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ULTRASTRUCTURAL ASPECTS AND FREQUENCY OF CILIATED FIBROBLASTS IN THE PERIODONTAL LIGAMENT OF INCISOR TOOTH OF RATS SUBMITTED TO DIFFERENT ERUPTION CONDITIONS

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ABSTRACT

The relationship between ciliated fibroblast found in the periodontal ligament of rodent incisor teeth and eruption rate was studied under different eruption conditions, using an estimation of eruption rate and ultra structural aspects of primary cilia. Inferior left incisor teeth were cut for 24 days to increase the rate of eruption. During this period, the rate of eruption doubled; however, after this period, the number and frequency of ciliated fibroblasts was not altered. This lack of change does not necessarily signify that ciliated fibroblast do not have a direct or indirect role in the process of eruption, as suggested by the characteristics of the periodontal ligament structure and emerging functions for the primary cilium found in other tissues.

INTRODUCTION

Cilia are cell surface, membrane bound projections that have a centriolar origin. In epithelial cells, the cilium axoneme consists of nine mobile doublet microtubules surrounding two central microtubules (9+2). The microtubules make contact with each other via dynein arms and by employing ATP recruitment that enables millions of cilia to execute their

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movement on the cell surface. Whilst some cells have multiple cilia, others cells, such as condrocytes and fibroblasts, have a single non-motile cilium that is denominated the primary or solitary cilium (Munger ,1958; Latta *et al.*, 1961; Wilson and Mc Whorter, 1966; Bari and Sorensen, 1965; Scherft and Daems, 1967; Dahl, 1967; Dingemans, 1969; Rash *et al.*, 1969; Federman and Nichols, 1974; Webber and Lee, 1975; Park *et al.*, 1988; Goranova and Chaldakov, 1989; Stubblefield and Brinkley, 1966; Wheatley, 1969, 1971, 1972; Archer and Wheatley, 1971). Generally, solitary cilia do not present a central pair of microtubules (9+0) and do not have dynein arms (for review, see Satir and Christensen, 2007). Solitary cilia can still present great variation in the number and arrangement of their microtubules, as first observed by Zimmerman (1898) in the rabbit renal tubule and in the human seminal vesicle.

During the early 1960s, some studies postulated that solitary cilia could participate in sensorial functions (Barnes, 1961; Sorokin, 1962) and many recent studies have reported the presence of specific receptors and ion channel proteins in the cilia membrane that could be involved in signaling pathways comparable to those described for motile 9+2 cilia (Moran and Rowley, 1983; Pazourot and Witman, 2003; Praetorius and Spring, 2005; Satir and Christensen, 2007). In contrast, Latta *et al*. (1961) suggested the solitary cilia to be a vestige of evolution. As such, the exactly role of solitary cilia in tissues, currently, remains unknown.

The presence of solitary cilia has been reported in fibroblasts in cell culture (Stsubblefield and Brinkley, 1966; Wheatley, 1969, 1971, 1972; Archer and Wheatley, 1971) and in oral tissues. Solitary cilia have also been observed in tooth pulp fibroblasts in dogs (Kubota, 1977) and in the periodontal ligament of the rodent incisor teeth presenting continuous growth and eruption (Bertseen *et al.*, 1975; Barbosa, 1999).

The eruption rate of incisor teeth is approximately 0.5mm/day under normal condition, however, when the tooth is shortened, eruption increases at double the rate of normal conditions (Michaeli et al, 1983). It has been hypothesized that teeth shortening produces a hypofunctional state and it is one of the best model for studies regarding eruption processes. On the other hand, in periodontal ligaments of incisor teeth, Barbosa *et al.* (2003) demonstrated that the number of ciliated fibroblasts is higher, compared to dermal and gingival fibroblasts and suggested that these increased numbers of ciliated cells could be associated with the metabolic activity of the periodontal ligament of the incisor teeth and, consequently, with the eruption process. Thus, to assess whether there is any relationships between the number and frequency of ciliated fibroblasts in periodontal ligaments and the eruption process, in this study the inferior incisor teeth were submitted to different eruption conditions.

MATERIAL AND METHODS

Fourteen male Wistar rats (150-200g body weight) were divided into two groups (7 rats/group). The left lower incisor tooth was shortened at the intergingival level using a diamond high-speed rotating instrument every two days for a total of 24 days to produce a hypofunctional eruption condition, while the right lower incisor tooth remained in a hyperfunctional eruption condition. The control group, had all left lower incisor tooth maintained in a normal functional eruption condition.

In the intact teeth, a reference mark was made with a diamond high-speed rotating instrument and the eruption rates were measured using a calibrated grid under a microscope eyepiece at 10x magnification every two days during the entire experimental period. The reference distances (mm) from the gingival margin to the marks made on the right teeth (normal and hyper functional groups), up to the end of the left incisor teeth (hypofunctional groups) were recorded. For these manipulations, all animals were lightly anaesthetized with halothane. The eruption rates were analyzed using the *Student t* test, at $P < 0.05$ level.

At the end of the experimental period, all rats received an intramuscular injection of Ketamine (80 mg/Kg) and Xylazine (8 mg/Kg), (Francotar and 2% Verbaxyl, respectively, Virbac, Brazil). An intracardiac perfusion with a solution of 2.5% glutaraldehyde in 0.1M phosphate buffer was carried out for 15 minutes. The mandibles were dissected and separated into hemi-mandibles, which were then immersed in 2.5% glutaraldehyde in 0.1M phosphate buffer for a further 3 hours at room temperature. Hemi-mandibles were then maintained in 5% EDTA in 0.1M phosphate buffer, pH 7.4, at room temperature, for 55 days with daily changes of solution. After complete demineralization, hemi-mandibles were cut transversely, taking the third molar as a reference and the samples were post fixed in osmium 1% tetroxide in 0.1M phosphate buffer, pH 7.4, for 3 hours, and washed in 0.1M phosphate buffer, before dehydrating with acetone and embedding in araldite resin. Ultra fine sections (60 to 90 nm) were obtained and contrasted with uranyl acetate and lead citrate. Sections were observed in an EM 10 electron microscopic (Carl Zeiss) at amagnification of 16 000X. For each animal and treatment, 600 fibroblasts were counted, totaling 4200 fibroblasts per group.

To the estimate of the real number of ciliated fibroblasts were used the ligament attached to the tooth as well as on the bone. The estimative were made by counting the number of cilia, basal bodies, basal body pairs, centriolar complexes, basal bodies/centriole pairs, centriole pairs and the basal bodies associated or not with the cilia. The real number of ciliated fibroblasts was estimated according to Beertsen *et al.* (1975), where the % = number of basal bodies-centriole pairs/number of centriole pairs + number of basal bodies-centriole pairs X 100; Archer and Wheatley (1971) and Wheatley (1972) where the % = number of cilia + basal bodies/centriolar complexes X 100; Wheatley (1971) where the % = basal bodies with cilia/basal bodies-centrioles (F) X 100 and Wheatley (1971) where the % = basal bodies with cilia + basal bodies without cilia/basal bodies-centrioles X 100. All data obtained were submitted to analysis of variance (ANOVA) and Tukey test at $P<0.05$ *level.*

RESULTS

The eruption rate during the experimental period is demonstrated in Figure 1. A significant increase in eruption rate was detected during the period by the Student *t* test ($p<0.05$).

Figure 2 (A and B) shows the ultrastructural aspects of the solitary cilia of fibroblasts in the periodontal ligaments of incisor teeth showed that the cilia extended from the distal end of the basal bodies (BB) and projected from the cell surface 3 shows and an accumulation of the filamentous proteins, which are denominated as basal foot (BF) or satellite were usually observed at the lateral side of the basal body. A single centriole (C) was generally connected

to the basal body by a cross-banded structure (CBE). This figure also shows a membrane invagination, (MI) which is deep laterally to the cilium.

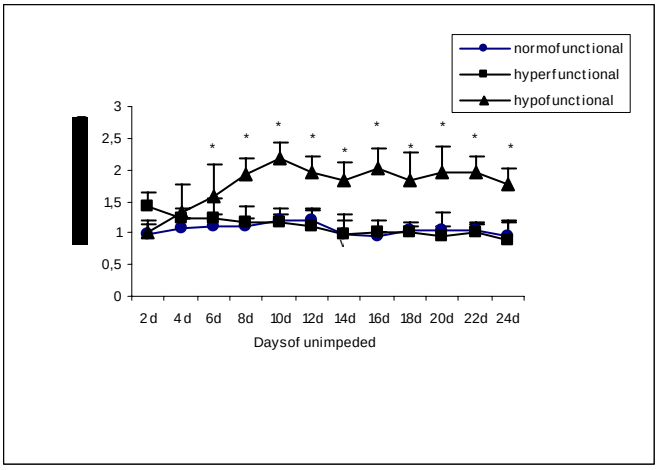

Figure 1. Eruption rate (mm) of the left inferior incisor tooth.
Mean ± DP. (*P<0.05)*

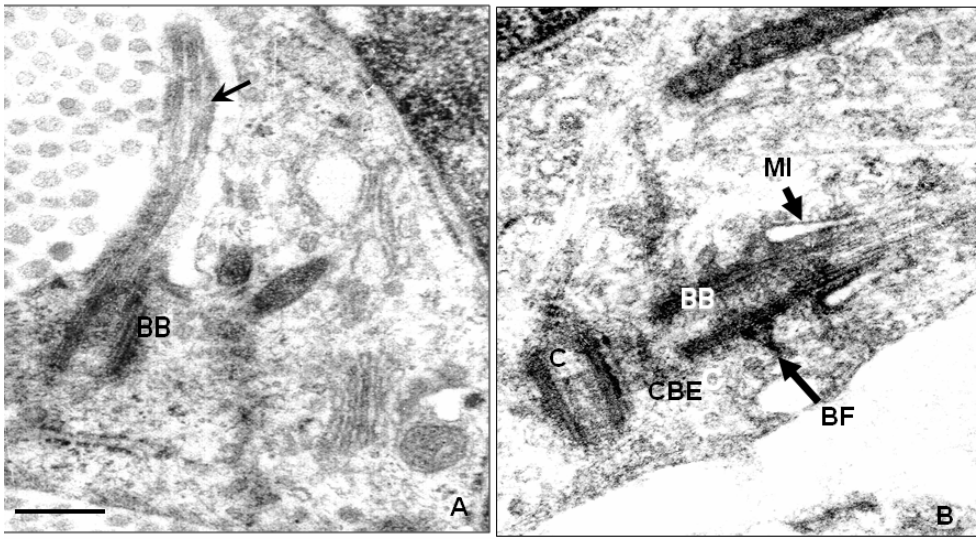

Figure 2. An electron micrograph of a longitudinal section of a solitary cilium of fibroblast in the periodontal ligament of the inferior incisor tooth. A-The solitary cilium extends from the distal end of the basal body (BB) and projected from the apical cell surface of the cell (arrow). B- present the body basal (BB), an membrane invagination (MI) and a single centriole (C) connected to basal body by a cross-banded structure (CBE). Scale bar = 0.28 μm.

The solitary cilia in cross section predominantly presented a 9+0 microtubular array, as shown in Figure 3. However, other microtubular solitary cilia arrangements were observed in cross sections; these included 8+2, 6+2, and 4+0 and are also shown in Figure 3.

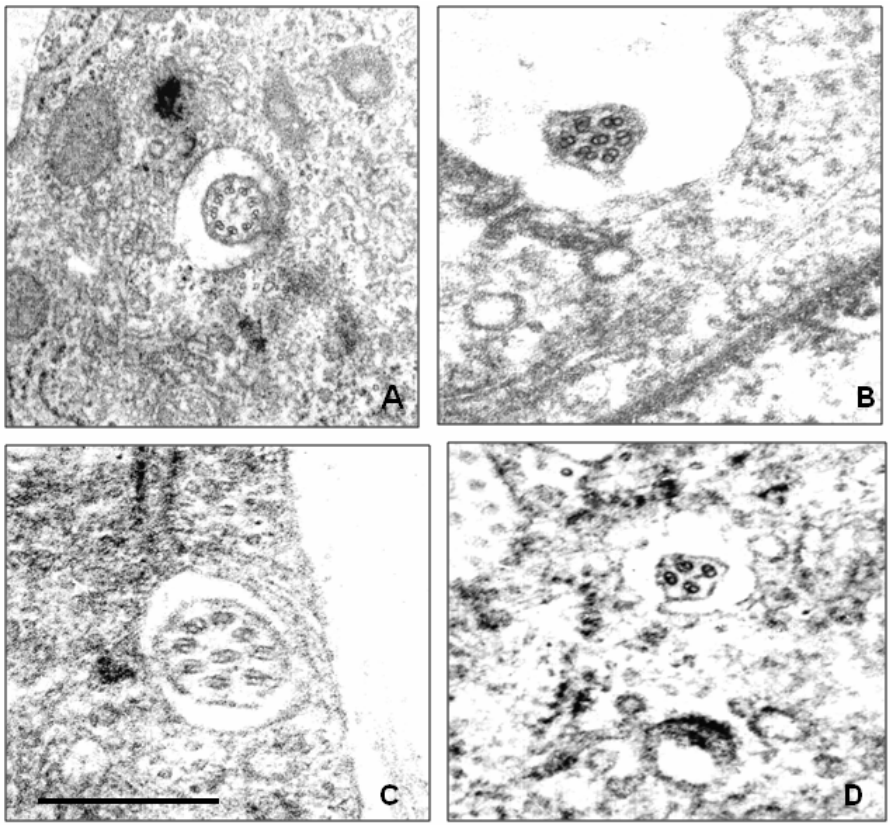

Figure 3. Electron micrographs of a cross section of a solitary cilium in the periodontal ligament showing irregular microtbular arrangements. A.9+0; B.6+2;C.8+2 and D.4+0. Scale bar=0,28µm.

