1m3 (Scorp F1)	Forward	TCWACWAATCATAAGACATTGGAAC	51.6
U1Sco (Scorp F2)	Forward	TCWACDAATCATAAGGATATTGGDAC	51.9
L1pyc (Scorp R1)	Reverse	CTATRATDGCRAATACDGCTCCTA	53.4

Table 4: List of primer combinations used for PCR.

Primer Combinations	Ta (˚C)
SPCOI F x SPCOI R	56
NANCY x LCO1490	48
U1m3 x L1pyc	49
U1Sco x L1pyc	49
SPCOI F x NANCY	44

Purification and sequencing

The amplified DNA product was purified with Wizard Gel and PCR clean up system (Promega, Madison, WI). For the samples, positive for amplicon, 25µl of membrane binding solution was added to the entire product volume. The mixture was transferred to a column and left undisturbed for 2-3 mins. The column was spun at room temperature for 90secs at 13400rpm. Then, 700µl of membrane wash solution was added to the column and the column was spun for 90sec at room temperature at 13400rpm. The waste collected in the collection tube was discarded. Another wash with 500µl membrane wash solution was done. The waste obtained in the collection tube was discarded and the sample was given a dry spin. The final product

was eluted with 50µl of nuclease free water and kept undisturbed for 5-7 mins. The final product was collected in a new collection tube by spinning the column at 13400rpm for 90sec. The purified product thus obtained were stored at -20ºC.

Purified products were sequenced using BigDye Terminator v3.1 cycle sequencing kit and ABI prism 3730 sequencer (Applied Biosystems, Foster City, CA).

Molecular Analysis

Acquired raw sequences were first checked and trimmed using BioEdit (version 7.2.5) (Hall 2013). The sequences were then run through BLAST tool (Altschul et al. 1990) to verify the translation product. These sequences were then used for the genetic analysis. Additional sequences were downloaded from NCBI database. Seventeen sequences from our earlier published work (Suranse et al. 2016) were used for better phylogenetic reconstruction and understanding of Indian scorpion populace. The sequences generated in the current study are in the process of submission to GenBank archive. Compiled sequences were aligned using MUSCLE (Edgar 2004). MEGA7, a BioInformatics tool was used for editing aligned sequences (Kumar et al. 2016). Entire data was partitioned into three codons positions and right partitioning scheme was determined by applying greedy strategy (Lanfear et al. 2012) using IQ-TREE (version 1.5.3) (Nguyen et al. 2015) based on minimum Bayesian Information Criterion (BIC) value (Schwarz 1978). Nucleotide substitution model for partition scheme was determined using IQ-TREE. Model with minimum BIC score was selected and best nucleotide substitution matrix was used for Maximum Likelihood analysis using IQ-TREE. Reliability of phylogenetic tree was assessed by performing 1000 bootstrap iterations. Ultrafast Bootstrap algorithm was used for creating the ML tree (Minh et al. 2013). FigTree (version 1.4.3) (Rambut 2007) was used for editing the tree.

For studying the intra-specific genetic diversity in *Hottentotta tamulus*, eight populations were studied (Table 2). Median Joining (MJ) tree was created based on COI of eight populations. POPART (version 1.7) (Leigh & Bryant 2015) was used for making the MJ tree. The input file format for POPART is NEXUS format. To convert the aligned FASTA sequences to NEXUS, Seaview (version 4.6.1) (Gouy et al. 2010) was used. MJ tree was created with

setting the ‘ε’ parameter ‘0’. The parameter is set to zero in order to get the tree with minimum spanning.

Raw genetic p-distances were calculated using MEGA 7. Only the actual sequence differences present in the existing state of the sequences are considered for distance calculation. The raw distances were converted into percentages and box plots were plotted for comparing the three groups of *H. tamulus* populations. Intra and inter population genetic trends were studied for the H. tamulus group.

Venom Extractions

Hottentotta tamulus was selected as the species for venomic study because of its abundance, wide distribution, medicinal importance and ease of venom extraction. Five locations were selected. From each of these 5 locations, a minimum of 5 specimens were collected. Each of these specimen was subjected to electrical stimulation to instigate venom release. Testronix 92C DC power supply (0-32V, 0-5amp) was used for administering a mild electric shock ranging from 15V-32V. Throughout the tenure of the extraction, the scorpions are starved and subjected to normal day-night cycles. All the extractions are stored in 1:1 mixture of acetonitrile and water at -20ºC. Extraction scheme was: Once the first extraction(E1) is carried out, the second extraction (E2) is performed 24hrs post E1. Third extraction (E3) is carried 48hrs post E2. Fourth extraction (E4) is performed 72 hours post E3. The last extraction (E5) is carried out a week after E4.

Venom protein analysis

Venom samples were lyophilized with lyophilizer. The lyophilized sample was resuspended in 6µl water. 2x SDS Dye was added to the sample and heated at 99ºC for 10min. Sample was then spun at 13000rpm for 10min. 10µL of sample was loaded onto 15% SDS-PAGE gel. The gel was run at constant voltage of 230V till the dye front reaches lower edge of gel plate. The gel is composed of 5% stacking gel and 15% resolving gel. Composition of Stacking gel (4ml): MilliQ – 2.8ml, AB Mix (30%) – 0.66ml, 1.5M Tris (pH=6.8) – 0.5ml, 10%SDS – 0.04ml, 10%

APS – 0.04ml and TEMED – 0.004ml. Composition of Resolving gel 15% (10ml): MiliQ – 2.2ml, Acrylamide/Bis-acrylamide (30%/0.8% w/v) – 5ml, 1.5M Tris (pH=8.8) – 2.6ml, SDS 10% (w/v) – 0.1ml, 10% (w/v) ammonium persulate (AP) - 100µl and TEMED - 10 µl

Venom Mass Spectrometry

Applied Biosystems 4700 proteomics Analyzer 284 was used to conduct MALDI/TOFMS of the venom samples. Four different matrices were tried, α cyano-4-hydroxy cinnamic acid (CHCA), 2,5-dihydroxy benzoic acid (DHB) and sinapinic acid (SA). The m/z values acquired were used for further analysis.

Results and Discussions

We studied total 196 scorpions under 5 families and 11 genera. Photographs of some live specimens are provided in Figure 3.

Genetic Analysis of Indian Scorpions

A total of 5 sets of primers were used from amplification of mitochondrial COI subunit I gene. From all the primers used, only two sets viz., SPCOI F x SPCOI and LCO1490 x Nancy amplified the gene of interest successfully. From a total of 196 collected specimens, 67 specimens yielded positive results for PCR (Table 1) and those products were sequenced. The acquired sequences were used for the phylogenetic analysis. Seventeen sequences were added from our earlier published work (Suranse et al. 2016). Other sequences used for analysis were added from NCBI Database.

Our published work (Suranse et al. 2016) mainly focused on the evolutionary relationships of the members of Buthidae family based on very few number of samples. In the current study, members of 3 different families of scorpions found in India were studied using molecular analysis. Nucleotide substitution model for full partition scheme were as follows: 1) Part1 (COI first codon position) - TN+G4 (BIC = 6714.097), 2) Part2 (COI second codon position) - TIM2+R2 (BIC = 3588.784) and 3) Part3 (COI third codon position)- TPM2+G4 (BIC = 25773.937). For the full partition scheme, the overall model score was BIC= 36939.230 (lh=-15898.877, df=785). For merged partitions, the best model was: The final best model for merge Part1 + Part2 +Part3 was GTR+R6 (BIC = 35119.389, lh=-16665.667, df=273), which was used for maximum likelihood analysis.

Maximum Likelihood tree (Figure 4) was created using the appropriate nucleotide substitution model determined using IQTREE. Sequences from Ricinoididae family were used as outgroup. The *Hottentotta tamulus* samples that were collected from Western and Eastern parts of the Western Ghats and the ones collected from Jalna (Dry Region) clade together to form a single monophyletic group although there were many genetic variations in these populations.

