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Research Article

Study on Prevalence of Tardigrades in Tamil Nadu and Species Identification Using Pan-PCR

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Abstract

Tardigrades (water bears) are organisms, capable of surviving in very extreme conditions. These organisms are known to be ubiquitous and are mostly studied for their extremotolerant properties. Many on-going researches in other countries have shown that some of the extremotolerant properties of Tardigrades can be exploited for medical and experimental applications. But it was also observed that, these properties differed in different species of Tardigrades and also depended on their geographical location. In previous geological studies it was observed that the presence of Tardigrades in tropical region was minimal which contradicted their well-known ubiquitous nature. Therefore, in this paper, we examined the prevalence of Tardigrades in some tropical areas of Tamil Nadu and distribution of different species along with studying their different temperature toleration capacity. Thirteen out of twenty-two moss samples collected from different region of Chennai, Coimbatore and Tirunelveli, were screened positive for the presence of Tardigrades. Based on the morphological identification of claws and buccal pharyngeal apparatus, the isolated Tardigrades were distinguished as three different species. Most of the species was distinguished as *Milnesium sp.,* and others as *Murrayon sp.* and *Macrobiotus sp.* using light and phase contrast (PC) microscopy. The phenotypically identified species were genotypically characterized as *Milnesium tardigradum, Macrobiotus sapiens* and *Paramacrobiotus richtersi* respectively using *18S rRNA* sequencing. The isolated species were reared in 2% agarose plates, in order to explore their reproducing ability in fluctuating room temperature. Their survival ability to different temperatures were tested to observe their toleration limit in terms of their capacity to revive after exposure. From the above experiments, it was observed that *Milnesium tardigradum* displayed higher tolerance to all the different conditions exposed and was concluded that *Milnesium tardigradum* was highly tolerant when compared to *Macrobiotus sapiens.*

Keywords: *Milnesium tardigradum; Paramacrobiotus richtersi; Macrobiotus sapiens*; Extremotolerant; Cryptobiosis; Tun State; 18S rRNA Sequencing

Introduction

Tardigrades are small eukaryotic multicellular micro-animals [1] with more than 1200 existing species, which are also known as water bears or moss piglets [2,3]. They are microscopic invertebrates, that belong to the superphylum of Ecdysozoa similar to *Caenorhabditis elegans*, yet belong to a separate phylum Tardigrada in the Animalia kingdom. They are mostly found in marine, freshwater and terrestrial habitats on substrates such as mosses, liverworts, algae and lichens [4,5]. They are popularly studied for their

extremotolerant properties that they undergo by a process called cryptobiosis.

Cryptobiosis is a phase, in which an organism alters its metabolic activity in response to stressful surroundings, similar to a bear hibernating to escape cold winters. Through previous studies, it is observed that freshwater and terrestrial Tardigrades in particular exhibit extremotolerant properties to various stresses compared to marine Tardigrades [4-9]. During exposure to extreme conditions such as temperature (-253˚C to 151˚C), pressure (6 to 7.5 MPa), ionizing radiations (X-Rays, UV and Gamma radiations), space vacuum and noxious chemicals, they undergo cryptobiosis by transforming themselves into a reversible tun and halting their metabolic process to enter a slowly emerging dormant-like state [8,10-13].

Tardigrades are omnivorous. They feed on mosses, lichens, algae, bacteria, rotifers and nematodes. Some of the species even exhibit cannibalism [14,15]. These micro-animals are observed to be cosmopolitan except for tropical conditions and their survivability factors majorly depends on climatic factors such as humidity, pressure, temperature and also pollution level [5]. They are placed next to lichens for the indication of pollution. The lifespan of Tardigrades is around 2 years in normal surroundings which exceeds up to 30 years during cryptobiosis [12,16-18]. It is also observed that their bodily contractions are preserved up to 120 years which exhibits their unique mechanisms to cope with extremities [19].

Many studies reported several molecular mechanisms involved in the toleration properties of Tardigrades. Some of the studies showed that the production of trehalose like compounds were responsible for survival during desiccation [19,20]. It was also observed that many proteins played specific role during cryptobiosis [10] such as Dsup proteins to resolve single and double stranded DNA breaks [13,21], and IDPs which forms a waxy coat under the epicuticle to restore fluids during desiccation. Subsequently, many families of heat shock proteins (Hsp 70 and Hsp 90), LEA (Late Embryogenesis abundant) proteins and antioxidants are produced to tolerate extreme conditions until favorable conditions were restored [11,20,22,23].