The structural parameters of the ciliated fibroblasts of the periodontal ligament obtained for each eruption condition were established by different methods and are demonstrated in Table 1 and in Table 2.

Table 1. Number of cellular structures used to estimate the frequency of ciliated fibroblast in the periodontal ligament of the lower tooth of mouse submitted to different eruption conditions (Hypo: function diminished of the left lower incisor tooth by shortened; Hyper: right inferior lower tooth with hyperfunction maintained; Normal: left lower incisor tooth with normal function). The data was submitted to analysis of variance (ANOVA) and Tukey test at level of *P<0.05*

Eruption conditions	A- Centriolar complex	B Basal Body	C Solitary cilia	D Basal body pair	E Centriole pair	F Basal/body centriole	G Basal Body with cilia	H Basal body without c
Hyper	255	26	113	18	16	66	15	26
Hypo	293	19	121	16	22	59	20	19
Normal	268	11	121	14.5	13.5	59	21	11

Table 2. Frequency and estimative (%) of the real number of the ciliated fibroblast in the periodontal ligament of the lower tooth of mouse submitted to different eruption conditions (Hypo: function diminished of the left lower incisor tooth by shortened; Hyper: right lower incisor tooth with hyperfunction maintained; Normal: left lower incisor tooth with normal function) calculated by distinct methods. The data was submitted to analysis of variance (ANOVA) and Tukey test at level of *P<0.05*

Real number estimative of the ciliated fibroblast					
Euption condition	Cilia frequency	Beertsen *et al.* (1975)	Archer & Wheatley (1971,1972)	Wheatley (1971)	Wheatley (1971)
Hyper	2.8	53	54.5	22	62
Hypo	3.3	42	48	34	66
Normal	3.3	52	49	36	54

*Beertsen et al. (1975) where the % = number of basal bodies-centrioles pairs (D)/number of centrioles pairs (E) + number of basal bodies-centrioles pairs (D) X 100; **Archer & Wheatley (1971) and Wheatley (1972) where the % = number of cilia (C) + basal bodies (B)/centriolar complex(A) X 100; ***Wheatley (1971) where the % = basal bodies with cilia (G)/basal bodies-centrioles (F) X 100. ****Wheatley (1971) where the % = basal bodies with cilia (G) + basal bodies without cilia (H)/basal bodies- centrioles (F) X 100.

Statistical analysis did not demonstrate significant differences between the frequency and number of ciliated fibroblasts of the incisor tooth periodontal ligaments that were submitted to different eruption conditions and underwent different techniques. However, the frequency of ciliated fibroblasts for this tissue was relatively high, even during normal eruption.

DISCUSSION

Rodent incisor teeth constitute an appropriate model for studies regarding the eruption process, since they demonstrate continuous growth and eruption. Thus, this characteristic allows their submission to different eruption experimental conditions. To increase the eruption rate of the incisor tooth, the shortening treatment has been shown to be the most efficient methodology. This technique increases the eruption rate by twofold, compared to impeded conditions (Michaeli et al, 1983, Gerlach et al., 2000). In the present study, this method was found to increase the eruption rate throughout the experimental period (Figure 1). It is also believed that the continuous growth and eruption of the incisor tooth produced by the shortening treatment leads to a higher turnover of the periodontal ligament components of the extracellular matrix and cells. It has been reported that glycosylaminoglycan is increased during accelerated eruption processes (Kirkham et al, 1993). On the other hand, the fibroblast is the principal cell found in the periodontal ligament. Ciliated fibroblasts found in the periodontal ligament under normal eruption conditions correspond to 70% (Beertsen et al.;1975) of the periodontal ligament. This assessment was confirmed by Barbosa et al (2003), in a study that demonstrated that the frequency of ciliated fibroblasts was higher in periodontal ligaments compared to other connective tissues. From these findings, we hypothesized that the acceleration of the eruption process by the shortening treatment could alter the frequency and number of fibroblasts solitary cilia. For this evaluation, four methods used elsewhere (Beertsen *et al*,1975; Archer and Wheatley, 1971; Wheatley, 1971 and Wheatley, 1971) were applied. These methods are based on different ultrastructural

parameters observed in fibroblasts. Thus, Table 2 presents an altered frequency of ciliated fibroblast following the shortening treatment, however, when results were submitted to statistical analyses, no significant differences in the frequency and numbers of ciliated fibroblasts were found for incisor teeth submitted to different eruption conditions.

These analyses also demonstrate that any of the methods used could have been applied to this study. Since the methods used, herein, are based on different and independent ultra structural aspects, the results of the study were not found to be significant. Results of the study show that the frequency and number of fibroblasts remain constant after the period of shortening treatment. Thus, we cannot affirm that the ciliated fibroblasts, which correspond to 70% of the cells of the ligament, did not play any role in gradual eruption processes since, unfortunately, this approach was not carried out in this study. We suggest that possibly, there is a moment during the increase in eruption, as shown in Figure 1, in which the fibroblast frequency also increases, however this must be further studied to estimate the frequency of fibroblast each two days along of shortened treatment. However, the frequency of ciliated fibroblasts in the periodontal ligament, as shown by different methods, was considered to be higher in all eruptions conditions, as previously demonstrated by Barbosa et al (1999).

The ultrastructural aspects of the solitary cilia found in cells of the periodontal ligament of rat incisor teeth, submitted to different eruption conditions, were similar to those of previous literature descriptions, i.e., presence of a basal foot or satellite in the lateral side of the basal body and a centriole connected to the basal body by a cross-banded structure (Hagiwara *et al.,* 2002); however, no changes were detected in the hypofunctional eruption condition. A microtubular array of 9+0 was also observed in this study, although other variations in numbers of axoneme microtubules were also observed (8+2, 6+2 and 4+0). These variations in numbers of axoneme microtubules of the solitary cilia have already been reported in the dental pulp by Kubota (1977), who suggested a relationship between the microtubules of axoneme and the cell origin; e.g.., epithelial cells have a normal pattern array of 9+2, while tissues of mesenchymal origin could present distinct microtubular arrays. This may occur due to the fact that the periodontal ligament has a mesenquimal origin. A variation in the number of microtubules was also observed in the solitary cilia of the periodontal ligament fibroblast (figure 3); however, whilst this finding is of interest, the exact role of these differences in microtubular arrangement in the solitary cilium in different tissues is unclear and worthy of further study.

Chailley *et al.* (1989) and Boisvieux-Ulrich *et al.* (1990) proposed that the basal foot may promote the movement of cilia by rotation or rearrangement of the basal body in cells of the epithelial oviduct. Another functional explanation for the basal foot in solitary cilia was proposed by Tenkova and Chaldakov (1988) and Poole *et al.* (1997). These authors suggested that the cilia could determine cell polarity, since the basal foot is linked to the Golgi apparatus and may be necessary for exocytosis. In other tissues, a potential role of solitary cilia in sensorial functions has been suggested (Poole *et al.,* 1985; Roth *et al.,* 1988; Schwartz *et al.,* 1997; Wheatley, 1995; Wiederhold, 1976). Solitary cilia were suggested by Munges (1958), Goranova and Chaldakov (1989) to resemble a chemoreceptor after they observed this organelle in cells of the Langerhans island and in smooth muscular fibers in the uterus, respectively. Maraspin and Boccabella (1971) demonstrated that the production of solitary cilia was inhibited by estradiol in the muscular uterus, indicating a chemoreceptor function.

Studies published recently by Anderson et al (2008) and Whitfield (2008) reported mechanical and chemical functions for solitary cilia with a focus on skeletal biology. The

solitary cilia deliver intracellular signals, such as Ca(2+) produced by compression of cartilage fluid that triggers a cascade of intracellular events that include appropriate gene activations to maintain the strength of bone and cartilage. Furthermore, authors demonstrated that the polycystic protein has a mechanically sensitive function and serves as a flow sensor in cultured renal epithelium.

Although this study did not aim to evaluate the periodontal ligament as a tissue submitted to constant masticator forces, it is possible that solitary cilia could have a specific sensorial function in maintaining the homeostasis of the extra cellular environment of the tooth during the eruption process. Solitary cilia may directly influence this process, since the periodontal ligament seems to demonstrate a higher and constant turnover of its components, including cells, collagen fibers and collagenases involved in the remodeling process. It is possible that solitary cilia of the periodontal ligament could also be involved in mechanical or chemical functional responses to the masticator forces that occur during dental function. Such a hypothesis opens new perspectives as regards the functional properties of the periodontal ligament and appropriate studies concerning solitary cilia function in this mechanism are required.

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ZOOPLANKTON COMPOSITION IN AN ESTUARINE AREA OF THE STATE OF MARANHÃO, NORTHEASTERN BRAZIL

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ABSTRACT

The Gulf of Maranhão is the largest and more important estuarine complex in the state of Maranhão, Brazil. São Marcos Bay located in the northeast region of the gulf, contains many small estuaries. The water circulation is due mainly to the tidal currents. This report describes the structure of the zooplankton community in relation to hydrographic conditions in the area, during the dry season/2000. The zooplankton samples were obtained in horizontal tows using a net with 200 µm mesh size. Temperature, salinity, dissolved oxygen and chlorophyll-*a* were measured in water samples. Temperature and salinity were homogeneous throughout the water column. The highest zooplankton densities were observed in São Marcos Bay (up to 19,500 ind.m^{-3}), and the lowest were recorded in the Cachorros River and Coqueiros Strait (less than 8,000 ind.m^{-3}). Fifty zooplankton taxa were identified. The copepods, decapods and chaetognaths represented 99% of the total density. *Parvocalanus crassirostris*, *Acartia tonsa*, *Subeucalanus pileatus* and *Oithona hebes* were the most abundant species. *Parasagitta tenuis* and *Ferosagitta hispida* were also abundant. Zooplankton diversity varied from 1.33 to 1.71 bits.ind^{-1}. The diversity index followed the variations in the density, and was similar to those found in other coastal systems of northeast Brazil.

Keywords: Zooplankton, Spatial variation, Estuarine area, São Marcos Bay, Brazil.

1. INTRODUCTION

The Brazilian coast has many estuarine regions of different origins and geomorphology (Bonecker et al. 2007). The Gulf of Maranhão is the largest and most important estuarine

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complex in the state of Maranhão. The gulf is located between the coast of the state of Amazonas and the northeastern coast of Brazil. In its central region, the São Luís do Maranhão Island separates the gulf into two great bays: the São Marcos Bay in the west, and the São José Bay in the east (Stride 1992). São Marcos Bay is a tropical estuary, and is unusual because of the wide range of tidal amplitude (Stride 1992). Similar characteristics to those of São Marcos Bay are found in the Rance estuary in northwest France, where tidal amplitudes of 13 m occur; and also at Pico Island in the Azores (Bonecker et al. 2007).

The study site is defined by the region formed by the São Marcos Bay, the Mosquitos Strait and the Cachorros River at 02°18'-02°47' S and 044°20'-044°25' W. Since 1980, many studies have been carried out in the region of the Gulf of Maranhão, the majority of these on aspects of the biology and fishery of shrimp and fish (Castro and Emerenciano 1978, Ramos-Porto et al. 1978, Coelho and Ramos-Porto 1980, Porto 1983/84).

While numerous studies exist on the zooplankton community structure in the neritic or epipelagic zone, information about the northern Brazilian region is extremely limited. Lopes (1988), Gonçalves de Lima (1996), Silva et al. (2003; 2004), Gonçalves et al. (2004) and Krumme and Liang (2004) studied the northern and northeastern coast of Brazil. The composition and occurrence of zooplankton in the Coqueiros and the Mosquitos straits and the Cachorros River were evaluated by Paranaguá et al. (1984) and Lopes (1986).