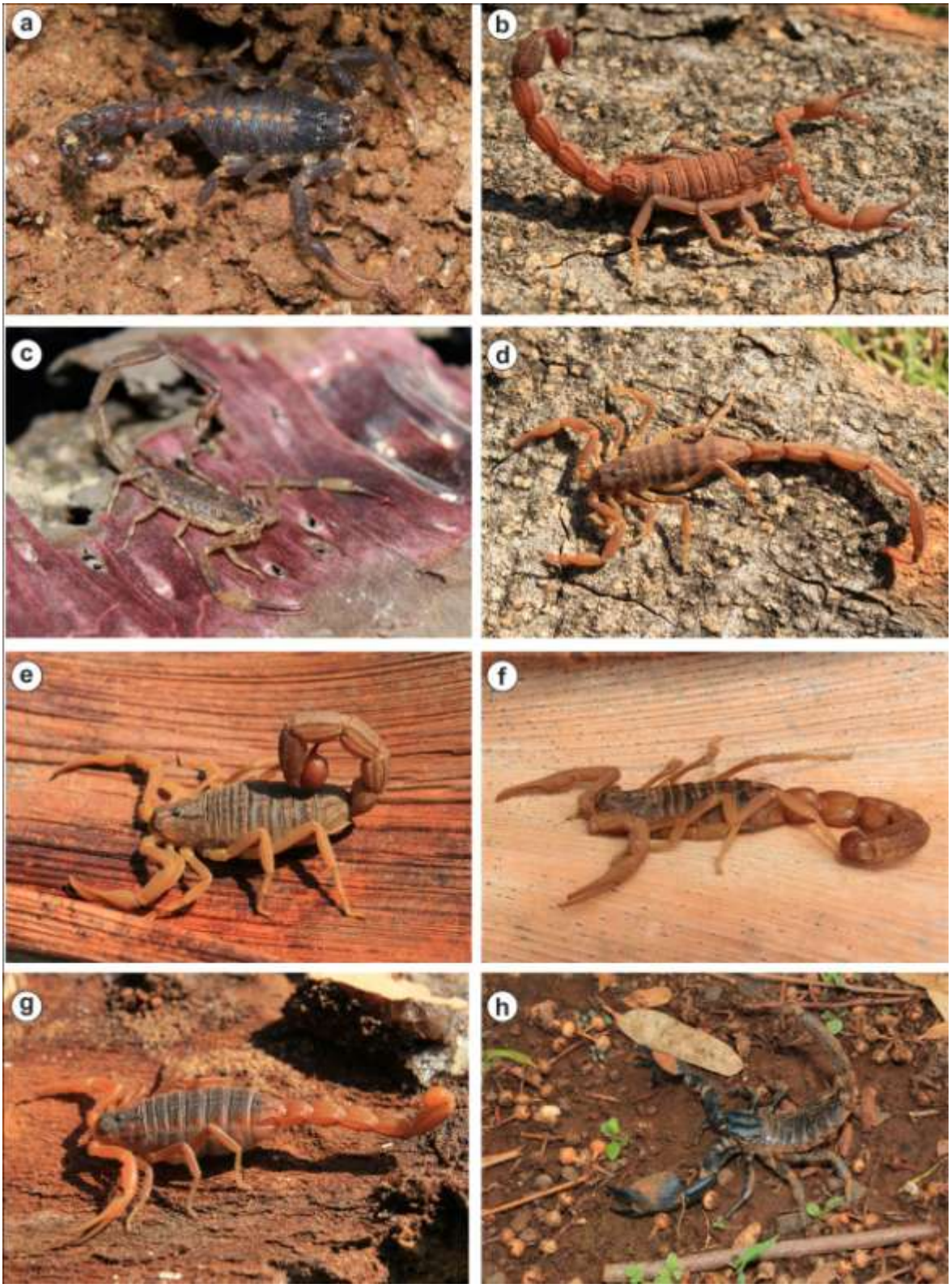


Figure 3: Some scorpions used for the study. (a) *Charmus* sp. from Matheran, (b) *Hottentotta* sp. from Talere (c) *Isometrus* sp. from Tamhini, (d) Lychas sp. from Tambdi Surla, (e-g) *Hottentotta tamulus* from Kalyan, Sangameshwar and Bhatye plateau respectively, and (h) *Heterometrus phipsoni* from Ajara.

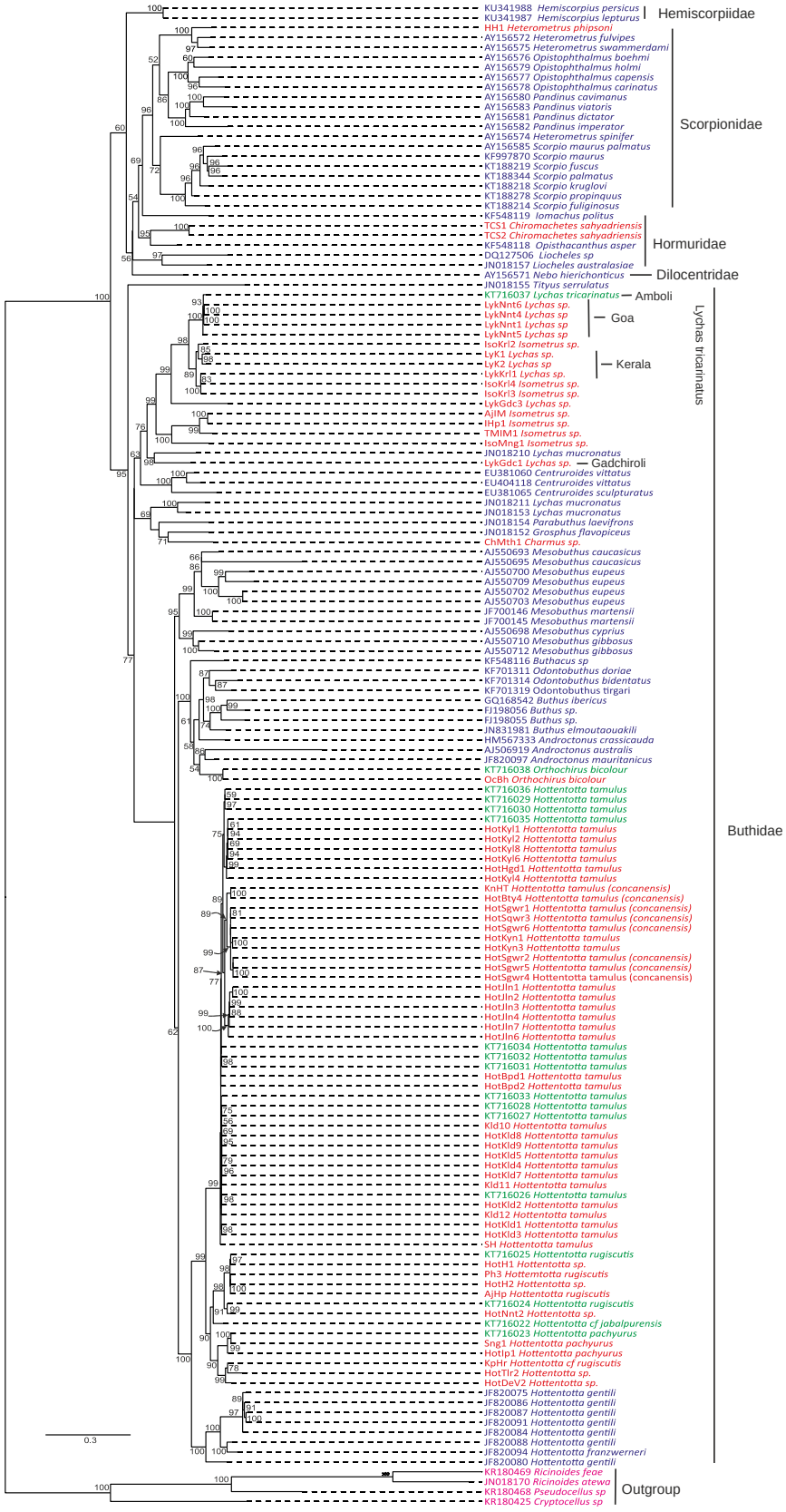


Figure 4: Maximum likelihood tree. Labels in red refer to specimens studied in current study, green refer to samples from Suranse et al. (2016) and blue labels refer to sequences from NCBI. Samples in pink are used as outgroup.