Cryptobiotic model systems are extensively studied using certain species of Tardigrades, which are: *Milnesium tardigradum, Macrobiotus richtersi, Hypsibius dujardini and Paramacrobiotus sp*. [6]. *Milnesium tardigradum* is the most predominant and high stress tolerant species till observed, which has been sent to low orbital space for testing its survival rate and tolerance to cosmic radiations [10,22,23]. *Hypsibius dujardini* is the least stress tolerant species with optical clear bodies [6,10,32] whereas *Macrobiotus sp*. and *Paramacrobiotus sp.* are mostly known for their encystment to survive in rapidly changing environmental conditions such as pH, temperature and osmotic shocks [24-26]. The various tolerant mechanisms they exhibit to achieve cryptobiosis and their endemism are explored in many countries, but there are very less studies indicating their presence in India. Thus, in the present study, we investigated different samples collected from some tropical region of Tamil Nadu, India, to check its prevalence and distribution along with their temperature tolerant properties.

Methods

Sample collection and isolation

A total of 22 moss and lichen samples from tree barks, rocks and walls were collected from different locations of Chennai, Coimbatore and Tirunelveli as listed in table 1. The samples were scraped off using knife and scalpel, which were stored in paper bags at room temperature and transported to the laboratory until use. The collected samples were rehydrated using tap water for around 12-16h (overnight incubation if needed) [1,13]. The samples were then screened for Tardigrades after an interval of 2 hours, according to [14] and were isolated using pipettes.

Phenotypic identification of isolated tardigrades

The isolated Tardigrades were transferred to the glass slide and preliminarily identified based on their morphological structures using Magnus MLX Plus light microscope (Olympus, Japan) [6,27]. The genus of each tardigrade was differentiated based on the structures of buccal-pharyngeal apparatus, claws, armour plates and distance between each pair of legs by comparing previously noted morphologies of each species as per [1,8,33]. Further morphological structures such as length of the body, muscular tissues, buccal lamellae, buccal papillae, buccal-pharyngeal apparatus, legs and each claw were visualized using EVOS FLC Phase Contrast (PC) microscope (Invitrogen, UK) [27].

Genotypic identification: Primer designing and gene amplification

Twenty–two *18S rRNA* tardigrade gene sequences were retrieved from GenBank, NCBI listed in table 2. The following,

Sample no.	Coordinates	Place
TM01	8°56'45.9"N 77°12'54.3"E	Tirunelveli
TM02	8°58'56.8"N 77°13'16.9"E	
TM03	8°59'08.9"N 77°13'11.3"E	
TM04	8°59'10.8"N 77°13'12.1"E	
TM05	8°59'11.9"N 77°13'12.6"E	
TM06	8°59'13.2"N 77°13'13.4"E	
TM07	8°59'14.9"N 77°13'14.6"E	
TM08	8°58'54.7"N 77°13'15.2"E	
TM09	10°57'58.5"N 77°02'01.2"E	Coimbatore
TM10	10°57'58.2"N 77°02'01.2"E	
TM11	10°57'57.7"N 77°02'01.4"E	
TM12	10°53'51.1"N 77°00'34.2"E	
TM13	10°54'10.9"N 77°10'36.6"E	
TM14	10°52'46.7"N 77°11'29.5"E	
TM15	10°52'34.7"N 77°11'30.2"E	
TM16	10°52'19.1"N 77°11'35.0"E	
TM17	10°49'44.9"N 77°11'36.2"E	
TM18	10°44'48.3"N 77°04'17.4"E	
TM19	10°43'47.2"N 77°01'29.9"E	
TM20	10°49'44.8"N 77°11'36.1"E	
TM21	13°06'05.5"N 80°14'13.9"E	Chennai
TM22	13°06'05.1"N 80°14'15.7"E	

Table 1: Geographical data of samples.

Taxa	Genbank accession no.
Echiniscus granulates	DQ839606.1
Echiniscoides sigismundi	EU266960.1
Echiniscus testudo	DQ839607.1
Echiniscus viridissimus	AF056024.1
Halobiotus stenostomus	AY582121.1
Hebesuncus conjugens	AM500646.1
Hypsibius cf. convergens	AM500650.1
Hypsibius cf. convergens 1	AM500647.1
Hypsibius klebelsbergi	AM500648.1
Hypsibius scabropygus	AM500649.1
Isohypsibius cambrensis	AM500652.1
Isohypsibius granulifer	AM500651.1
Macrobiotus sp.	U32393.1
Macrobiotus hufelandi	X81442.1
Macrobiotus sapiens	DQ839601.1
Milnesium tardigradum	AY582120.1
Paramacrobiotus aerolatus	DQ839602.1
Paramacrobiotus tonollii	DQ839605.1
Paramacrobiotus richtersi	DQ839603.1
Ramazzottius oberhaeuseri	AY582122.1
Richtersius coronifer	AY582123.1

Table 2: Accession number of the retrieved sequences from NCBI.