The aim of this study is to describe the occurrence and abundance of the zooplankton community in the Gulf of Maranhão (São Marcos Bay, the Mosquitos Strait and the Cachorros River). This community is compared with others located in different coastal regions. The relationships of the zooplankton community to environmental factors are also examined. Few estuaries on the Brazilian northeastern coast have been surveyed, and this area is the subject of the fewest studies on the estuarine and coastal zooplankton communities (Brandini et al. 1997). Only abstracts, dissertations and theses exist, which increases the importance of this study.

2. MATERIAL AND METHODS

2.1. Study Area

São Marcos Bay is located on the central coast of the state of Maranhão, where the Maranhão Gulf is located (Figure 1). The bay is an estuary approximately 100 km long and up to 16 km wide. It receives several rivers, including the Grajaú, Mearim and Pindaré. Mangroves and marshes are dominant in this region (Juras et al. 1983, Camargo and Isaac 2003). The range of tidal amplitude can reach 7 m. In the bay mouth, the flood tide flows to the northwest and the ebb tide flows to the north, with a current speed of 3.9 knots (Stride 1992). The climate is typically equatorial humid, with a rainy season (November through June) and a dry season (July through October).

The mixing process of saline and continental waters, high primary and secondary production, and high concentrations of nutrients and suspended matter characterize the waters of this region as typically estuarine (Juras et al. 1983). This is the largest and most important estuarine complex in the state of Maranhão.

Figure 1. Locations of the sampling stations in an estuarine area in the state of Maranhão, northeastern Brazil.

South-southwest of São Luís do Maranhão Island (02°38’12’’- 02°43’14’’S and 044°23’35’’- 044°17’50’’W) are the Coqueiros and Mosquitos straits and the Cachorros River. The Mosquitos Strait (2^{o}38'12"S - 44^{o}23'35"W) separates São Luís Island from the continent.

This strait is 5 km long and 104 m wide, extending southeast-northwest, and linking Arraial Bay with São Marcos Bay (Nogueira and Ferreira-Nogueira 2001). The Coqueiros Strait is a north-south channel, limited to the north by São Marcos Bay and to the south by the Mosquitos Strait, which joins it to São José Bay. Draining into the northern part of the Coqueiros Strait and 3.5 km distant from São Marcos Bay, the Cachorros River is the main contributor of continental waters to the strait (Castro et al. 1989).

The hydrodynamism in the Coqueiros and the Mosquitos straits is the result of the interaction of the tidal waves proceeding from the São Marcos and São José bays. The strong currents of the region produce intense turbulence in the water column, causing mixing and consequent resuspension of fine material (Ferreira and Kjerfve 1981).

2.2. Sampling Method and Treatment of Samples

A sampling program was carried out during the dry season (July 2000) at six stations: four in the Coqueiros Strait (stations 1 to 4), one in the Cachorros River (station 5) and one in São Marcos Bay (station 6) (Figure 1). The zooplankton samples were collected during the day at ebb tide, with the exception of station 5, which was visited at flood tide because of logistical constraints. All the hauls were horizontal at the surface, using a cylindrical-conical net of mesh size 200 µm with mouth diameter 60 cm, fitted with a calibrated flowmeter. Samples were preserved immediately after the hauls in 4% buffered formalin.

The samples were divided into fractions with a Folsom Plankton Sample Splitter (McEwen et al. 1957), and replicated subsamples containing at least 100 individuals were taken for analysis (Frontier 1981) of the more abundant groups. Appendicularia, Copepoda, Doliolidae and Chaetognatha were identified by means of appropriate references (e.g., Björnberg 1981, Bradford-Grieve et al. 1999, Casanova 1999, Esnal 1999, Esnal and Daponte 1999, Bonecker and Carvalho 2006, Avila et al. 2006 and Dias and Araujo 2006). The other zooplankton groups were counted. Species composition and abundance were determined for all samples. Shannon's diversity index (H') was calculated to evaluate the degree of organization of the copepod community (Shannon 1948).

Local depth ranged from about 10 m at stations 1 to 5, between Tauá Mirim Island and the continent, to 20 m at station 6, in the bay. Water temperature and salinity were also determined from samples taken at the surface (0.5 m), middle depth (5~10 m) and near the bottom (15~20m) using a LabComp thermosalinometer (0.1 ºC). Dissolved oxygen and chlorophyll-*a* were measured according to CNEXO (1983) and Parsons et al. (1984), respectively.

Analysis of variance (ANOVA) tests were used at a significance level of p=0.05 to identify statistical differences among the sampling stations. All data were evaluated for normality and homogeneity prior to analysis. Data were log-transformed [log (x+1)] to obtain a homoscedastic data distribution (Legendre and Legendre 1983). In order to test the similarity in the abundances and compositions between stations, a cluster analysis on samples-species data matrix of the 6 stations was performed using the Euclidean Distances index.

3. RESULTS

3.1. Environmental Data

The water salinity and temperature were homogeneous ($p < 0.05$). The lowest values were observed at stations 1 and 2, between Tauá Mirim Island and the continent. At station 4, at the entrance of the Coqueiros Strait, differences between water salinity at the surface and near the bottom were greater than those found at the other stations (Table I).

During the survey period, water temperature varied more widely (6.2ºC) at the surface than near the bottom. At station 4, the temperature differed more between the surface and the bottom than at the other stations. This result is similar to that for the salinity. At stations 4 and 5, colder water was found at the surface (Table I).

Dissolved oxygen content ranged between 3.6 (station 3) and 5.1 mg.L^{-1} (station 6). The levels at the stations in the Mosquito Strait and Cachorros River were relatively lower (Table I). Chlorophyll *a* ranged between 3.4 (station 4) and 32.3 µg.L^{-1} (station 1) (Table I). Stations 1 and 2 showed higher levels than at the other stations.

Table I. Temperature (ºC), salinity, dissolved oxygen (mg.L^{-1}) and chlorophyll *a* (µg.L-1) sampled in an estuarine area of the state of Maranhão, northeast coast of Brazil

	Stations					
Parameter	1	2	3	4	5	6
Temperature - surface	28.5	28.8	28.9	22.7	25.0	28.5
Temperature - mid-depth	28.5	28.5	28.5	28.5	28.3	28.5
Temperature - bottom	28.4	28.5	28.4	24.2	28.4	28.3
Salinity - surface	16.4	17.0	21.9	22.7	22.5	20.3
Salinity - mid-depth	16.5	17.2	21.9	23.8	22.4	20.9
Salinity - bottom	16.6	17.2	22	24.1	21.6	21.1
Dissolved oxygen	3.9	4.4	3.6	3.9	4.1	5.1
Chlorophyll *a*	32.3	26.7	5.9	3.4	4.3	4.9

Table II. Minimum and maximum values, mean and standard deviation (ind.m^{-3}) of the zooplankton taxa sampled in an estuarine area in the state of Maranhão, northeastern coast of Brazil

Taxa	Min.	Max.	Mean	S.D
Order Foraminifera	0.04	0.82	0.16	0.32
Class Hydroidomedusae	0.11	1.71	0.49	0.62
Phylum Nemata	0.04	0.04	0.01	0.02
Class Bivalvia (larvae)	0.04	8.02	1.77	3.13
Class Gastropoda (larvae)	0.28	0.92	0.20	0.37
Class Polychaeta (larvae)	0.2	97.71	24.61	40.21
Order Calanoida	42.36	42.36	7.06	17.29
Acartia tonsa	407.12	2880.59	1316.52	988.20
Acartia lilljeborgi	42.36	425.33	189.93	156.41
Acartia spp.	296.53	2456.98	975.00	848.14
Parvocalanus crassirostris	1186.13	8221.99	3486.11	2553.20
Paracalanus sp.	42.36	101.78	24.02	41.69
Pseudodiaptomus acutus	42.36	219.25	57.72	86.08
Pseudodiaptomus richardi	42.36	42.36	7.06	17.29
Pseudodiaptomus sp.	30.38	30.38	5.06	12.40
Subeucalanus pileatus	84.72	4056.18	824.09	1606.30
Subeucalanus sp.	211.81	1315.52	383.17	488.51

Table II. (Continued)

Taxa	Min.	Max.	Mean	S.D
Temora stylifera	219.25	219.25	36.54	89.51
Family Pontellidae	50.89	50.89	8.48	20.78
Pontellopsis regalis	50.89	50.89	8.48	20.78
Nauplii	182.28	1315.52	573.83	560.27
Order Cyclopoida	50.89	84.72	22.60	36.61
Oithona hebes	60.76	931.96	434.90	285.26
Oithona sp.	84.72	84.72	14.12	34.59
Euterpina acutifrons	30.38	101.78	36.15	37.50
Class Cirripedia	0.12	3.39	0.84	1.27
Class Ostracoda	0.04	0.08	0.02	0.03
Order Decapoda	151.23	634.74	298.46	178.86
Order Amphipoda	0.04	0.1	0.04	0.04
Order Mysidacea	0.05	0.1	0.03	0.04
Order Isopoda	0.03	0.1	0.04	0.04
Ferosagitta hispida	0.46	88.82	22.03	34.09
Parasagitta tenuis	18.13	375	91.13	139.83
Chaetognatha - unidentified	0.95	25.42	8.14	11.37
Chaetognatha - juveniles	1.34	92.1	21.89	39.38
Oikopleura dioica	0.03	0.83	0.20	0.35
Oikopleura rufescens	0.12	0.24	0.06	0.10
Doliolum nationalis	0.03	0.1	0.02	0.04
Class Actinopterygii				
Eggs	0.03	0.05	0.04	0.02
Larvae	0.08	3.3	1.41	1.25

3.2. Species Composition and Abundance

Forty taxa were identified, including 10 species of copepods and 30 species of other groups (Table II). Copepods, decapod larvae and chaetognaths represented 99% of the total zooplankton abundance. Copepods were the largest zooplankton component in the study area, in terms of both numerical abundance and species diversity. Copepods accounted for at least 94% of the total zooplankton abundance.

The average abundance of zooplankton was 8811 ind.m^{-3} (SD=8336). The highest zooplankton abundance among the six sampling stations was 19,579 ind.m^{-3} at station 6 in São Marcos Bay, while the lowest was 5393 ind.m^{-3} at station 4 in the entrance of Coqueiros Strait (Figure 2). The Cluster Analysis grouped the stations situated between the Cachorros River and the continent, which were separated from the stations located in Coqueiros Strait and São Marcos Bay (Figure 3).

The zooplankton was dominated by estuarine-coastal copepod species, *Parvocalanus crassirostris, Acartia tonsa, Subeucalanus pileatus* and *Oithona hebes*.

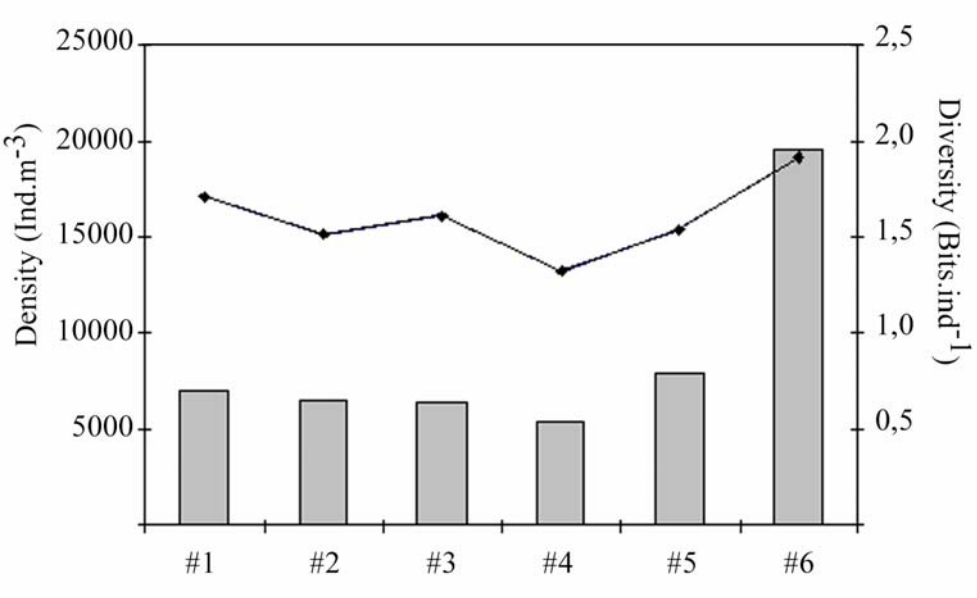

Figure 2. Total zooplankton density (Ind.m^{-3}), and species diversity values in an estuarine area in the state of Maranhão, northeastern Brazil (bar= Total zooplankton density; line= Species diversity).