The specimens that are currently identified as *H. pachyurus* and *H. rugiscuitis* are likely to be belonging to many different lineages. The current taxonomic classification seems inadequate to efficiently identify and classify this species cluster. Moreover, it is observed that H. *tamulus* has a very wide distribution, on the contrary, *H. pachyurus* and *H. rugiscuitis* populations are present in very localized packets.

Scorpions belonging to the genus *Charmus* are very shy creatures and very difficult to capture. Owing to that, very little information is available about these organisms. In the tree, the specimen that we collected (ChMth1) clusters together with *Grosphus flavopiceus*, a Buthid specie highly endemic to Madagascar. This suggests that *Charmus* could be a potential candidate to understand phylogeographic relationships of fauna in Africa and India, so also the famous Gondwana Split.

Orthochirus bicolour (OcBh) clusters together with our earlier published Orthochirus sequence. These specimens fall together in the cluster with Androctonus mauritanicus and Androctonus australis. Though they are distinct species yet, they may have had a common Gondwanan ancestor or their evolution has been dictated by Gondwana Split. Thus this group too could provide better insights into Gondwana Split.

Taxonomically, genus Isometrus and Lychas are distinguished by just a single apomorphic character i.e. a radial spur being present on the third and fourth leg in Lychas and its absence in Isometrus. Our analysis suggests that though these genera are different, they form monophyletic clades. Some specimens from genus Isometrus fall well inside the clade of Lychas suggesting that the apomorphic character defined to distinguish the two genera is not valid and thus the taxonomic system needs a revision.

Two different Lychas specimens were found in Gadchiroli. They differ in their carinae stuctures, one having two carinae and the other one with three carinae. Lychas tricarinatus is a species complex. Specimens from Amboli, Goa, Kerala and Gadchiroli can be identified as Lychas tricarinatus taxonomically, based on the presence of three carinae but they differ in many other characters and thus could be different species altogether. This entire species requires a revision.

Heterometrus is a group of scorpions famous for their robust body and huge size. Their phylogenetic position has not been studied. In the current study, the placement of Heterometrus phipsoni is determined.

Chiromachetes is an enigmatic genus belonging to the family Hormuridae. Very insufficient information is available regarding this genus. Specimens have been collected for the recently described *Chiromachetes sahyadriensis* by Mirza et al (2015). The phylogenetic placement of C sahyadriensis has been described for the first time in this study.

Population Genetics of Hottentotta tamulus

Hottentotta tamulus was selected as the specie for population genetics and venom analysis because they are distributed widely, abundant in number and relatively easy to handle during extractions. H *tamulus*, popularly known as 'The Indian Red scorpion' is believed to be the most venomous scorpion. Every year, there are incidents of envenomations and deaths due to this organism. However, the venom from this group could be a potential target for drug discovery and could be of immense medical and pharmacological value. Their venom can be studied for making more effective and generalized antivenins as well as therapeutic agents for diseases like Cancer, AIDS and many others.

The Median Joining tree (Figure 5) made for *H. tamulus* suggests that there is a lot of genetic variation for this species within a population, present in a single locality and even across different population from diverse locations. The *H. tamulus* population formed three different groups, region of high rainfall to the west of Western Ghats, rain shadow area to the east of Western Ghats and the dry region. Even though there are several mutations in the COI gene, all these mutations are null mutations. Moreover, for the protein product formed by the COI gene, the amino acid sequences are the same except for two samples collected from Jalna and two samples from Pashan. The protein product for these specimens differ in two amino acids each while the rest populations code for the same protein with same amino acid sequence. All these populations belong to same species and morphologically very similar as well. In fact, there was no population difference in the morphometric analysis (Figure 6).

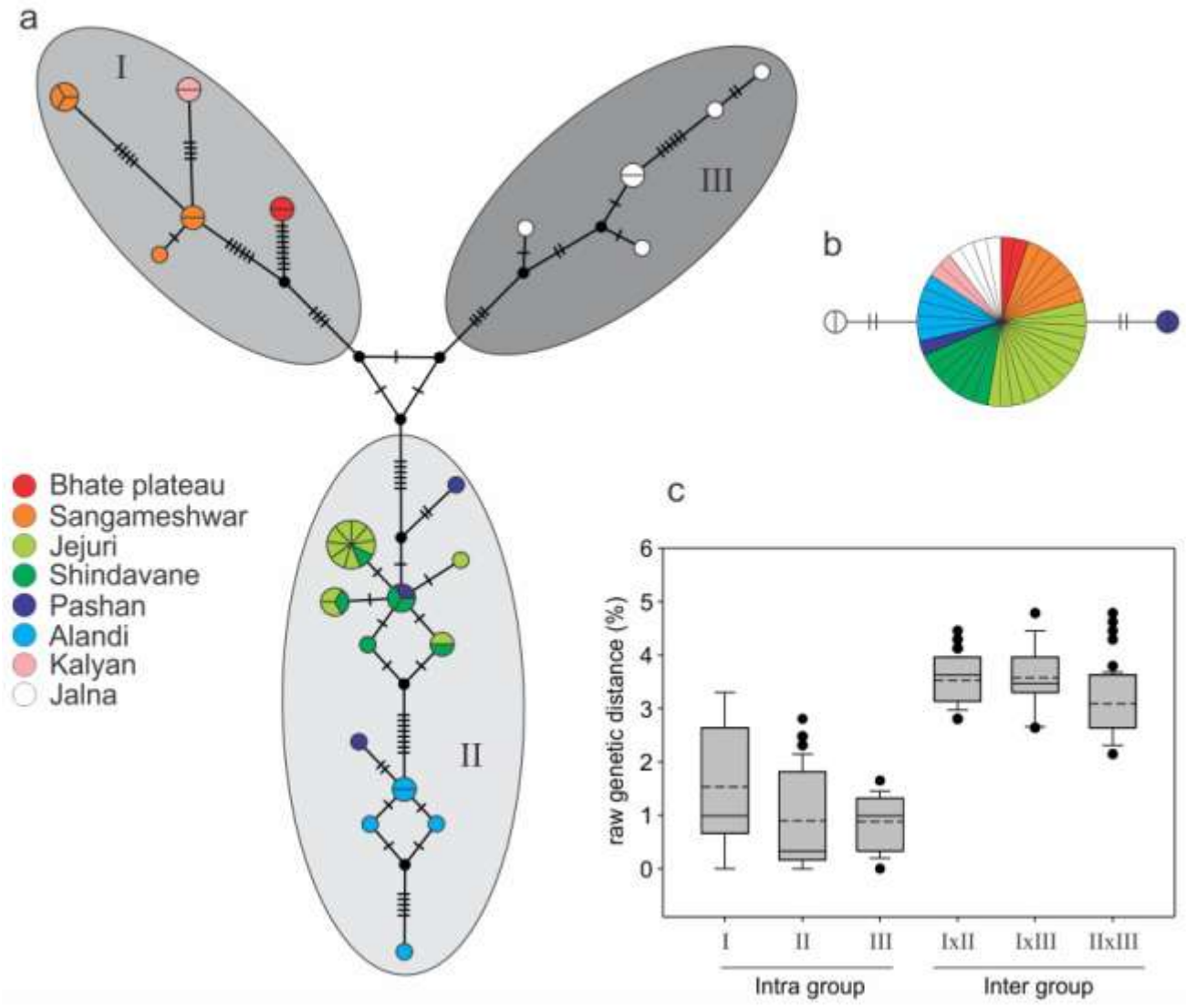


Figure 5: Population genetics of *H. tamulus*. (a) Median-joining network of COI gene sequences. Pie is divided into number of specimens of each haplotype. Number of mutations are shown with hash bars. (b) Median-joining network of COI protein sequences. (c) Raw genetic distances in intra and inter groups formed by three geographical areas.