EuT18S-F – forward (5'-AATGRGTACACTTTAAATCC-3') and Eu18S-R – reverse (5'-CTGTTATTGCTCAATCTCGTG-3') pan-primers were designed based on conserved and homologous region of the gene sequences using sequence alignment editor tool, BioEdit version 7.0.9 (Isis Pharmaceuticals)**,** and their specificity was checked using Primer Blast, NCBI. The primers were synthesized at Indigenous DNA (India). In the sequence design, R represent the degeneracy of the bases A and G in the particular position.

Genomic DNA was extracted from cultivated eleven similar triple washed adult Tardigrades using QIAGEN DNeasy Blood and Tissue kit (Qiagen, Germany) according to the manufacturer's protocol with prolonged heating time (~ 45min) for lysis. A pan-PCR targeting *18S rRNA* gene was performed for the isolated Tardigrades with 25µL reaction volume consisting of 1µL of forward and reverse broad-range Eutardigrades *18S rRNA* primers designed in the present study (10 pM of each primer), 10 mM of dNTP mix (Bio Basic Inc, Canada), 2.5 µL of 10× PCR buffer (15mM $MgCl_2$), 0.1 µL consist of 0.5U of *Taq* DNA polymerase (Genet Bio Co, South Korea), 1 µL DNA template and 16.8 µL PCR grade water.

The pan-PCR amplification was performed in Veriti 96-Well Thermal Cycler (Applied Biosystems, USA) with initial denaturation at 94°C for 5 min followed by 35 cycles of denaturation at 94°C for 45 s, annealing at 52°C for 30 s and extension at 72°C for 45 s, and with final extension at 72°C for 5 min. Known positive and negative controls were also included. To increase the yield of the PCR product a modified nested PCR was carried out using same primers as similar to the protocol of [34].

The obtained *18S rRNA* pan-PCR amplicons were resolved along with 100bp DNA markers (Genet Bio Co, South Korea) in 1% agarose with ethidium bromide (10 mg/mL) by gel electrophoresis for ~25min at 135 V using Mupid-exU system (Takara, Japan) and gel was analysed by BioGlow UV Transilluminators (Crystal Technology, USA).

18S rRNA gene sequencing and phylogenetic analysis

The *18S rRNA* pan-PCR amplicons were purified by gel excise method using FavorPrep GEL/PCR Purification kit (Favorgen Biotech Corp, Taiwan). Purified PCR amplicons of the *18S rRNA* gene was sequenced in both directions using the specific primers described. Sequencing was carried out at Macrogen Inc. (Seoul, Korea) using ABI PRISM®BigDye™ Terminator and ABI 3730XL sequencer (Applied Biosystem, USA).

The obtained forward and reverse sequences of *18S rRNA* gene pan-PCR product were trimmed, edited and aligned for contig using Bio-Edit software version 7.0.9 (Isis Pharmaceuticals). The *18S rRNA* gene sequences were compared with known sequences in NCBI Database by using BLAST analysis (http://www.ncbi.nlm.nih.gov/ BLAST/) and the sequences were deposited in the NCBI GenBank database and accession numbers were obtained.

Phylogentic cluster analysis of the present study *18S rRNA* gene sequences along with retrived database sequences was conducted with MEGA 7.0 (software) using the neighbourhood joining (NJ) method. Robustness of the nodes was tested by bootstrapping with 500 replicates.

Tardigrade culture

Isolated Tardigrades from sample were cultured on 2% agarose gel plates with KCM solution and was maintained at fluctuating temperature of range 20-25˚C. Around 10-15 individuals of Tardigrades were picked and transferred to the petri plates along with the addition of a mixture of *Scenedesmus sp.* and *Chlorella sp.* or cultured nematode isolated from TM08 as feed according to [15,22]. The Tardigrades growth progress was observed each day and recorded. Sub-culturing of Tardigrades was done once in 2 weeks [6,10,13,14,32].

Studies on temperature toleration of different species of Tardigrades

Cultivated species of *M.tardigradum* and *M.sapiens* were observed for their toleration capacity when exposed to different temperature by disturbing their ideal temperature (20-21˚C). The different conditions included exposure of Tardigrades to sudden heating, disturbing their thermostat to room temperature and overnight incubation at 4˚C [28].