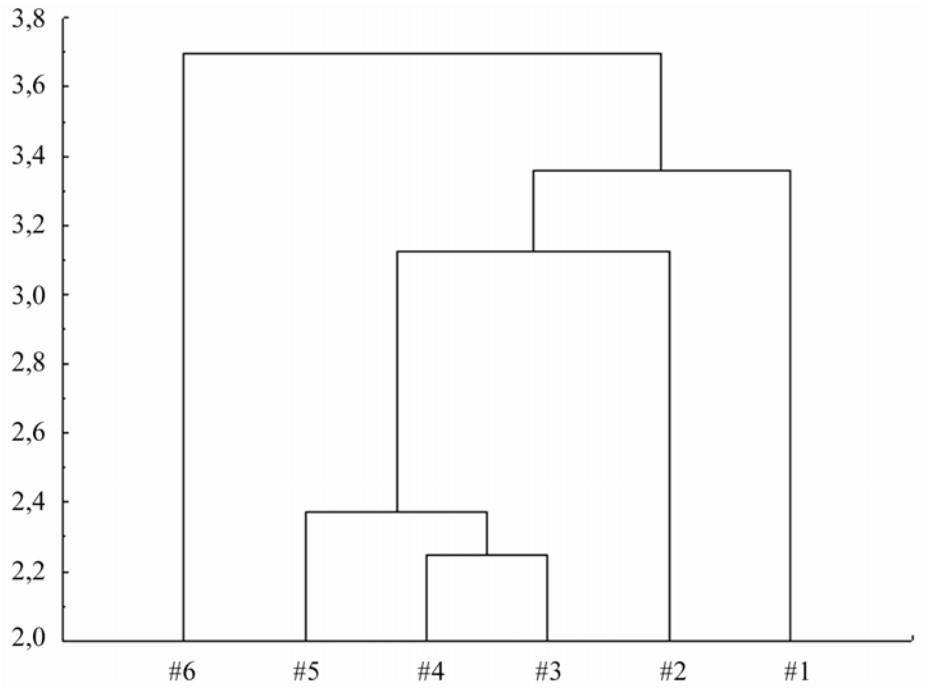

Figure 3. Cluster analysis on 15 species x 6 stations data matrix performed using the Euclidean Distances index.

Three genera of copepods (*Parvocalanus*, *Acartia* and *Subeucalanus*) dominated the zooplankton community, accounting for 81% of the total zooplankton abundance.

Parvocalanus crassirostris was the most dominant copepod in the estuarine area, constituting 40% of the total copepod abundance. It is an estuarine-marine species, and dominated in areas with salinities > 20, with the exception of stations 1 and 2, situated between Tauá Mirim Island and the continent. The marine species *Pontellopsis regalis* was found at the entrance of the Cachorros River (station 5). An estuarine species *Pseudodiaptomus richardi* was recorded at station 2 in the Coqueiros Strait.

The Crustacea Decapoda was the second most abundant group in all stations. The highest density values (635 ind.m^{-3}) were found at station 6 in São Marcos Bay (Table II).

Other important zooplankton species in this estuarine system were *Parasagitta tenuis* and *Ferosagitta hispida* (Chaetognatha), found in all stations; and *Oikopleura dioica* and *Oikopleura rufescens* (Appendicularia), found at the inner stations 1 and 2.

In relation to the Shannon's diversity of zooplankton, station 6 (São Marcos Bay) showed the highest value (1.91). At the other stations, diversity varied from 1.33 to 1.71, with the lowest value at station 4 (Figure 2). The diversity values followed the variation of total zooplankton density values. There was practically no variation in the number of species found at each of the six sampling stations.

4. DISCUSSION

The variations of the environmental variables (salinity, temperature, dissolved oxygen) are similar to those found in the same region and in other estuarine systems (Fernandes et al. 2002, Gomes et al. 2004). The homogeneity of the water column is normal for the region, where there is no strong stratification because of the hydraulic characteristics of the bay (NUCLEBRAS 1984).

Salinities were characteristic of brackish waters. The minimum salinities were observed at the inner stations with less influence of sea water, which enters during flood tide.

The water temperature varied more in the surface samples, which were probably directly influenced by the air temperature and solar incidence. The greatest stratification of temperature and salinity occurred at station 4. This station is directly influenced by the sea water from the Coqueiros Strait, entering to the mouth of the bay, as much as by the fresh water from the rivers that discharge in this strait.

Dissolved oxygen was relatively low, characteristic of locations that receive a large organic load. The highest values of dissolved oxygen found at station 6 probably indicate the greatest influence of clearer water from the sea, because this station is in the mouth of the São Marcos bay, rather than the Coqueiros Strait or the Cachorros River.

The higher values of chlorophyll *a* can be explained by the location of the stations closer to the interior of the continent, where the input of nutrients from rivers is higher and discharge into the Coqueiros Strait, leading to a greater increase in the amount of phytoplankton, reflecting the eutrophization potential. The higher values observed at the station in São Marcos Bay may be related to its location in an open area, where greater exchange with the adjacent coastal region possibly occurs.

The zooplankton was composed by estuarine and estuarine/marine species, similar to assemblages recorded in the same region (Paranaguá et al. 1984, Lopes 1986) and in other estuarine regions in São Marcos Bay (Gonçalves et al. 2004). These authors also recorded

copepods, decapod larvae and chaetognaths as the dominant groups. In relation to the Copepoda, *Parvocalanus crassirostris*, *Acartia tonsa*, *Subeucalanus pileatus* and *Oithona hebes* were the most important. The dominant species remained nearly unchanged, which demonstrates that the region is in balance. *Parvocalanus crassirostris*, an estuarine-marine species found in brackish waters of estuaries and mangroves (Björnberg 1981), was more abundant in this area. This species is frequent in Brazilian coastal waters (Björnberg 1981), and was best represented at the station in São Marcos Bay. The marine species *Pontellopsis regalis* was found at the entrance of the Cachorros River.

The Crustacea Decapoda was the second most abundant group. The high densities of decapod larvae in the area could be explained as a consequence of the concentration of zoeae in estuarine and coastal waters. The high numbers of some larvae, juveniles and post-larvae at the entrance of the Cachorros River may be related to the collections having been made during flood tide.

This study provides the first data on species identifications of Chaetognatha, Appendicularia and Doliolidae in the region. Consequently, it is not possible to compare the results presented here with past data. Previous reports (Paranaguá et al. 1984) identified the Chaetognatha and Appendicularia to the genera *Sagitta* and *Oikopleura*, respectively. Gonçalves at al. (2004) reported the occurrence of the species *Flaccisagitta enflata* and *Oikopleura dioica* in the estuarine waters of São Marcos Bay.

The highest diversity values were found in São Marcos Bay. The values of diversity are similar to those found in other coastal systems of the northeastern Brazilian coast **(**Silva et al. 2003), and to those related by Margalef (1983) for coastal regions.

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MORPHOLOGY AND DISTRIBUTION OF FOUR PILL MILLIPEDES (*ARTHROSPHAERA*) OF THE WESTERN GHATS

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ABSTRACT

Pill millipedes, belonging to the genus *Arthrosphaera* (Family: Sphaerotheriidae) are endemic giant millipedes found mainly in the upper moist soil horizons of the forests of Southern India and Sri Lanka. They are also known as rollers as they roll into sphere or pill on conglobation as defense behavior. The bulldozing shape of body of pill millipedes helps to till and mix the soil with litter fragments, transfer organic matter into soil through burrows and uplift organic matter from underneath to generate moder humus. They hibernate into the subsoil surface on the onset of summer and attain quiescent state. Their large body size confers several advantages like tolerance to elevated moisture stress and increase the burrowing ability. The large body size is also known to affect energy acquisition and utilization, and in turn the rates of organic matter decomposition. The order, Sphaerotheriida characterized by 13 body segments (tergites) and 21 pairs of walking legs. The tergites are strongly curved and horny. Each tergite (except for pygidium) is overlapped slightly by the preceding tergite. In the process of conglobation, the head and ventral structures are completely covered by the pygidium, which helps in protection of the head and sense organs. Further, special closing ledges (locking carinae) are located on the inner side of the tergites. Stridulation (rubbing the last legs against the sides of last tergite generate sound) is an interesting phenomenon in *Arthrosphaera*. Stridulation behavior was seen usually in females and occasionally in male *Arthrosphaera* of the Western Ghats. The male stridulatory organ is the harp and in female it is washboard located at the anterior telopod and subanal plate respectively. Systematic descriptions of the order Sphaerotheriida are lacking, the current classification scheme lacks sufficient description and demands more morphological features to define monophyletic groups in *Arthrosphaera*. The current study, in addition to available morphological descriptions, emphasizes other essential features to revise the description of four species of *Arthrosphaera* (*Arthrosphaera dalyi, A. davisoni, A. fumosa* and *A.*

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magna) found in the Western Ghats along with their distribution pattern. The morphological description such as color, head, antennae, mouthparts, gnathochilarium, collum, thoracic shield, tergites, endotergum, anal shield, legs, female sexual features and male sexual features have been furnished.

Keywords: Pill millipedes, *Arthrosphaera*, morphology, distribution, Western Ghats.

INTRODUCTION

The body of millipede is generally long and cylindrical consists of three major parts head, trunk (with four simple segments) and rest of the body (with rings composed of two segments each carrying two pair of legs) (Hopkin and Read, 1992). The head capsule is heavily calcified to facilitate burrowing between soil particles, fragments of leaf litter and rotting wood. The head bears mouthparts and a number of sensory structures (antennae, Tömösvary organs and eyes). Collum, the first trunk segment behind the head is legless and each of the next three segments carries one pair of legs. Single pair of antennae possesses unique sensory cones on the last antenomere. At the base of the antennae, Tömösvary organs are present and their functions are not clearly known (Hennings, 1906; Sierwald and Bond, 2007). Median eyes are absent and the lateral patch of ocelli found in most of the millipede groups are considered as compound eye derivatives. The mouth parts consist of a pair of mandibles and the plate like gnathochilarium. According to Hennig and Mickoleit (1986) and Kaestner (1963), gnathochilarium is the fused first maxilla, while others (e.g. Kraus and Kraus, 1994) claim it to be formed by the fusion of both maxillae. The gnathochilarium consists of several sclerites that vary among orders and the homology is uncertain (Hilken and Kraus, 1994). On the ventral side of the body, structures associated with sperm transfer are present. All chilognath millipedes have a calcified cuticle (Hopkins and Read, 1992). The cuticle consists of three layers: a thin epicuticle, exocuticle and endocuticle. The cuticle of each segment consists of dorsal tergite and a ventral sternite with lateral pleurites. However, in many groups, the structure of each ring has been modified extensively. In Sphaerotheriida, tergites, pleurites and sternites are separate but in all other millipedes the pleurites are fused to the tergites to form the pleurotergal arch. Most millipedes are fairly dull in appearance or show cryptic coloration. Many species of the order Sphaerotheriida are attractively marked (e.g. red, orange and yellow spots).

The clade Pentazonia (to which pill millipedes belong) possess modified legs in males at the caudal body end (telopods) that function in sperm transfer as well as clasping the female. The structure of the gonopods is species specific and often reliable means of identification (Hopkin and Read, 1992). The pill millipedes are short compared to other millipedes with only 11-13 body segments. The Order Sphaerotheriida consists of giant pill millipedes of southern hemisphere and they possess 13 segments and roll (conglobate) into almost spheres. This Order is further organized into finer groupings including the Family Sphaerotheriidae and Zephronidae. Individuals of the order Glomerida of the northern hemisphere are also known as pill millipedes possessing 13 segments. Their second and third tergites are fused to from a very large plate. In members of both the orders the legs originate from the ventral part and consist of seven podomeres (coxa, trochanter, prefemer, femer, postfemer, tibia and

tarsus). The gonads open on or behind the second leg pair. These structures form the apertures by which sperms are introduced into the female.

The systematic treatments of the order Sphaerotheriida are scanty. However, various authors addressed this issue employing different morphological parameters for classification and same character has been named differently (Attems, 1897; Jeekel, 1974; Silvestri, 1917; Verhoeff, 1927, 1928). Recently, Wesener and Sierwald (2005a) gave a systematic scheme with comprehensive account of morphological characters used by different authors with emphasis on further standardization. In view of detailed assessment of different morphotypes of *Arthrosphaera* obtained from the Western Ghats and west coast of India in the current study, the scheme proposed by Wesener and Sierwald (2005a) has been followed. The details documented in this study hoped to fill the gaps and facilitate future studies on systematics of *Arthrosphaera*.