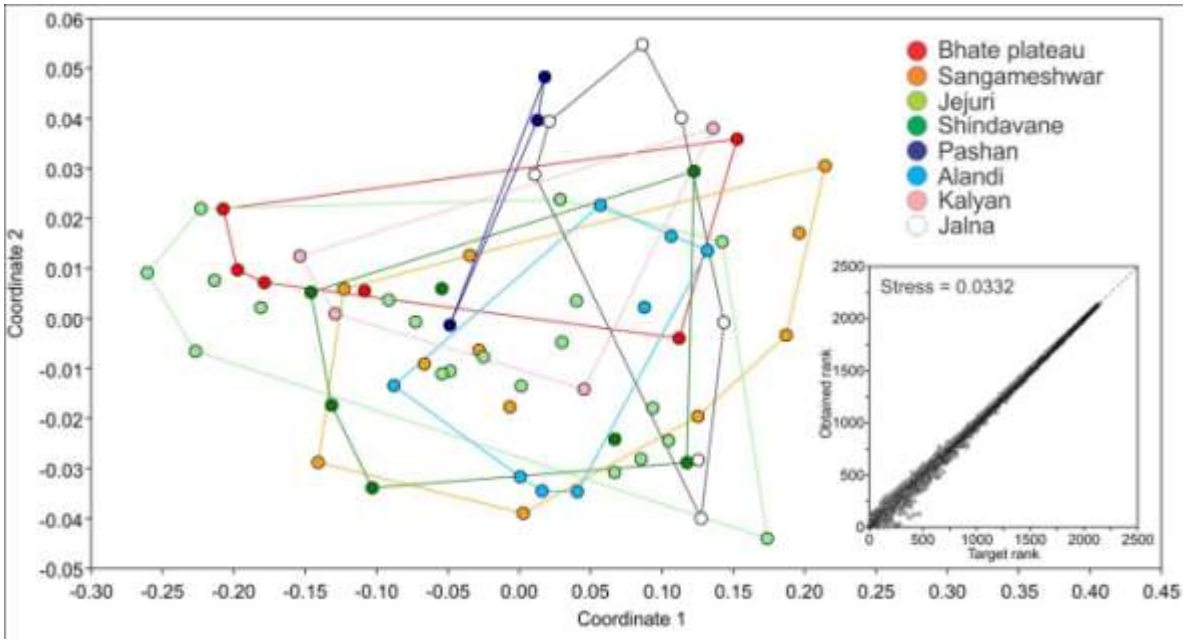


Figure 6: NMDS plot of morphometric data. NMDS is a good fit for the data as the based on the Shepard plot (inset) and low stress value of 0.0332.

It is interesting to note that during venom extractions, we found characteristic differences in the venom release behaviours of these different populations. The samples collected from Sangameshwar were very aggressive and their venom amount released was the highest from the entire lot. A very high voltage stimulation (25V-30V) was required to agitate the scorpions to release venom. The venom was released from a small orifice at the tip of the stinger and was more of a spray. A similar behavior was observed for scorpions collected from Kalyan. Scorpions from Jalna were very agile, inherently agitated and released the venom with a very small stimulus. Specimen from Khalad were surprisingly docile and even their venom release was in the form of drops and not a spray.

It has been established earlier by (Newton et al, 2007) that there is variation in venom with variation in genetics of *H. tamulus*. Our study has more sampling as compared to the earlier work and thus is very relevant in better understanding the relationship between genetic variation and variation in venom. In order to determine the fragment sizes, present in the venom, an SDS-PAGE electrophoresis was carried out (Figure 7).

Venom analysis of Hottentotta tamulus

As the solvent was acetonitrile-water, samples directly could not be used for SDS-PAGE, so the samples were first lyophilized and then resuspended in MiliQ before actually running the gel. Two such attempts were made to check for resolution of the venom. As the initial concentration of venom was meagre and more loss during lyophilization, nothing conclusive could be determined from the gel image. Markers of size 10kDa, 12kDa, 15kDa, 17kDa, 23kDa and 36.8kDa. Neither the marker, nor the sample were resolved properly by the gel electrophoresis, yet it can be noted from the image that the venom is composed of many small stretches and a few large stretches of amino acids.

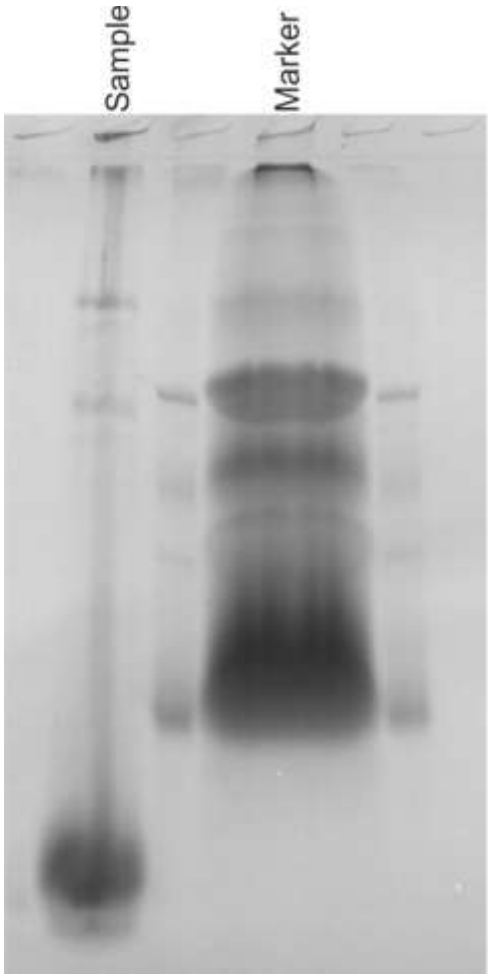


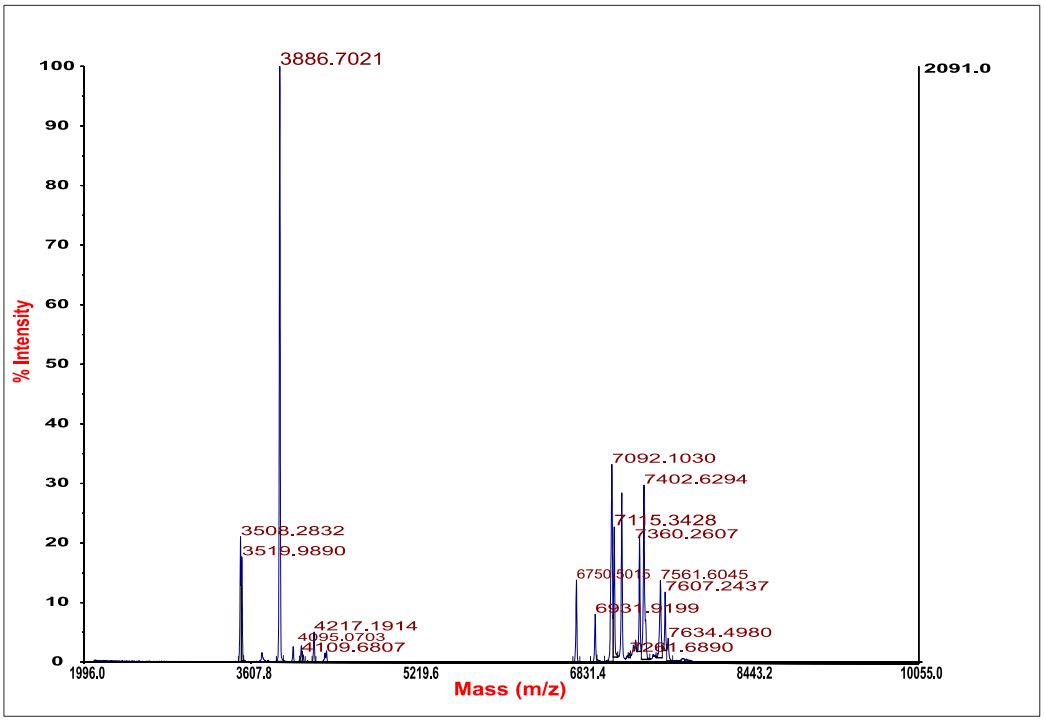
Figure 7: SDS-PAGE image for venom sample from *Hottentotta tamulus* from Sangameshwar (HotSgwr2 E1).