During sudden heat application, triple washed cultivated Tardigrades, were placed on microscopic slides and were exposed to fire from beneath the slide for about ~20-35 sec until the sample droplet dried containing Tardigrades. For disturbing their thermostat to room temperature, 10-15 Tardigrades were reared in fluctuating conditions of temperature, where they were kept in chamber with ideal temperature for first 12 hours and then in room temperature for the next 12 hours. Similarly, to induce cold conditions, around 10-15 Tardigrades were incubated at 4˚C overnight. After exposure of Tardigrades to all the above conditions, before and after the restoration of normal conditions were observed under Magnus MLX Plus light microscope (Olympus, Japan).

Results

Screening test results

Thirteen samples were screened positive with three different groups of species identified as *Milnesium sp., Murrayon sp.,* and *Macrobiotus sp*. (Table 3) morphologically. Around 84% of the positive samples showed similar phenotype as of *Milnesium sp.*

Phenotypic characteristics of isolated Tardigrades

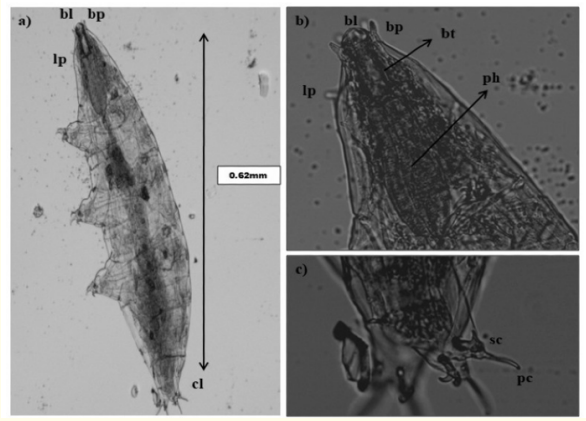
Milnesium sp. was identified by their unique reddish brown pigmented slender bodies with protruding buccal lamellae, buccal papillae and two lateral papillae [4]. Their claw morphology was distinguished with two primary claws and six secondary claws on each leg (Figure 1(a-c)). TM10 sample consisted of both *Murrayon sp.* and *Milnesium sp.,* where *Murrayon sp.* was dominant when compared to latter. *Murrayon sp*. was identified by their plump bodies with transparent guts and 'V' like shaped claws present on legs (Figure 1(d-f)) [29]. TM21and TM22 samples consisted of *Macrobiotus sp*., which was identified by their plump and large bodies with protruding peribuccal lamellae with a buccal ring as illustrated by [33]. They had L-shaped claws (Figure 1(g-i)) [1]. Both *Murrayon s*p. and *Macrobiotus sp*. lacked buccal and lateral papillae.

Genotypic identification of tardigrades

The *18S rRNA* specific pan-PCR products visualized after gel electrophoresis, showed an expected band size of 936bp (Figure

Sample no.	Type of sample	Screening test	Phenotypic identification
TM01	Liverworts	Negative	-
TM02	Mosses from *Ficus religiosa*	Positive	*Milnesium sp.*
TM03	Mosses from *Ficus benghalensis*	Positive	*Milnesium sp.*
TM04	Mosses from *Ficus benghalensis*	Positive	*Milnesium sp.*
TM05	Mosses from *Ficus benghalensis*	Positive	*Milnesium sp.*
TM06	Mosses from *Ficus benghalensis*	Positive	*Milnesium sp.*
TM07	Mosses from *Azadirachta indica*	Negative	-
TM08	Mosses from *Ficus religiosa*	Positive	*Milnesium sp.*
TM09	Algae grown on soil	Negative	-
TM10	Mosses from *Ficus religiosa*	Positive	*Murrayon sp. & Milnesium sp.*
TM11	Bryophytes from walls	Negative	-
TM12	Mosses from *Azadirachta indica*	Positive	*Milnesium sp.*
TM13	Mosses from stones	Negative	-
TM14	Mosses & lichens from *Cocus nucifera*	Positive	*Milnesium sp.*
TM15	Mosses from *Arecaceae*	Positive	*Milnesium sp.*
TM16	Mosses from *Albizia lebbeck*	Positive	*Milnesium sp.*
TM17	Mosses from *rocks*	Negative	-
TM18	Mosses from *rocks*	Negative	-
TM19	Mosses from Arcea catechu	Negative	-
TM20	Mosses from *Tamarindus indica*	Negative	-
TM21	Mosses from *Azadirachta indica*	Positive	*Macrobiotus sp.*
TM22	Mosses from *Azadirachta indica*	Positive	*Macrobiotus sp.*

Table 3: Screening test results.