MATERIALS AND METHODS

Individuals of *Arthrosphaera* were collected from different locations of the Western Ghats, foothill and west coast during post-monsoon season (October-January, 2005 and 2006). The locations encompass *Arthrosphaera* species include: Kadaba (124 m); Basrikallu (1387 m); Madikeri (1147 m); Adyanadka (91 m); Adoor (110 m) and Peraje (124 m). Representative millipedes obtained were immobilized using ethyl acetate, straightened and preserved in 70% ethanol.

The dimensions of body (length, breadth and diameter on conglobation) were recorded. Morphological details of each morphotype were assessed based on observations under low and high powers of microscope.

Further details of each morphotype were evaluated on dissection and observation of various body structures after cleaning in KOH (4%): a) antennae), b) mandibles, c) gnathochilarium, d) section of endotergum, e) second leg pair in females and males, f) ninth pair of legs in females and males, g) subanal plates with washboard in females and h) anterior and posterior pairs of telopods. Detailed morphological features of each morphotype have been documented. Photographs of moving animals, conglobated animals and different segments of each morphotype have been furnished.

RESULTS

The coloration of body and different parts, specific features of head, antennae, mandibles, gnathochilarium, collum, thoracic shield, tergites, anal shield, legs, female sexual characters and male sexual characters have been given for each morphotype. The dimensions such as body length, breadth, diameter of conglobated animal (widest and narrow axis) for males and females were also provided (Tables 1-4). Distribution and ecology of each morphotype has been discussed based on the literature and the present study.

Table 1. Weight (g) and dimensions (mm) of males and females of *Arthrosphaera dalyi* (n=10, mean; range in parenthesis)

Weight/Dimension	Male	Female
Weight	7.77 (5.4-11.9)	8.94 (5.4-18.55)
Length	45.42 (39-49)	45.00 (39-50)
Breath	20.80 (16-24.0)	20.04 (15-27)
Conglobated diameter		
Widest axis	29.58 (20.2-33.8)	30.94 (27.3-34.5)
Narrow axis	23.05 (17.4-25.4)	24.05 (19.0-28.2)

Table 2. Weight (g) and dimensions (mm) of males and females of *Arthrosphaera davisoni* (n=12, mean; range in parenthesis)

Weight/Dimension	Male	Female
Weight	13.49 (8.1-16.1)	12.33 (7.3-15.1)
Length	49.83 (38.4-56.2)	45.89 (34.2-52.4)
Breadth	25.60 (18.7-31.4)	23.33 (18.4-25.7)
Conglobated diameter		
Widest axis	33.53 (26.4-38.3)	32.06 (24.3-35.7)
Narrow axis	29.26 (21.3-30.2)	26.13 (20.0-29.7)

Table 3. Weight (g) and dimensions (mm) of males and females of *Arthrosphaera fumosa* (n=09, mean; range in parenthesis)

Weight/Dimension	Male	Female
Weight	13.64 (10.80-15.1)	12.94 (9.48-15.4)
Length	48.04 (42.3-52.3)	46.27 (41.32-51.48)
Breadth	25.01 (23.6-27.0)	24.92 (23.2-27.2)
Conglobated diameter		
Widest axis	30.68 (23.8-35.0)	31.28 (26.1-35.2)
Narrow axis	26.51 (23.2-31.4)	26.16 (22-28.6)

Table 4. Weight (g) and dimensions (mm) of males and females of *Arthrosphaera magna* (n=11, mean; range in parenthesis)

Weight/Dimension	Male	Female
Weight	9.75 (7.9-13.75)	9.16 (7.8-11.75)
Length	41.18 (35-53)	39.16 (28-47)
Breadth	20.90 (16-28)	20.33 (15-26)
Conglobated diameter		
Widest axis	25.80 (19.2-33.8)	25.53 (18.4-32.43)
Narrow axis	22.69 (16-30.1)	21.93 (16.5-27.2)

Arthrosphaera dalyi Pocock (Figure 1)

Color – The body is uniform deep olive brown with a reddish posterior margin of each tergite. Head and collum are dark brown, while antennae and legs are green.

Head – Polished and smooth with numerous hairs and setiferous pits around the central part and the lateral sides of the eyes. Posterior margin of the head possesses very few hairs.

Antennae – Very long with thin, long and cylindrical joints. Relative length of the antennomere: 1>2>3>4 = 5<6, the 6^{th} antennomere is the longest and oval tip bearing 35-40 sensorial cones. Numerous hairs are present all over the surface of each antennomere.

Mouthparts – Molar plate process of the mandible with a small furrow near the apical end with 5 pectinate lamellae with long and thin teeth and number of teeth declining proximally.

Gnathochilarium – Posterior surface with many hairs, hairs on the lingual lamella are less and a few sensorial cones are seen on the lateral of palpi.

Collum – The collum is polished, smooth with a few isolated long hairs and posterior margin with a tuft of hairs at the centre.

Thoracic shield – A few hairs are present in the concave lateral extension of the thoracic shield and marginal brim a little broaden anteriorly.

Tergites – Pubescent and punctured in front, perfectly smooth and polished in their hinder half. Anterior paratergite depression of the anterior tergites has several ridges and ridges are not seen posteriorly. Tips of the paratergite project posteriorly.

Endotergum – There is a single row of marginal bristles. Inner area is covered with a few depressions (pits).

Anal shield – It is rounded, punctured and pubescent throughout. The ventral surface carries two black locking carinae on each side. A small invagination exists at the middle of the carinae.

Legs – Tarsi of first 3 leg pair with a few ventral spines, claws only weakly curved and without an apical spine. Tarsi of the remaining pairs of legs have 6-8 ventral spines with an apical spine and curved claw. Ninth leg pair without coxal lobe. All the podomeres possess long hairs.

Female sexual characters – Second pair of legs without coxal lobe. Operculum of vulvae is long and slender, anterior margin without indentation and lower margin with indentation at the centre. Inner plate of vulvae long and broad and its anterior margin reaches over the operculum. Exterior plates are not as long as inner plate. Anterior margin of the exterior plate extends below base of the operculum. Subanal plate semicircular, hairs are present at the periphery and a dense area of hair mesially. The washboard possesses four symmetrical stridulation ribs.

Male sexual characters – *Anterior telopods*: First joint (prefemer) with a harp like structure with two stridulating ridges (one displaced to the lateral side). Femoral process situated behind the tibio-tarsus is blunt. Tibia and tarsus are distinctly separated and tibia possesses a lobe above. Tarsal process has three lateral blunt cones. *Posterior telopods:* Syncoxite with a few hairs. Coxal horns (inner horns) are dark brown and have a pointed tip. Movable finger possesses two white lobes with row of crenulated teeth. Opposite finger has crenulations juxtaposed the crenulated teeth of the movable finger.

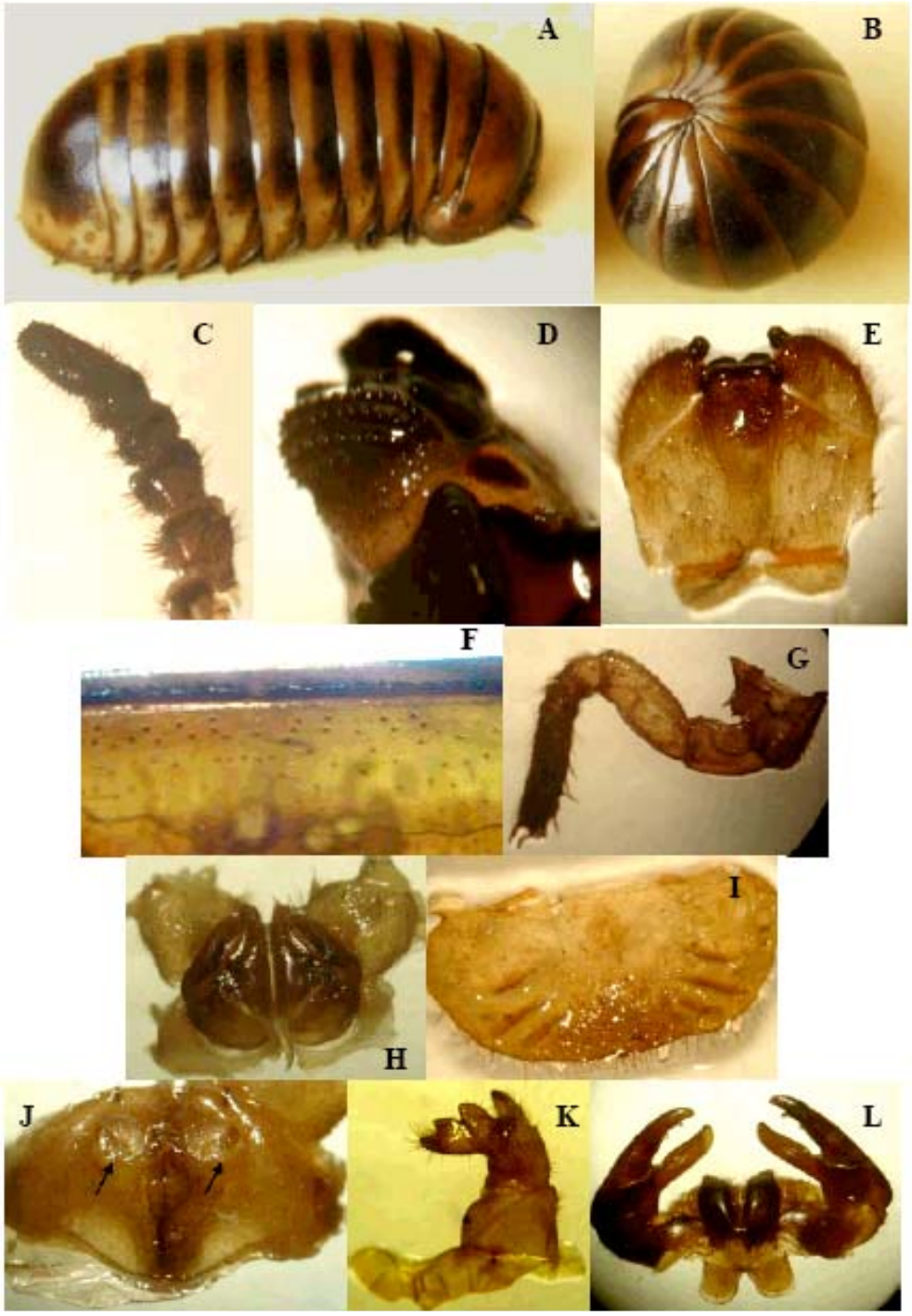

Figure 1. *Arthrosphaera dalyi* – Whole animal (A), conglobated (B), antenna (C), mandible (D), gnathochilarium (E), endotergum (F), 9th leg (G), vulvae (H), washboard (I), male gonopore (arrowed) (J), anterior telopod (K) and posterior telopod (L).

Distribution and ecology – The Specimens were collected from Kadaba (Western Ghat foothill location, Karnataka) at an attitude of 124 m. These individuals are abundant in

organically managed *Areca* plantation and nearby semi-evergreen forest. The animals appear immediately on onset of monsoon (end of May or early June) and could be collected in post-monsoon season up to December or January. Comparatively animals are abundant in plantation than the nearby forest. Interestingly, the number of animals could be recovered from other nearly plantation is significantly less. Probably the agricultural practices play an important role in colonization of these pill millipedes. Pocock (1995) reported this millipede from Palani Hills (1828 m) and Kodaikanal (2133 m) (Tamil Nadu). Achar (1986) has collected these animals from Alagarkovil Hills (Tamil Nadu). Chowdaiah and Kanaka (1974) have also found these animals from Southern India.

Arthrosphaera davisoni Pocock (Figure 2)

Color – Body is black. Posterior portion of the tergite has a yellow band. Head is olivaceous. Collum and thoracic segment are yellowish. Antennae are olivaceous with yellow sensory area. Legs are golden brown with olivaceous tarsal segments.

Head – Possess many hairs and pits (coarsely and closely punctured). Around central clypeus and lateral sides of the eye possess hairs. The posterior margin of the head is devoid of hairs.

Antennae – Possess six joints and length of the antenomere: 1>2>3>4 = 5<6, 6^{th} antenomere is large and oval with more than 50 sensorial cones.

Mouthparts – Mandibles: Molar plate process with 2 steps, 7 pectinate lamellae with long teeth and number of teeth declining proximally.

Gnathochilarium – Possesses many hairs and number of hairs on the lingual lamellae is less. Lingual lamellae has 15-20 pits with hair (resembles sensory cones).

Collum – Anterior margin with some isolated hairs and posterior margin devoid of hairs.

Thoracic shield –Lateral extension of the thoracic shield has a few hairs and rim around anterior margin slightly broader than rest of the region.