Based on the results from SDS-PAGE, we decided to conduct mass spectrometry for the venom samples to check the presence of peptides in the venom samples collected. From the four matrices tried, SA matrix yielded the best results for MALDI-TOFMS, and was selected as the matrix for further spottings. The samples were check for two mass ranges, (1) Low mass range and (2) High mass range.

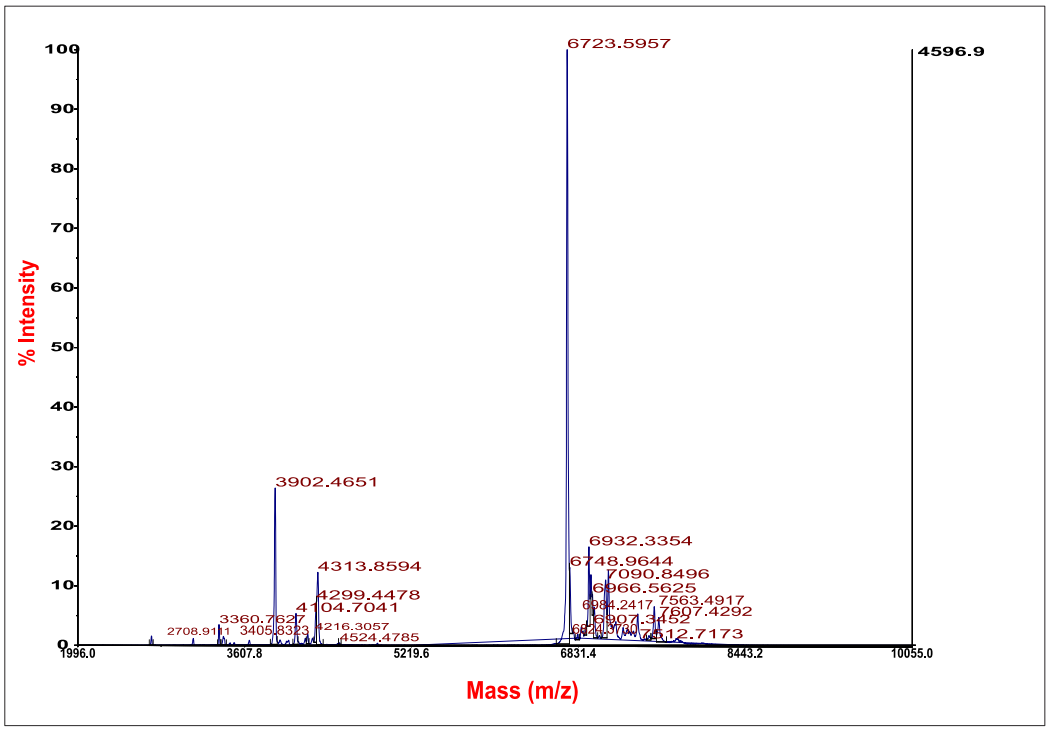
The results of MALD-TOFMS for *H. tamulus* venom for low mass range are shown in Figure 8. There is a characteristic peak (~3800) was observed for all the specimens. It is present in almost all the samples surprisingly; its relative abundance is very high in case of sample from Jalna. Another peak (~6600) is present absolutely in all samples except the sample from Jalna. Despite the specimens being from three different groups, these common peak suggests that the venom concoction might contain peptides or fragments that have similar sequence or structural homology and thereby comparable masses. Further investigation is required to thoroughly understand the composition of the venom as well as the variations and similarities in its various components.

The results of MALD-TOFMS for *H. tamulus* venom for low mass range are shown in Figure 9. A similar result was observed in case of the high mass range of mass spectrum. A characteristic peak was observed in the range of ~13000 to 14000. Venoms are generally a cocktail of diverse peptides and thus the peaks obtained in the mass spectrogram may be different peptides, so also, fragmentation that might occur while performing mass spectrometry cannot be ruled out. Tryptic digestion followed by MALDI-TOF/TOF MS need to carried out to determine the sequence of the peptide(s) present in the venom.

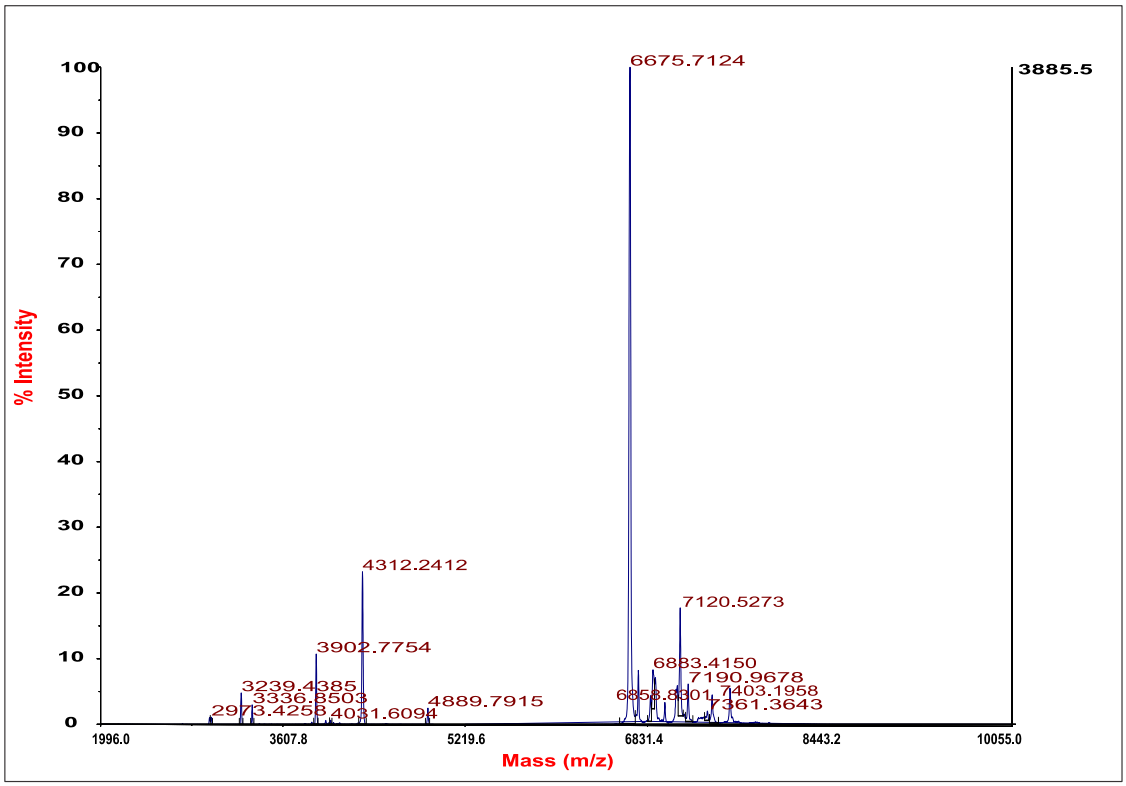
a


3886.7021
2091.0
100
90
80
70
60
50
40
30
20
10
0
% Intensity
7092.1030
7402.6294
7115.3428
7360.2607
3508.2832
3519.9890
7561.6045
7607.2437
6931.9199
4217.1914
4095.0703
4109.6807
7634.4980
1996.0
3607.8
5219.6
6831.4
8443.2
10055.0
Mass (m/z)