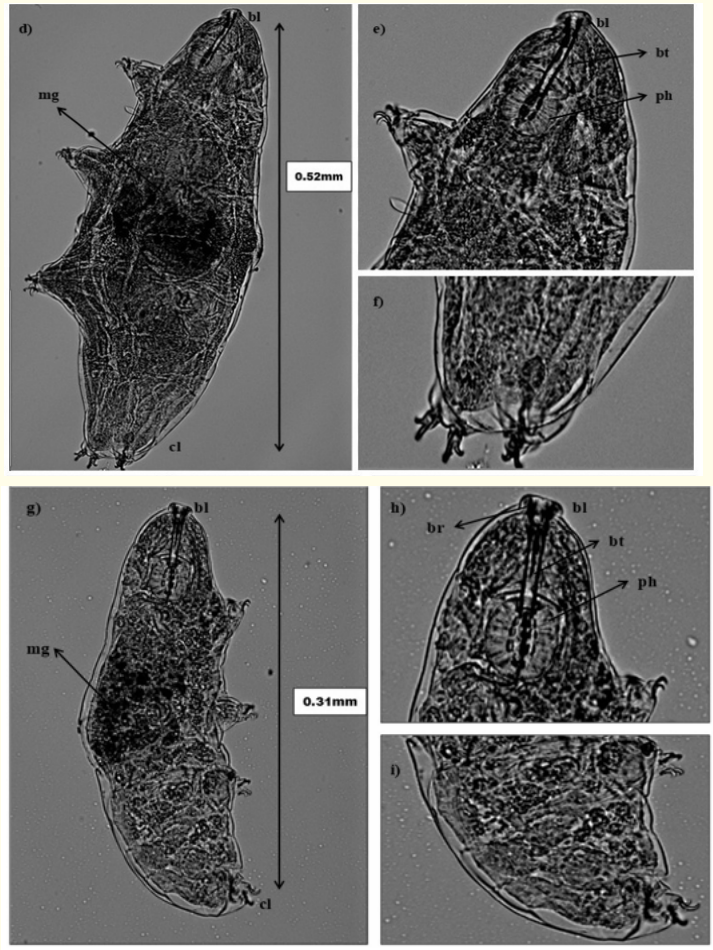


Figure 1: Phase contrast images of isolated Tardigrades (a-c): a) *Milnesium tardigradum* phenotypically identified as *Milnesium sp.*; b) buccal-pharyngeal apparatus of the specimen; c) claw morphology with distinct primary and secondary claws. (d-f): d) *Macrobiotus sapiens* phenotypically identified as *Murrayon sp.* e) buccal-pharyngeal apparatus f) claw morphology. (g-i): g) *Paramacrobiotus richtersi* phenotypically identified as *Macrobiotus sp.* h) buccal-pharyngeal apparatus i) claw morphology. Note: bl=buccal lamellae; bp=buccal papillae; br=buccal ring; bt=buccal tube; cl=caudal legs; lp=lateral papillae; mg=midgut; pc=primary claw; ph=pharynx; sc=secondary claw.

2). The phenotypically identified *Milnesium sp., Murrayon sp.* and *Macrobiotus sp*. were genotypically (*18S rRNA* sequence) characterized as *Milnesium tardigradum, Macrobiotus sapiens* and *Paramacrobiotus richtersi* respectively using NCBI BLAST tool. The 18S rRNA sequences of *M. tardigradum, M. sapiens* and *P. richtersi* was deposited in NCBI Genbank database with the accession number MK567640, MK680800 and MK685669 respectively.

The phylogenetic cluster analysis showed that the sequences of both *M. tardigradum* and *M. sapiens* were homologous with the database sequence and had trivial genetic variations. Whereas the

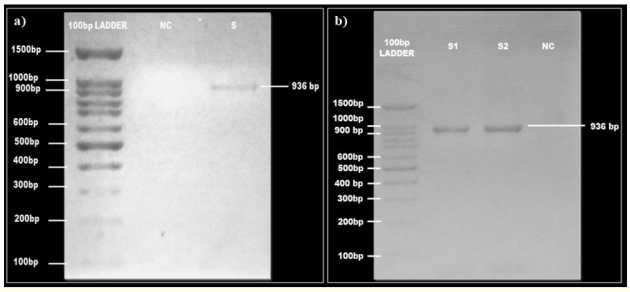


Figure 2: Representative gel for modified nested pan-PCR a) first round pan-PCR product b) second round nested pan-PCR products with different template volume. Note: S=sample; S1=sample 1 with template volume 1µL; S2=sample 2 with template volume 2.5µL; NC=negative control.

sequence of *P. richtersi* formed a different lineage, without forming a node with its synonym *Macrobiotus richtersi* (Figure 3).