Tergites – The middle of the upper surface of terga densely punctured and less densely in front and behind. The posterior tergites are pubescent in front.

Endotergum - Anterior paratergite has depression with a few hairs and ridges. Tips of the paratergite process project posteriorly. Posterior half of the terga not polished.

Anal shield – Rounded, densely punctured throughout and a pit like depression on the anterior inferior angle. Ventral surface carries two black locking carinae on each side.

Legs - First few pair of legs without coxal lobe, 9^{th} leg has a coxal lobe with two spine-like projections and tarsi with 4-6 ventral spines and apical spine has curved claw. All the podomeres have long isolated hairs.

Female sexual characters – Second pair of leg is without coxal lobe and operculum of the vulva is long without central invagination. Lower side has an indentation, which is black. Inner plate of vulvae long and broad, and its anterior margin reach above the end of operculum. Exterior plate is not as long as inner plate and subanal plate rounded. The washboard has 5 ridges on one side and 6 stridulation ridges on the other side. Hairs are present at the periphery and also at the central portion of washboard.

Male sexual characters – Gonopores present in the second leg pair without coxal lobes. *Anterior telopods*: First joint has small harp-like process having 2 stridulatory ridges. Femoral process situated beside the tibio-tarsus, small and stout. Tibia and tarsus are

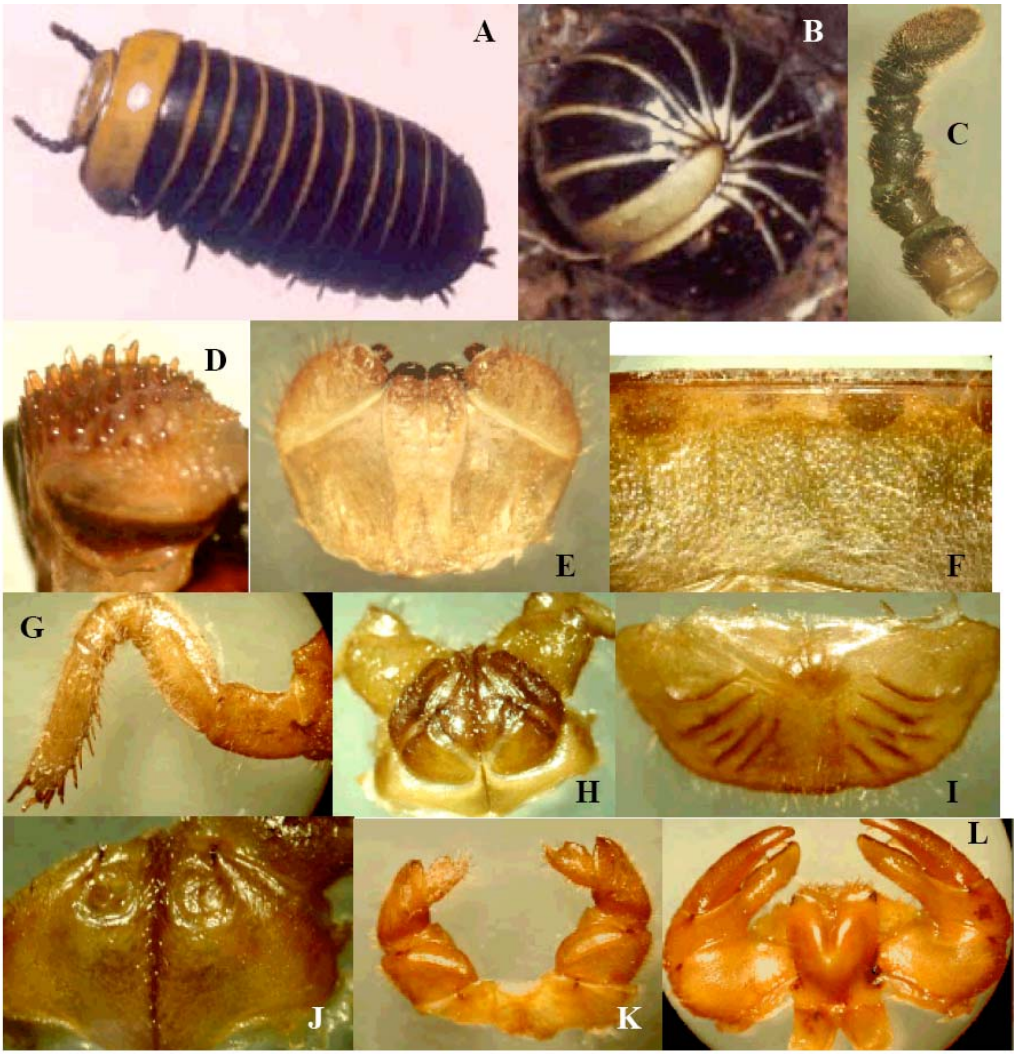

Figure 2. *Arthrosphaera davisoni* – Whole animal (A), conglobated (B), antenna (C), mandible (D), gnathochilarium (E), endotergum (F), 9th leg (G), vulvae (H), washboard (I), male gonopore (J), anterior telopod (K) and posterior telopod (L).

separated, former has single lateral lobe, and the latter with 2 lappets and thick spines on the inner edge. *Posterior telopods*: Coxal horns of the syncoxite of the posterior telopods with a dark lateral claw at the apex and inner horn is hairless. Movable finger has crenulations with specific dentition. Two white lappets are present on the movable finger.

Distribution and ecology – In our study, this species was found at Basrikallu plantation, Kudremukh region (Karnataka). The plantation is situated on the sloppy landscape of the Western Ghats and consists of *Areca*, cardamom, pepper and coffee. The abundance in nearby forest is lesser than the plantations. These animals have been reported from Coimbatore, Annamalai Hills (1432 m) (Tamil Nadu) (Attems, 1936). Achar (1986) collected this species from Khandala hilly tracts of Maharashtra.

Arthrosphaera fumosa Pocock (Figure 3)

Color – Pale olivaceous body, posterior half of the tergites except for the second and anal shield are smoky black, legs and antennae are deep olive to green. Nuchal plate (collum) with pale blotches and black spots.

Head – With numerous hairs and setiferous pits around the clypeus. Posterior margin of the head bears short hairs.

Antennae – It bears 6 antennomers, 6th one is the largest and broadest bearing numerous sensorial cones, the 6th joint bears terminal disc of oval or oblique shape, length of the antennomeres 1> 2> 3 = 5<6.

Mouthparts – Mandible with seven rows of pectinate lamellae, number of teeth declining from apical to proximal. Molar plate process with two steps near apical end.

Gnathochilarium – Ventrally it has a few hairs, group of sensorial cones present on the lateral margin of palpi. Central pads bear hair and different types of sensory cells, and some with central pit and some without pits.

Collum – Anterior margin possesses a few short hairs and remaining portion is bald.

Thoracic shield – A few short hairs present in the lateral extension of the thoracic shield. Anterior marginal brim is only a little broader than rest of the brim, polished and minutely punctured.

Tergites – Anterior portion of tergites is rugose and punctured, posterior portion is smooth and highly polished. Anterior paratergite depressions have a few short hairs. Anterior paratergite depressions of the a few anterior tergites consist of ridges. Tips of the posterior margins of paratergites are slightly projecting posteriorly.

Endotergum – Endotergum has one row of marginal bristles, and internal section with short spines and a few isolated bristles and nodules.

Anal shield – Rounded, densely and closely punctured with many small hairs. Ventral side has two black locking carinae on both sides.

Legs – Tarsi of first two legs with a few ventral spines (3-4) and possess weakly curved claws. Tarsi of following leg pairs with 9-10 ventral spines, and apical spine and curved claw. Coxae of the 9th pair of legs devoid of lobe but the outer rim have many small black triangular spines. Podomeres possess many long hairs.

Female sexual characters – Subanal plate with washboard consisting of well-developed stridulation ribs. They are elongated and three ribs are on one side and four on the other side. Vulvae are large, covering more than two-third of the coxae. Operculum is very broad and long. Anterior margin is devoid of indentation. Exterior and inner plates of vulva are below the operculum and surrounding the basal margin of the operculum. Inner plates are long and broad, reaching almost the tip of operculum.

Male sexual characters – Male gonopore is located at inside margin of 2nd coxae. *Anterior telopods*: First joint with a small harp and two stridulation ridges. The 2nd joint (femoral process) is broad, tibia and tarsus coalesced, tibial part has a superior cone-shaped lappet and tarsal part (4th joint) has two flat lappets. Along the sides of all the four joints there are many hairs. *Posterior telopods*: Telopod is syncoxite nearly hairless. Femoral process

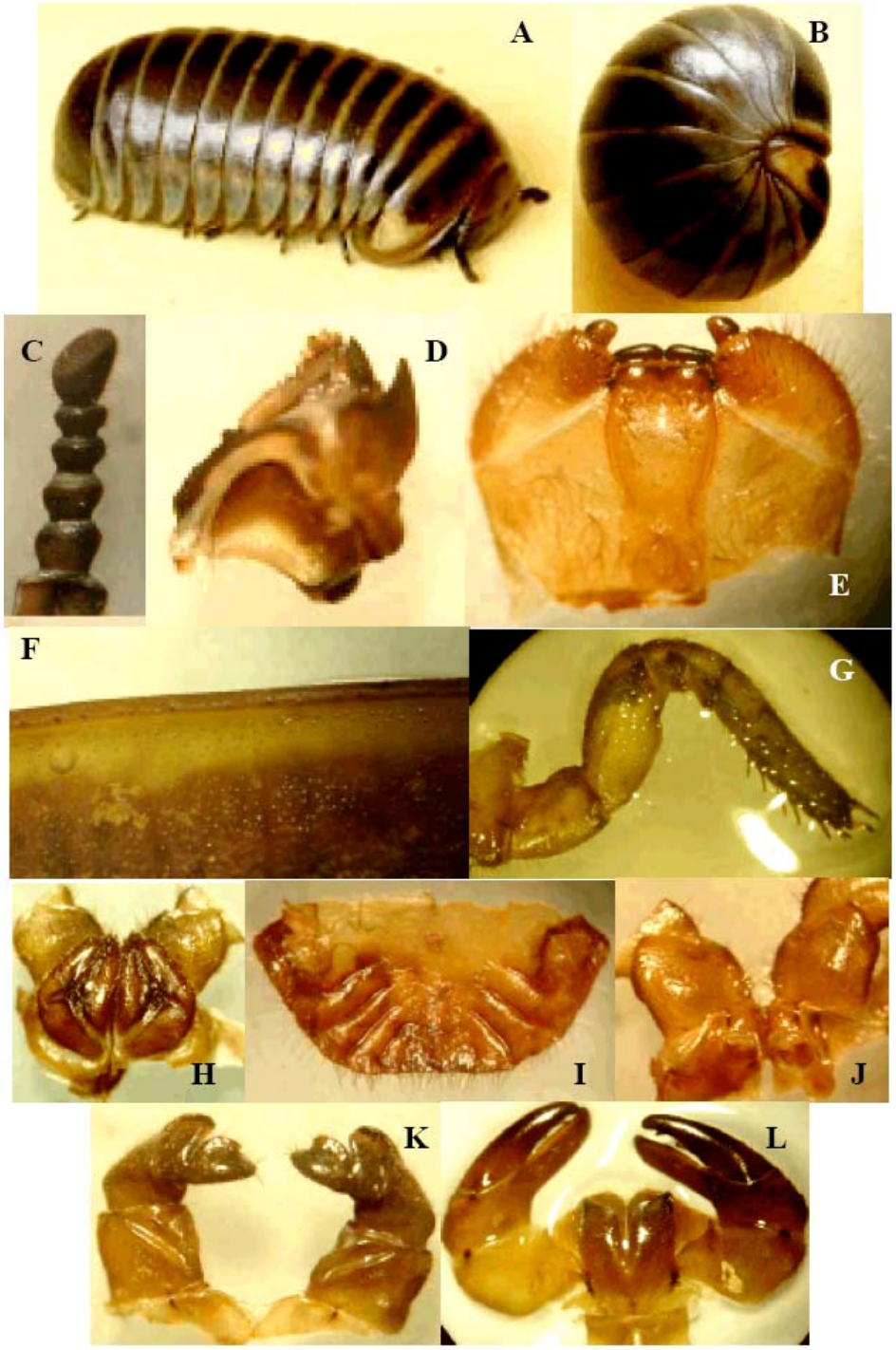

Figure 3. *Arthrosphaera fumosa* – Whole animal (A), conglobated (B), antenna (C), mandible (D), gnathochilarium (E), endotergum (F), 9th leg (G), vulvae (H), washboard (I), male gonopore (J), anterior telopod (K) and posterior telopod (L).

club-shaped, tibia with two white lappets and a row of numerous tubercles. The movable finger is slightly dark. The opposite finger has crenulations juxtaposed crenulated teeth of the movable finger. Inner horn of syncoxite has a pointed tip.