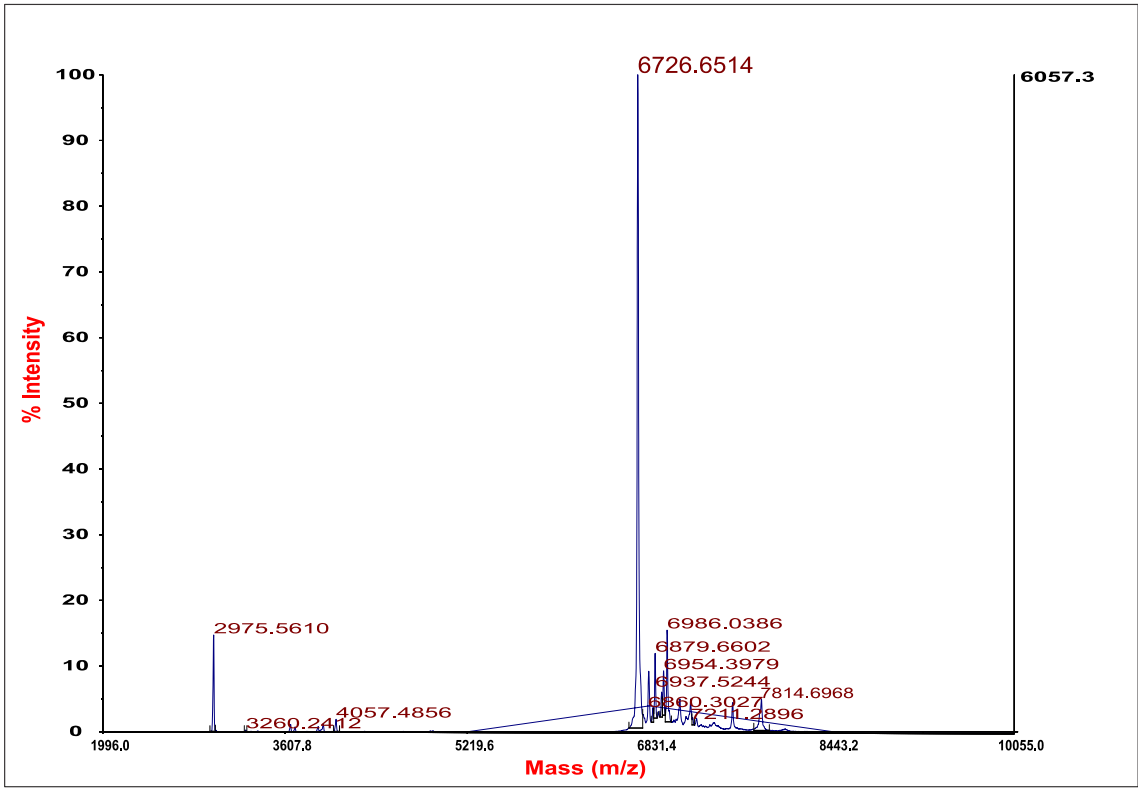
b


6723.5957
4596.9
100
90
80
70
60
50
40
30
20
10
0
% Intensity
3902.4651
6932.3354
6748.9644
4313.8594
7090.8496
6966.5625
4299.4478
4104.7041
7563.4917
7607.4292
3360.7627
2708.911
4216.3057
4524.4785
1996.0
3607.8
5219.6
6831.4
8443.2
10055.0
Mass (m/z)

c


6675.7124
3885.5
100
90
80
70
60
50
40
30
20
10
0
% Intensity
4312.2412
7120.5273
3902.7754
6883.4150
7190.9678
3239.4385
3336.3503
2973.4258
4031.6094
4889.7915
7403.1958
7361.3643
1996.0
3607.8
5219.6
6831.4
8443.2
10055.0
Mass (m/z)

d


6726.6514
6057.3
100
90
80
70
60
50
40
30
20
10
0
% Intensity
2975.5610
6986.0386
6879.6602
6954.3979
6937.5244
7814.6968
4057.4856
3260.2412
7241.2896
1996.0
3607.8
5219.6
6831.4
8443.2
10055.0
Mass (m/z)

e

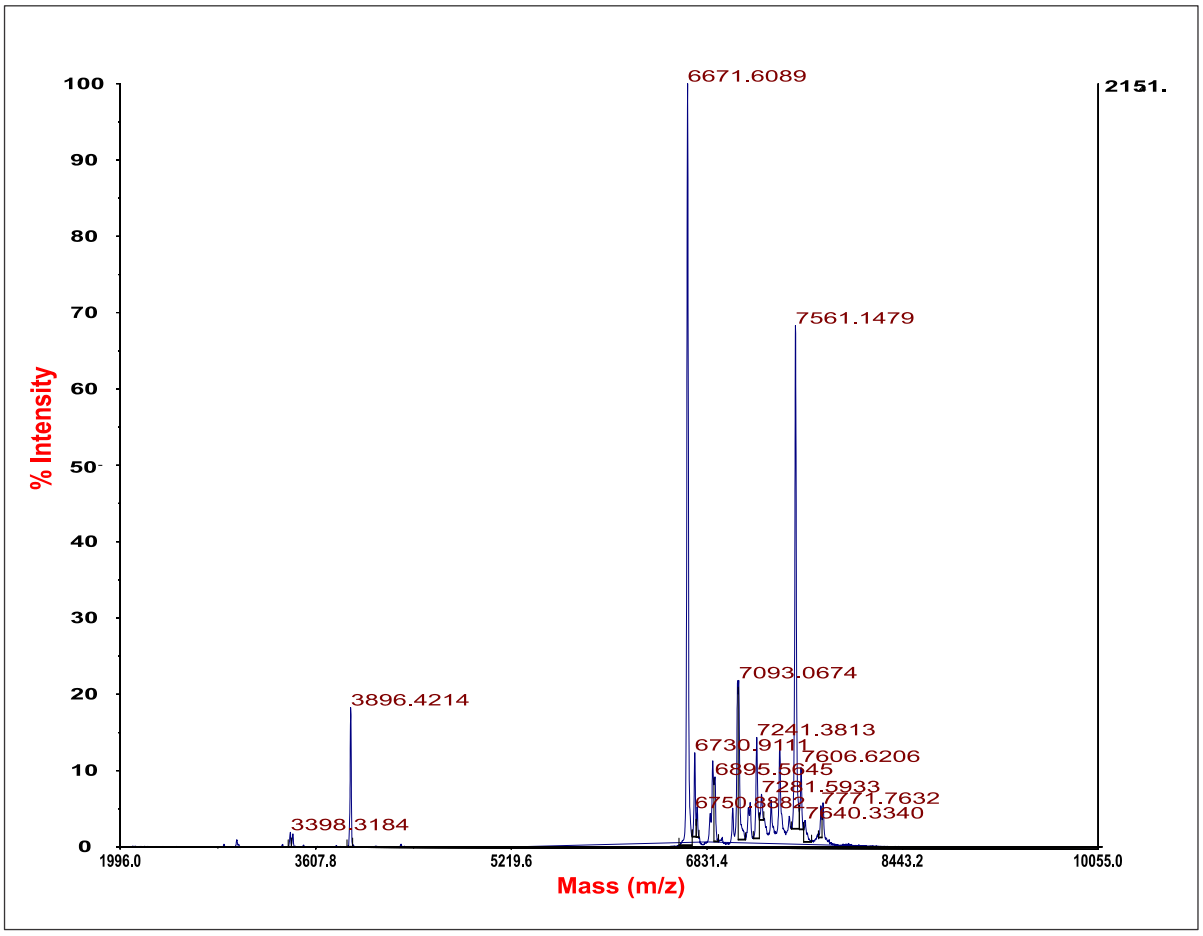
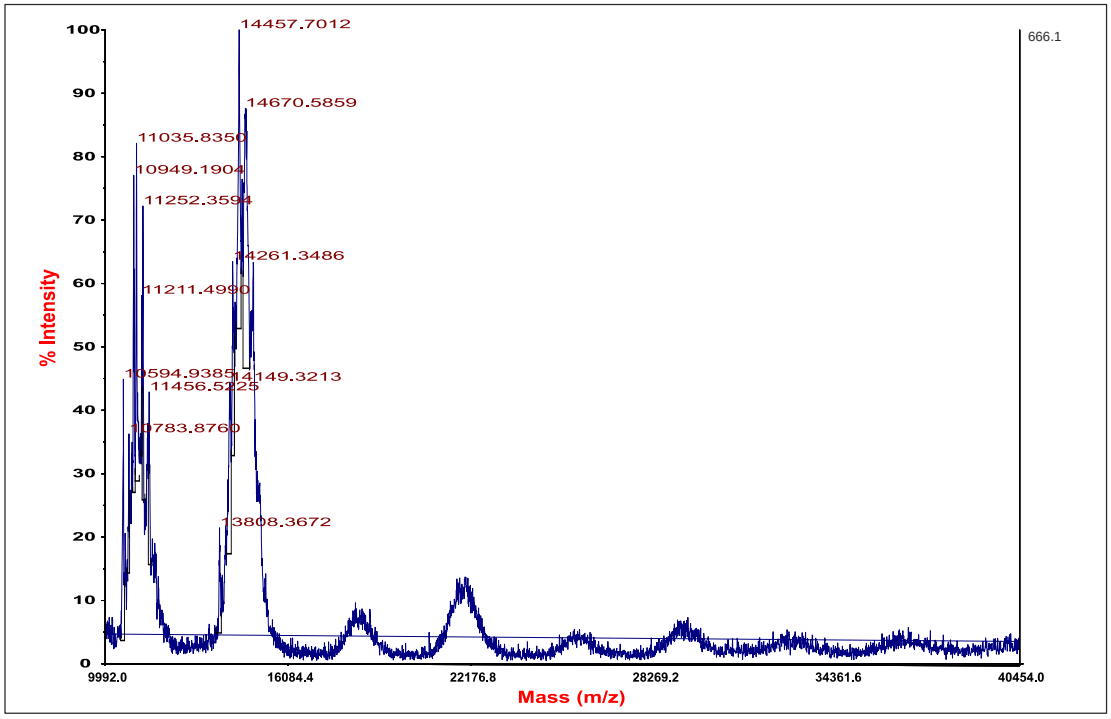
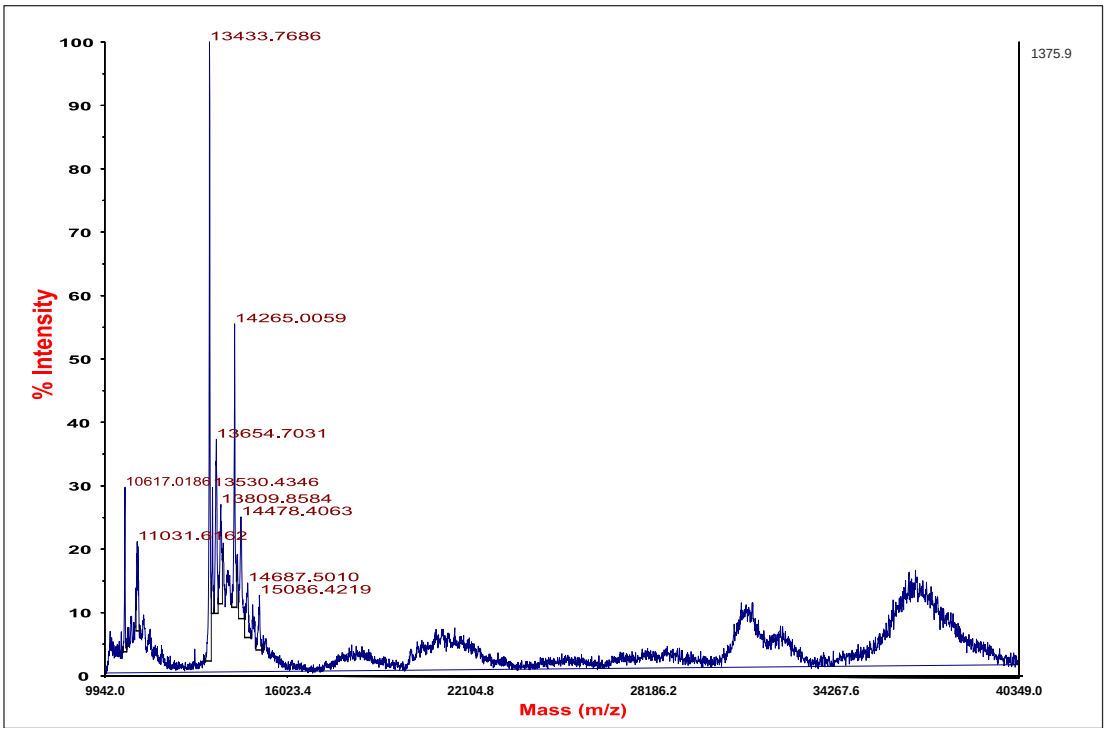