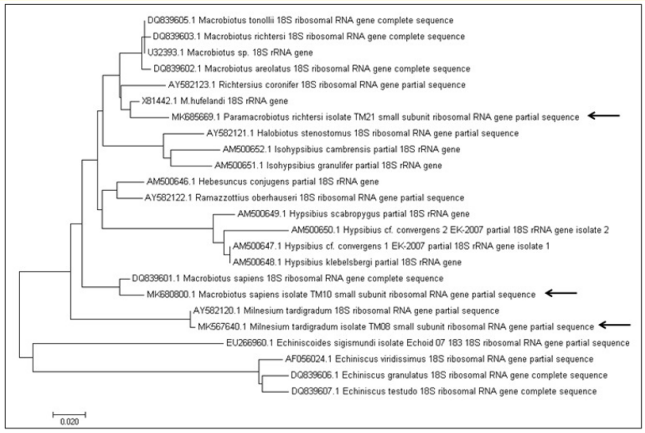


Figure 3: Phylogram obtained from present study *18S rRNA* sequenced sequences and sequences retrieved from GenBank, NCBI (Table 2) using Neighbor-joining (NJ) method.

Culturing of tardigrades

M. tardigradum and *M. sapiens* were isolated from TM08 and TM10 for understanding the temperature tolerance capacity of a high and less tolerant species respectively. Both the species were reared on 2% agarose gel plates with a mixture of both *Scenedesmus sp.* and *Chlorella sp*. but based on the feeding behavior of *M. tardigradum,* they were also fed with nematodes cultured from respective sample. Their feed intake of algae was very low and a shedded skin was only noted after a week.

In comparison, *M. sapiens* feed intake was appreciably noted with the greenish appearance of the gut and within 3 days of inoculation, the culture plates were recorded with shedded skin. Around 2-4 eggs were observed in the sheds along with eggs dispersed on the gel surface after one week. After 2 weeks, a juvenile tardigrade was observed and the initial count of individuals increased by one. This represented that *Macrobiotus sapiens*, reproduction capacity was not affected with the fluctuating room temperature but *Milnesium tardigradum* needed very stringent temperature control and their ability to reproduce was hampered with fluctuating conditions.

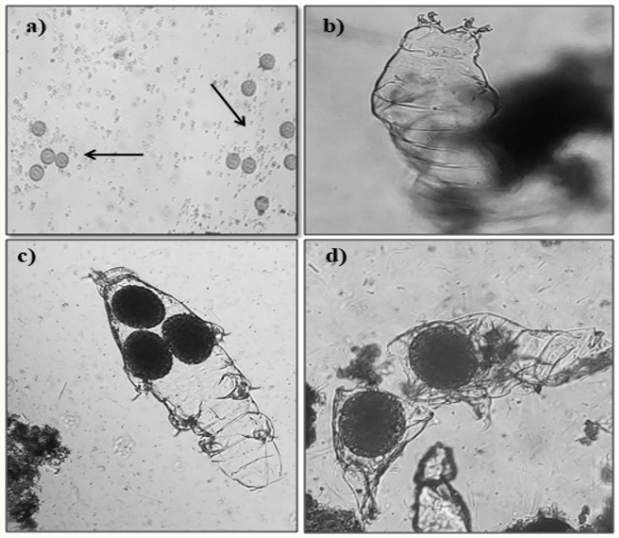


Figure 4: Light microscopic images of a) eggs dispersed over the gel surface; b) shedded skin of *M.sapiens*; c) shedded skin of *M. tardigradum* bearing three eggs; d) shedded skin of *M. sapiens* along with two eggs.

Temperature tolerant properties of *Milnesium sp.* and *Macrobiotus sp.*

Both *M. tardigradum* and *M. sapiens* showed varied resistant capacitance to different temperatures. *M. tardigradum* was more

resistant than *M. sapiens* and was able to regain alive after exposure to stress.

Sudden heating (>150˚c)

M. tardigradum when exposed to sudden heating underwent encystment as similarly illustrated by Guidetti *et al*. (2011), by shrinking their body inside their outer cuticle (Figure 5 (a)). When rehydrated the body cells regained its original shape and started to bulge out. After resurrection of its original shape, they started to move. Whereas *M. sapiens* shrinked their body, after exposure without noticeable reduction in their size. After rehydration they were not able to regain their original structure and were dead (Figure 5 (b)).