Distribution and ecology **–** Found in the semi-evergreen to evergreen forests of Madikeri and nearby locations of Karnataka. Immediately after heavy rainfall during southwest monsoon these millipedes start moving around. Interesting observation is that in addition to consuming the decomposed leaf litter, these millipedes scrape the mat of green algae and mosses attached to boulders and walls. They were recovered from Coimbatore (1432 m) (Tamil Nadu) by Pocock (1899). The animals collected resemble those collected by Achar (1986) from coffee estates of Madikeri (Karnataka).

Arthrosphaera magna Attems (Figure 4)

Color **–** Body dark brown, posterior border of tergites yellowish forming a stripe. Head, collum and thoracic shield are yellowish brown. Antennae and legs are olive green.

Head – Head has a few setiferous pits and clypeus is not with many hairs and pits. The posterior margin of the head towards the collum has a few isolated hairs.

Antennae **–** Consists of 6 joints, 6^{th} joint is expanded laterally, terminal disc is oval and oblique with more than 40 sensorial cones. The length of the antennomeres: 1>2>3>4>=5> 6.

Mouthparts **–** Mandible with 4-5 rows of pectinate lamellae, number of teeth declining proximally and molar plate process has a condoyle with a single step.

Gnathochilarium – The lingual lamella with a few bristles and palpi with a few long hairs. Sensorial cones are present on the central pads and on the palpi. Sensory cells are of different type and some are with pits as well as central hairs, and the others with hairs.

Collum **–** Anterior margin with a row of setiferous punctures, similar row is present near the posterior border and rest of the surface is not punctate.

Thoracic shield **–** Polished, not punctate, anterior zone in the middle of dorsum with a few scattered punctures and each puncture possesses short hairs.

Tergites **–** Anterior half of the tergite is densely punctate and hairy. Posterior half is smooth and shiny with only a few punctures near the posterior margin. The anterior paratergite depressions are densely covered with hairs and tips of posterior margin of paratergite project posteriorly.

Endotergum – It shows a row of marginal bristles. The area between the marginal ridge and the internal area has many black scleretized spots. First sternite lobe is long and reaching beyond the length of the coax, covered with many long hairs and curved towards the leg pair.

Anal shield **–** Rounded entirely punctate, several punctures are united to form common shallow depressions. Anterior zone is more densely punctate with hairs. Ventral surface carries two black locking carinae on each side. They are separated by distinct suture. A small invagination in seen at the end of the suture.

Legs **–** Tarsi of first two legs with 3-4 ventral spines and weakly curved claw. Tarsi of next pairs of legs possess 8-11 ventral spines and a curved claw. Coxae of all legs at the inside margin densely covered with many long hairs, also on the following leg joints at the

inside margin there are some very long isolated hairs. The 9th pair of legs possesses a small tooth.

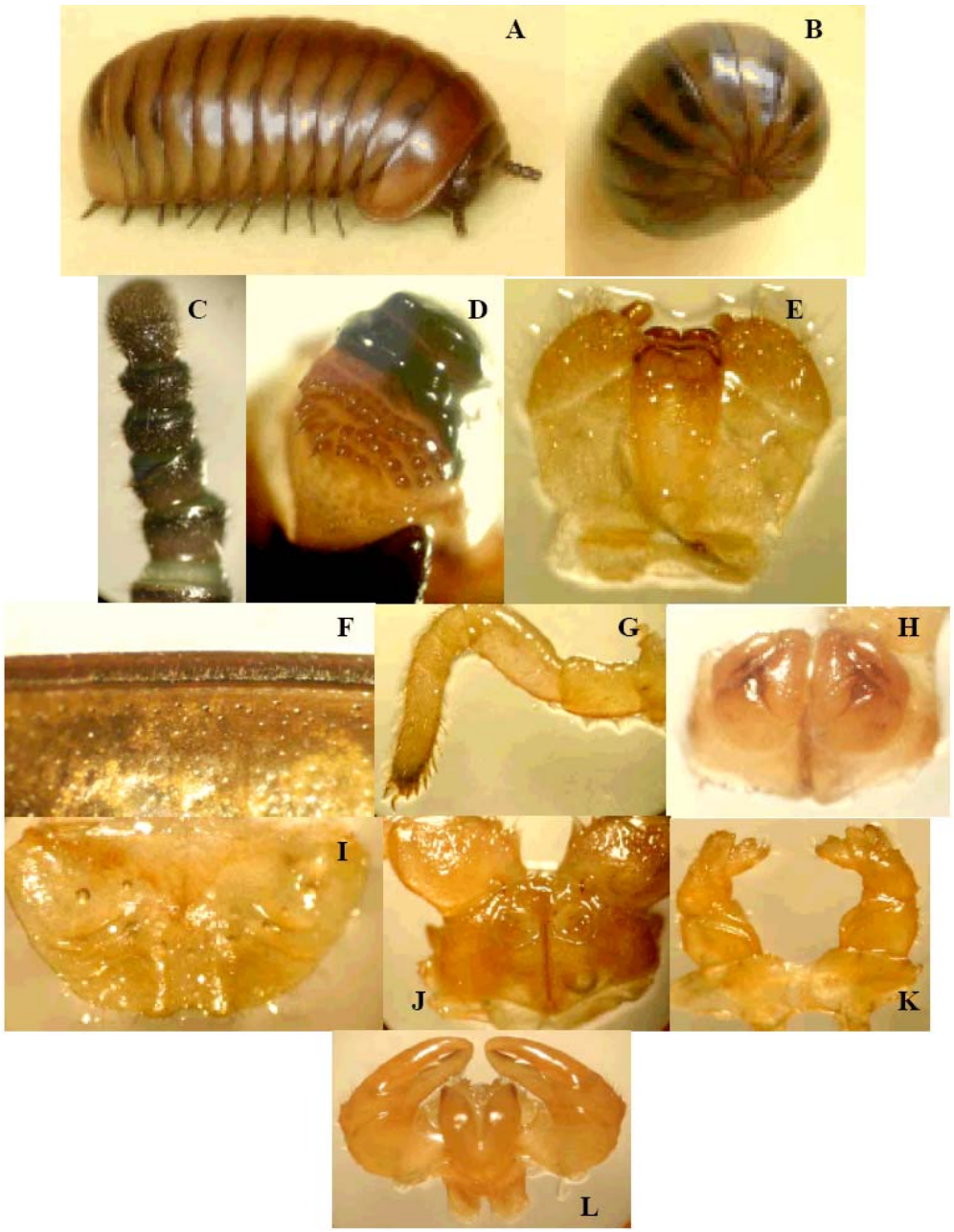

Figure 4. *Arthrosphaera magna* – Whole animal (A), conglobated (B), antenna (C), mandible (D), gnathochilarium (E), endotergum (F), 9th leg (G), vulvae (H), washboard (I), male gonopore (J), anterior telopod (K) and posterior telopod (L).

Female sexual characters – Coxae of 2nd pair of leg of female has a small lateral cone. Vulva consisting of 3 pieces, basal part is bipartite. Exterior and inner plate of the vulvae is

broad, and also the operculum is large and broad. Subanal plate is rounded and the washboard has 3 symmetrical stridulation ribs.

Male sexual characters – Second pair of leg in males is devoid of coxal lobe. Male gonopore exists on the coxae of 2nd leg pair covered with scleretized plate. *Anterior telopods*: Possess four joints and distally synoxite. Inner margin of first joint consisting one each of long and short stridulation ridge. Femoral process (projection) is short, blunt and situated behind tibio tarsus. Tibia and tarsus (3rd and 4th joint) distinctly separated, tibia has a superior rounded lobe, tarsus with a similar lobe, and then narrowed and cylindrical with long bristles at the apex. On the posterior side it has a small knob and a small spine.

Posterior telopods: Telopod is synoxite and densely covered with hairs and coxal horns (inner horns) large, thick at the base, densely hairy on the medial side, apex is rounded and dark and thorny. Femoral process, (2nd joint) longer than tibia, crenulated teeth present on inner side. Tibia has two short broadly rounded lappets and posterior edge has numerous rounded tubercles.

Distribution and ecology – These are abundant in the forests and plantations of Western Ghats foothill. In our study, these millipedes were recovered from Adyanadka (Karnataka) mixed plantation and nearby semi-evergreen forest, and they were also found in mixed plantations of Adoor and Peraje (Karnataka). All these locations are situated in foothill of Western Ghats at an average altitude of 91-124 m. *Arthrosphaera magna* were reported from other foothill locations of the Western Ghats (Hosangadi, Mundaje, Gundya and Sampaje) (Karnataka) (Ashwini and Sridhar, 2008). Individuals of *A. magna* recovered from Mundaje and Sampaje were larger and heavier than Hosangadi and Gundya locations. Achar (1986) collected *A. magna* from *Areca* plantations of Karkala (Karnataka). There are earlier reports on collection of this millipede from Khandala Hills and north of Phenda Ghat, Kolhapur (Maharashtra), Shevaroy Hills and Woodhouse Rajamundry (Tamil Nadu).

DISCUSSION

Diplopod classification seems to be over split. More than 12,000 described species are assigned to 2947 genera in 145 families (Shelley, 2003). Among these, 68% of genera possess single or two species and 715 generic names have been considered synonyms (Sierwald and Bond, 2007). As seen in many groups of millipedes, systematic treatments are very scanty with the order Sphaerotheriida and needs specific systematic treatments. Verhoeff (1927), Attems (1897) and Silvestri (1917) gave an over view of all aspects of Diplopoda and particularly the Sphaerotheriida. Jeekel (1974) discussed the group taxonomy and geography of the Sphaerotheriida. VanderSpiegel *et al*. (2003) also attempted a revision of some of the oldest species of the genus *Sphaerotheriium*. Wesener and Sierwald (2005a) revised the genus *Sphaeromimus* based on the electron microscopic study. They are of the opinion that *Sphaeromimus* shares certain characteristics with members of the Indian genus *Arthrosphaera* (e.g. four jointed anterior telopod, six jointed antennae and, flat and broad sixth antennomere), on the other hand, presence of harp on the anterior telopod relates them to the genus *Zoosphaerium*. As pointed out by Wesener and Sierwald (2005a), the morphological details of the current classification scheme are insufficient and more characters are necessary

to define precisely the species and subspecies within Sphaerotheriida. These common characters clearly indicate that the Sphaerotheriidae once inhabited the countries between Indo-Australian region and Madagascar-South Africa and subsequently they disappeared in the intermediate countries due to unfavorable climatic conditions. This is also supported by the fact that Sphaerotheriida are distributed currently in these two widely separated regions. However, the genera differ from each other in these areas with a few common characters.

Pocock (1899) listed a few characters diagnostic to the genus *Arthrosphaera* of Indian pill millipedes. These characters include: broad, truncate apex of the legs and upper angle bearing a long spine above the claw and thus a considerable space exists between the claw and the spine. In female, the vulva is composed of three distinct pieces and they are placed together in the form of a cone. In the male, the movable digit of the hinder pair of copulatory feet is composed of a single segment. These characteristics have been summarized by Attems (1936) in the key to the Indian genera of Sphaerotheriida with additional characters (coxae of the second legs of female is separated, tarsal part of the tibio-tarsus of the anterior telopods are broadly rounded lobe, margin of the lobe beset with knobs and tibia of the posterior telopods has two white lappets).

Four species of *Arthrosphaera* found in the present study are distinguishable from each other based on the characters given in Table 5. *Arthrosphaera dalyi* was reported from Alagarkovil hilly tracts of Tamil Nadu and the specimen measured 36 mm length and 29 mm width (Achar, 1986) and Palani hills (Pocock, 1895). This species collected in our study showed an average length of 45.21 mm and width of 20.42 mm. *Arthrosphaera davisoni* was collected from Basrikallu plantation near Kudremukh forest (Karnataka) in our study. The average length and breadth are 47.86 mm and 24.45 mm respectively. In the earlier studies, this species was collected from Khandla hilly tracts of Maharashtra (Achar, 1986) and Coimbatore, and Annamallai hills (Tamil Nadu) (Pocock, 1895). Pocock (1899) described this species with mean length of 31 mm for female and 22 mm for male. *Arthrosphaera fumosa* was reported from Coimbatore (Tamil Nadu) by Pocock (1895). In the present study, it was found from the forests of Madikeri and nearby forest localities of Karnataka. The average length and width of these millipedes were 47.15 mm and 24.96 mm respectively, while Pocock (1899) gave a dimension 35 mm and 16 mm. *Arthrosphaera fumosa* collected in our study resembles *Arthrosphaera* sp. (M) collected by Achar (1986) from the coffee estates of Madikeri (Karnataka). *Arthrosphaera magna* was collected from three different plantation localities at the foothill of Western Ghats. This species was reported from Karkala (Achar, 1986) and Adyanadka (Ashwini and Sridhar, 2005) of Karnataka. This species was also reported from the Western Ghats of southern India: Khandala, Phenda Ghat and Kolhapur (Maharashtra), Shevaroy hills (Tamil Nadu) and Rajamundry (Tamil Nadu). These animals were reported to measure 63 mm in length and 18.5-34 mm width (Attems, 1936), while Ashwini and Sridhar (2008) indicated as 61.57 mm length and 28.62 mm breadth. In the present study, average length and breadth are 40.17 mm and 20.61 mm respectively.