Figure 8: MALDI-TOF MS results for *H. tamulus* venom (low mass range) of fifth extraction for (a) HotJln6, (b) HotKld3, (c) HotKyl3, (d) HotKyn3 and (e) HotSgwr6.

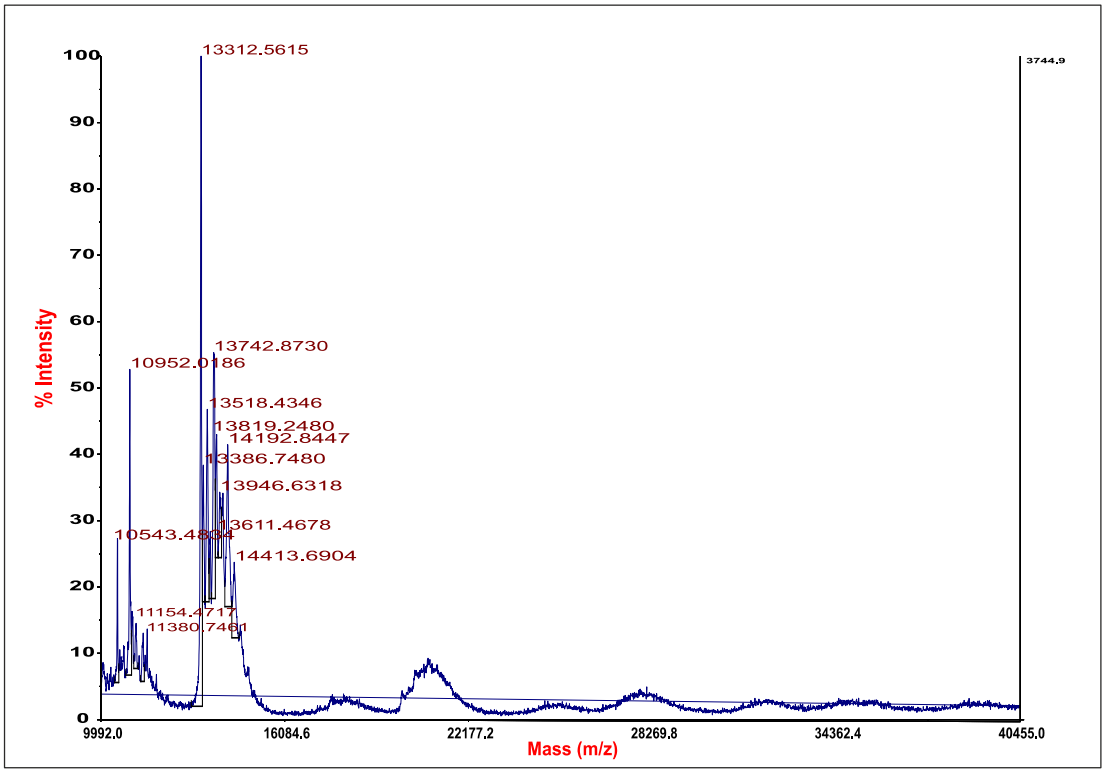
a


14457.7012
14670.5859
11035.8350
10949.1904
11252.3594
14261.3486
11211.4990
10594.9385
14149.3213
11456.5225
10783.8760
13808.3672
% Intensity
Mass (m/z)
9992.0
16084.4
22176.8
28269.2
34361.6
40454.0
666.1