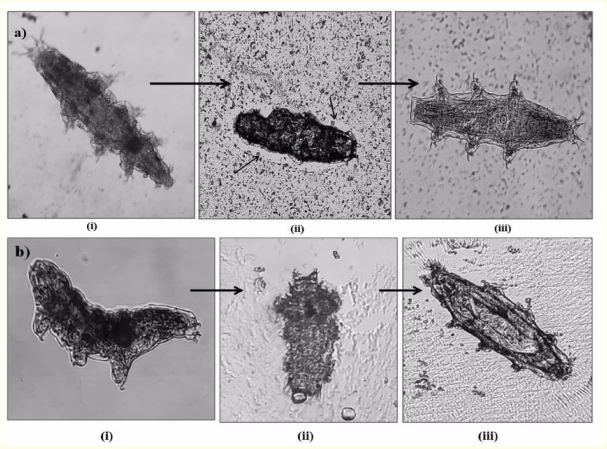


Figure 5: a) (i) *M. tardigardum* before exposure to heat (ii) Cyst formation by shrinking body inside the outer cuticle indicated with arrow marks (iii) After rehydration; b) (i) *M. sapiens* before sudden heating (ii) After exposure to heating (iii) Dead individual after rehydration.

Indian room temperature

M. tardigradum when incubated in Indian room temperature by disturbing their ideal temperature, their activity started to slow down and after 2-3 days their bodies formed a cyst (Figure 6) [11]. After incubation in ideal temperature, their cyst started to grow out to normal body structure. Contradictorily *M. sapiens* was highly active when incubated in Indian room temperature without any noticeable effect in their activities. They were able to feed, grow and shed skin as normal. Hence, the following observation indicated *M. sapiens* more tolerable to slight variations in the temperature when compared to *M. tardigardum.*

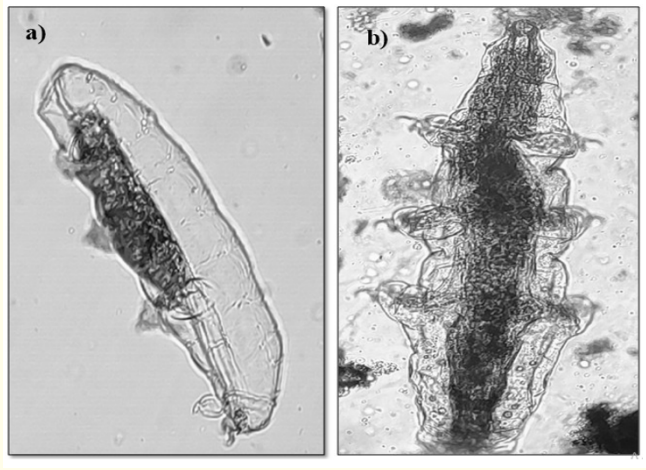


Figure 6: (a) Encyst of *M. tardigardum* after exposure to Indian room temperature; (b) after restoring ideal condition.

Cold temperature

M. tardigradum when incubated at 4˚C overnight, they transformed into a tun and started to crawl back its original structure by extending their claws and legs out of the tun, after restoration of the normal condition (Figure 7(a)). Whereas, *M. sapiens* were highly active for the first 8h after incubation. After 8h they transformed in to a cyst rather a tun. When conditions were restored, they were not able to regain back alive and started to exudate their internal components (Figure 7 (b)).

Discussion

The current study aimed to check the prevalence and distribution of Tardigrades in Tamil Nadu, along with their temperature tolerant properties. Out of 22 collected samples, only 13 samples showed their presence, which mostly included mosses and lichens from tree barks. One of the possible reasons for their absence could be due to the texture of mosses found in the rocks and walls being thick [1], that made Tardigrades unable to use their stylets to feed. Also, there are higher chances of displacement of Tardigrades from a particular region to another due to rain or some other climatic conditions [3]. When the samples were rehydrated for 2-3 days,

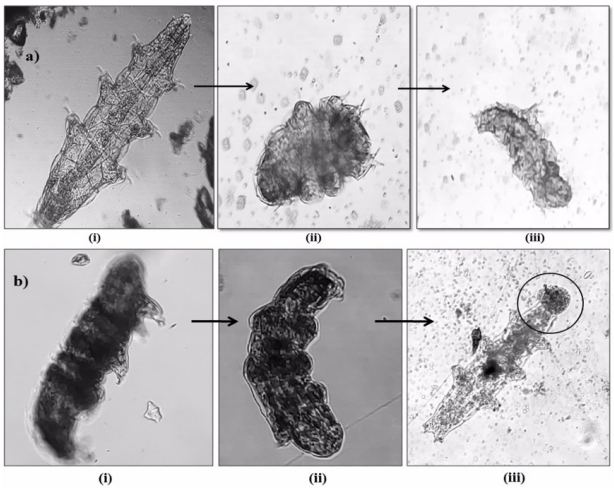


Figure 7: (a) (i) *M. tardigradum* before incubation at 4˚C (ii) Tun formation after overnight incubation (iii) After restoration of normal condition; (b) (i) *M. sapiens* before incubation at 4˚C (ii) After 8h of incubation (iii) Exudation of internal compounds after persistent overnight incubation and restoration of normal conditions (Exudation marked by circle).

Tardigrades bulged out and attained their full body length. This condition occurs mainly due to one of the three reasons: (i) oxygen depletion (ii) pseudo-simplex stage I (iii) dead [1,8,28]. Another reason could be that the Tardigrades might have died because of starvation; since resurrected after unknown number of days of tun state. Even though, they are known to survive anhydrobiosis, Tardigrades cannot survive after reviving, if their food or storage reserves are depleted to maintain their metabolism over years [16]. Consequently, most of the samples consisted of *Milnesum sp*., indicates their survival ability and higher toleration capacity to various geographical region and stress.

The positively screened sample TM10, consisted of two species *M. sapiens* and *M. tardigradum*. But when the population ratio was taken into account, *M. tardigardum* had low count when compared to *M. sapiens*. This observation might represent a dominance of a particular species over an area or refer *M. tardigradum* in higher tropical level as most of the studies report *Milnesium sp.* as carnivores as well as a cannibal. Sample TM21 and TM22 were collected from the localities of Chennai, was observed with *P. richtersi*. The number of individuals found were less and were structurally small when compared to screening of other samples, which may be an indication of pollution level in the particular area [8], as Chennai stands as one of the most polluted cities of India.

The phenotypic identification of *Milnesium sp.* showed a very unique morphology as stated by [1]. Their buccal region contains peribuccal lamellae and lateral papillae, which are not present in other species of Eutardigrades. The phenotypically identified *Murrayon sp.* was genotypically characterized as *M. sapiens*. This shows a remarkable morphological similarity between the two species which cannot be determined when identified phenotypically [31] as both the species are from the same family Macrobiotidae. The difference in their morphology is stated by the difference in the shape of claws as V-shaped and L-shaped for *Murrayon sp.* and *Macrobiotus sp.* respectively [8] which cannot be distinguished easily under the microscope. Thus, the identification of Tardigrades using phenotypic methods is not accurate as many genera exhibit similar morphology and there are chances of misidentification of the genus as done in the present study. Therefore, it is necessary to promote genotypic methods for the correct identification of the species.

The phylogenetic tree construction, showed a different lineage for *P. richtersi*, different from its synonym group *M. richtersi*. This raises a question for the authenticity of the sequence obtained or present in the database. Or which can be explained by variation of some bases in the sequence collected during sequencing.

During exposure to sudden heating, *M. tardigradum* was able to come alive after restoration of normal conditions, but their activity was greatly reduced which is in accordance with the results [20]. Also, [20] reasoned this fact by the incapability of producing heat protectants by Tardigrades in a very short period of time, which is quite lethal. Documenting it again; it goes in accordance with previous literature. Further *M. tardigradum* tries to adapt to different conditions for survival which, provides a direct evident that it is universally prevalent. Future studies and experiments can be done to understand about their survival mechanism and how their proteins are involved for adaptations.

In an overview, the temperature tolerant studies between *M. tardigardum* and *M. sapiens* showed that *M. tardigradum* was more

capable of toleration when exposed to different temperature as similar to [22]. *M. tardigradum* underwent different types of cryptobiotic states, as per the conditions exposed and was able to regain alive after conditions were restored. Comparatively, *M. sapiens* were very sensitive to high temperature, but were much tolerant to a particular range of mesophilic conditions, without switching on to cryptobiotic state immediately.

Conclusion

The present study indicates the prevalence of Tardigrades in TamilNadu, and shows that *Milnesium sp.* are highly distributed when compared to other species. The comparison between phenotypic and genotypic studies of the isolated Tardigrades reveals that, species under the same family share similar body structures which cannot be identified morphologically and shows the advantage of genotyping method. The studies conducted for the temperature tolerant capacity indicates *M. tardigradum* are highly resistant to any change in temperature and survive alive after restoration of the ideal conditions when compared to *M. sapiens*.

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Volume 5 Issue 11 November 2021