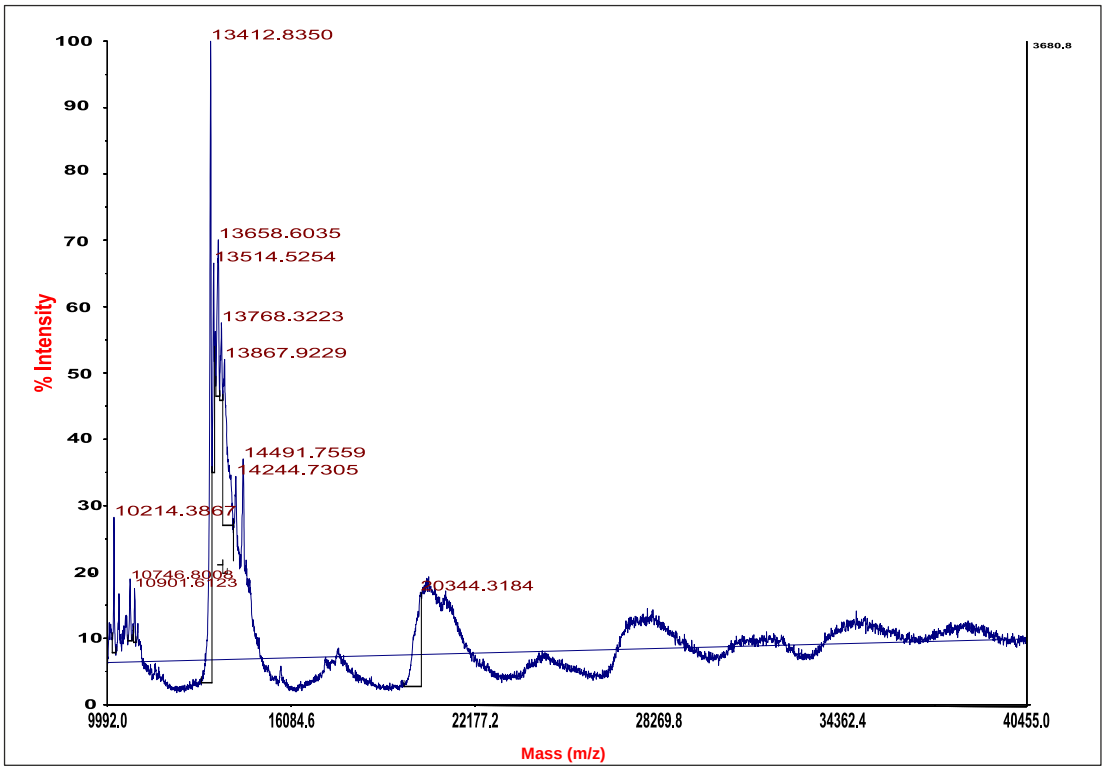
b


13433.7686
14265.0059
13654.7031
10617.0186
13530.4346
13809.8584
14478.4063
14687.5010
15086.4219
% Intensity
Mass (m/z)
9942.0
16023.4
22104.8
28186.2
34267.6
40349.0
1375.9

c


13312.5615
3744.9
% Intensity
13742.8730
10952.0186
13518.4346
13819.2480
14192.8447
13386.7480
13946.6318
13611.4678
10543.4864
14413.6904
11154.4717
11380.7461
Mass (m/z)
9992.0
16084.6
22177.2
28269.8
34362.4
40455.0

d


13412.8350
3680.8
% Intensity
13658.6035
13514.5254
13768.3223
13867.9229
14491.7559
14244.7305
10214.3867
10746.8008
10901.6123
10344.3184
Mass (m/z)
9992.0
16084.6
22177.2
28269.8
34362.4
40455.0

e

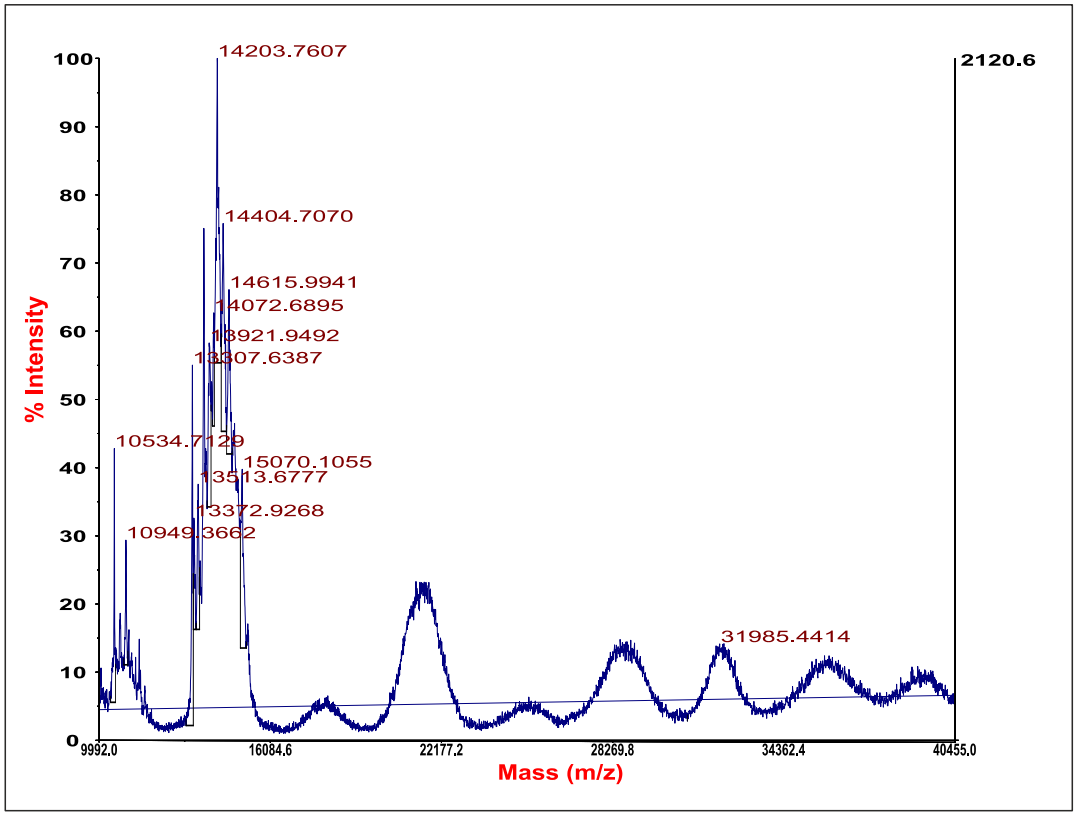


Figure 9: MALDI-TOF MS results for *H. tamulus* venom (high mass range) of fifth extraction for (a) HotJln6, (b) HotKld3, (c) HotKyl3, (d) HotKyn3 and (e) HotSgwr6.

Limitations of current work and future directions

This study is a very novel approach towards a parallel between Evolutionary studies and Venomics, thus, very little background information is available. The genetic analysis is carried out based on a single gene. Here the constraint is that for a gene like COI which is very conserved we observed several mutations in intra and inter population gene pool and thus designing primers for amplification is very difficult. To perform multigene analysis standardization attempts were made earlier, but funds being the limiting factor, we could not proceed with the standardization.

For venom analysis, the general way of collecting venom is performing multiple extractions and then pooling all the extractions to carry out experiments. In our study we wanted to address that is there any change in the venom composition for an individual over time and thus the amount of venom available for analysis was meagre. Venom was dissolved in acetonitrile-water, this solvent environment is not compatible for SDS-PAGE and thus gel electrophoresis could not be applied detection of venom components. An attempt was going to be made for peptide sequencing but due to some technical issues, this idea could not materialize.

This project is an extensive work and one-year time frame is inadequate to effectively complete the entire project. Prior to this study, during semester projects, population genetics markers were used to study population variation but we could not obtain amplification for the markers.

We are still in possession of the venom extracts. We will try to apply different techniques to identify and sequence the components present in the venom concoction and try to conduct a thorough analysis.

Conclusions

First general molecular phylogenetic tree of Indian scorpions has been made and evolutionary relationships amongst different individuals have been established. It has been observed that H. *tamulus* is very widely distributed and all the members are actually a single specie but with enormous mutation that are null in nature. Even morphologically, there are no evident differences in this cluster. H pachyurus and H rugiscuitis have been observed to be in local packets. These species seem to have descended from multiple distinct lineages. Lychas and Isometrus group identification on the basis of the single apomorphic character is inadequate as a few scorpions from Isometrus group fall well within the Lychas clad. Scorpions that are currently identified as Lychas tricarinatus might be a cluster of different species. In this study, the phylogentic placement of Heterometrus phipsoni, Charmus and Chiromachetes sahyadriensis (Mirza et al. 2015) is explained for the first time.

Mass spectrometry results suggest that there might be conserved peptides in venoms across different population thereby challenging the existing knowledge. But before leaping to any conclusions, more experimentation is required.

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