The presence of two locking carinae in some of the *Arthrosphaera* spp. reported in our study resembles the members of *Zoosphaerium* of Madagascar (Wesener and Sierwald 2005b), while one locking carina resembles the members of *Sphaeromimus* of Madagascar (Wesener and Sierwald 2005a). The subanal plates with washboard have not been described so far for the Indian species.

Table 5. Differences between four *Arthrosphaera* spp. found in the Western Ghats, India

Species	Color	Length (mm)	Width (mm)	Molar plate	Rows of teeth in pectinate lamellae	Locking carinae	Suture between carinae	Coxal lobe of legs	Stridulation ridges in the washboard	Nature of tibia and tarsus of anterior telopod
Arthrosphaera dalyi	Olive brown with reddish posterior margin	45.21	20.42	Absent	5	2	Present	Present	4	Separated
A. davisoni	Black with a yellow band posteriorly	47.86	24.46	2 steps	7	2	Present	Present	5 on one side, 6 on the other	Separated
A. fumosa	Pale olivaceous with smoky black posterior half	47.15	25.46	2 steps	7	2	Present	Absent	3 on one side, 4 on the other	Coalesced
A. magna	Dark brown with yellowish band at the posterior end	40.17	20.61	1 step	4-5	2	Present	Coxal tooth	3	Separated

Further minute details like the sensorial cones on the 6th joint of antennae, central pads of lamellae lingual, palpi of gnathochilarium and details of mandibles are lacking for *Arthrosphaera* spp. Thus, detailed study using scanning electron microscopy is warranted to throw some light on further details of Indian Sphaerotheriidae.

The stridulatory organ is considered to be absent in the *Arthrosphaera* by Attems (1936). But, in most of the descriptions of *Arthrosphaera*, the first joint of anterior telopods possesses two sharp ridges. In the present study too these ridges have been seen in all the four *Arthrosphaera* spp. Further, specimen with these stridulatory organs was treated under other genera of Sphaerotheriida. To separate tribes and subfamilies, Jeekel (1974) employed characters such as the shape of female vulva and the stridulatory organ. The shape of the bursa embracing the operculum in *Arthrosphaera* is the main synapomorphy of the genera of the family Sphaerotheriidae. Jeekel (1974) considers the presence of the female stridulation organ, the washboard, as the synapomorphy for the subfamily Arthrosphaerinae to which the genes *Arthrosphaera* belongs. The two Malagasy sphaerotherid genera, *Zoospharium* and *Sphaeromimus* from Madagascar have been classified in the Tribe Zoosphaeriini based on the presence of the harp in the males. The genus *Arthrosphaera* is placed under Arthrosphaeriini as it devoid of this character (Jeekel 1974). Attems (1936) in his key to Indian genera of Sphaerotheriidae clearly indicated that the characters such as basal part of the vulva bipartite and presence of two white lappets in the tibia of the posterior telopods are the important diagnostic features of *Arthrosphaera*. If the vulva is undivided and the posterior telopods or both the telopods possess stridulatory organs, they belong to other genera of Sphaerotheriidae. In the present study, pill millipedes showed the presence of ridge on the first joint of anterior telopods, vulvae are bipartite and the first joint of anterior telopod of all the species consists of two ridges (stridulatory?). The position of the ridges in anterior telopod exhibited considerable variation. So far, these ridges are not described as harp by the earlier investigators. Among the *Arthrosphaera* spp. recovered in our study, both males and females of at least a few species stridulate. The homology between the stridulatory organ (harp) and the ridge on the anterior telopods of *Arthrosphaera* need further investigation.

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REPRODUCTION AND LIFE HISTORY IN THE TWO LAND SNAILS *MONACHA CARTUSIANA* (MÜLLER) AND *EOBANIA VERMICULATA* (MÜLLER) (HELICIDAE: MOLLUSCA) IN THE LABORATORY

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ABSTARCT

Mating, oviposition, post-oviposition period, development, and generation period of the two land snail species *Monacha cartusiana* (Müller) and *Eobania vermiculata* (Müller) were studied in the laboratory in Egypt during the two seasons of 2003/4 and 2004/5. Data revealed that mating is essential for both snail species to lay eggs, thus it occurs when snails reached their sexual maturity (from adulthood till mating) during a period averaged 190.3 and 228.6 days for *M. cartusiana* and 231.6 and 568.3 days for *E. vermiculata* after adulthood in the two different seasons respectively. Pre-oviposition period (from mating till laying first egg) lasted an average of 2.7 and 8.2 days for *M. cartusiana* and 3.5 and 9.2 days for *E. vermiculata* during the two seasons respectively.

Oviposition period (from first laying egg till last one) averaged 22.5 and 64.1 days during which the snail of *M. cartusiana* deposited an average of 63.0 and 177.5 eggs during the two seasons respectively. On the other hand this period averaged 15.2 and 24.9 days for *E. vermiculata* during which the snail individual deposited and total average of 89.6 and 162.0 eggs during 2003/4 and 2004/5 respectively. While, post-oviposition period (from last egg till death) lasted 142.4 and 71.9 days for first snail species and 372.5 and 660.1 days for the second one during the same seasons respectively. The generation period (from egg to egg) extended an average of 369.8 and 402.6 and 481.7 and 868.7 days for both species during the same seasons respectively.

Keywords: *Monacha cartusiana* – *Eobania vermiculata* – Mating – Oviposition– Generation period – Sexual maturity.

INTRODUCTION

Land molluscs can cause considerable damage to field and horticultural crops in the world. Runham and Hunter (1970) reported that gastropod pests fed on cereals, potatoes,

vegetables, lettuce, maize, carrots, beetroot, clover, cabbage, turnip, as well as horticultural crops and ornamental plants. Management and control of such pests requires basic information on their life histories and habits.

In Egypt, the snails *Monacha cartusiana* and *Eobania vermiculata* are two the most important pest species, which the first infested 38 different host plants of field crops (7), vegetables (11), fruit trees (4), ornamental plants (5) and weeds (11) ; while the second species was found on 17 ornamental host plants and two fruit trees at different localities.

The main objective of this investigation was to spot the more light about oviposition, life cycle, life span, and reproductive potentiality of these pests in the Egyptian lands.

MATERIAL AND METHODS

50 adult individuals of each the glassy clover snail *M. cartusiana* and the brown garden snail *E. vermiculata* were collected by hand from infested host plants during their active period in November 2003 for rearing during season 2003/4 and repeated again in November 2004 for season 2004/5 and both were observed till death. Members of each species were kept in plastic boxes 15 x 7 x 11 cm and 27 x 27 x 25 cm contained moist soil to a depth 8 – 10 cm and provided with fresh lettuce leaves *Lactuca sativa* L. as a main source of food; then covered with muslin cloth fixed with rubber bands to prevent snails from escaping.

These cultural boxes were examined daily; fresh food and moisture were supplied as required, and the soil was searched for new clutches of eggs. Newly deposited clutches were removed then placed in prepared pots (13 x 10 cm) with moist soil.

They were observed daily till hatching to determine the incubation period as well as hatchability. Juveniles of both species were placed singly each in plastic cups (10 x 6 cm) filled with moist clay soil of about five centimeters and provided with fresh lettuce leaf disc then covered to avoid snail escaping and examined daily; fresh food and moisture were added as required until reached maturity. Mortality ratio among these juveniles averaged 14% for *M. cartusiana* and 18% *E. vermiculata* before reaching adulthood.

Adult snails of each reared species were paired in prepared plastic pots (13 x 10 cm) and observed several times daily till mating occurs to determine their sexual maturity period and mating behavior. After mating every pair was separated and each individual placed in a new prepared plastic pot to determine the pre-oviposition period, oviposition period, number of clutches per individual, clutch size, as well as post-oviposition period.

RESULTS

A. Mating

Both these species are hermaphrodites, and, as is normal for Helicids, self-fertilization does not occur. Mating usually took place when individuals reach their sexual maturity during a period which differed according to season and species. This period averaged 190.3 and 228.6 days for *M. cartusiana* during season 2003/4 and 2004/5 respectively, while it averaged 231.6 and 568.3 days for *E. vermiculata* during the same seasons respectively after adulthood

was attained (Tables 1 and 2). These results obtained from 10 and 20 and 13 and 12 pairs were involved in each set of observations for the two species in the same seasons respectively. (Figure 1)

Table 1. Duration of different stages of *M. cartusiana* during two seasons

Season	Average period in days ± SD							
	Incubation period	Juveniles	Life cycle	Sexual maturity	Pre - Oviposition	Oviposition	Post - Oviposition	Life span
Average ±SD for 10 snail/pair 2003/4	19.3 ± 3.6	185.8 ± 61.8	204.1 ± 59.5	190.3 ± 35.3	2.7 ± 1.4	22.5 ± 23.8	142.4 ± 105.6	497.7 ± 69.6
Range	12-25	124-319	136-344	153-237	1-5	1-76	20-362	376-708
Temp. ± SD	18.1± 1.6°C	19.8± 1.8°C	18.6± 0.9°C	19.2± 1.8°C	17.4± 0.3°C	17.9± 1.2°C	18.2± 0.7°C	21.8± 3.4°C
Average ±SD for 20 snail/pair 2004/5	18.1 ± 3.7	141.3 ± 12.1	156.5 ± 15.3	228.6 ± 12.4	8.2 ± 4.6	64.1 ± 25.6	71.9 ± 62.8	529.4 ± 78.6
Range	13-29	112-154	125-183	216-259	2-29	9-103	10-225	376-760
Temp. ± SD	18.7± 0.9°C	19.2± 1.3°C	18.8± 1.1°C	20.2± 2.1°C	19.8± 0.8°C	19.8± 0.8°C	20.1± 1.3°C	22.6± 3.7°C

During the reproductive season (mainly from mid November till mid January); mating in both species usually occurs at night and its timing differed according to species, in *M. cartusiana* from 5.0 p.m. till 11.0 p.m. with a maximum during the period from 8.0 p.m. to 10.0 p.m., while in *E. vermiculata*, mating usually occurred between 10.0p.m. and 3.0 a.m.

During mating process two snail individuals faced each other and attached together strongly from the fleshy parts. Copulations ranged from 90 to 225 minutes with an average of 143.0 ± 48.5 (± SD) minutes for *M. cartusiana*, while it ranged from 300 to 420 minutes with an average of 325.5 ± 43.0 minutes for *E. vermiculata*. After this period, disengagement takes place gradually over a period of averaging 22.5 ± 1.6 minutes for *M. cartusiana* and 35.0 ± 4.1 minutes for *E. vermiculata*.

Table 2. Duration of different stages of *E. vermiculata* under laboratory conditions

Season	Average period in days ± SD							
	Incubation period	Juveniles	Life cycle	Sexual maturity	Pre-oviposition	oviposition	Post-oviposition	Life span
Average ±SD for 13 snail/pair 2003/4	21.0 ±3.8	185.3 ±86.6	210.1 ±84.1	231.6 ±106.6	3.5 ±1.9	15.2 ±8.3	372.5 ±113.3	920 ±161.3
Range	11-25	77-371	104-396	111-445	1-6	1-26	139-550	631-1367
Temp.± SD	19.6± 1.7°C	20.6± 1.4°C	20.8± 1.9°C	19.3± 1.2°C	17.4± 0.2°C	18.2± 1.7°C	23.2± 2.7°C	21.6± 2.4°C
Average ±SD for 12 snail/pair 2004/5	21.1 ±4.7	239.4 ±127.1	293.7 ±147.0	568.3 ±284	9.2 ±8.9	24.9 ±23.3	660.1 ±272.7	1532.4 ±490
Range	11-30	101-597	122-620	138-1074	1-24	1-57	300-1066	603-2760
Temp.± SD	18.9± 0.8°C	19.8± 1.8°C	21.0± 1.4°C	20.6± 1.9°C	20.8± 0.5°C	20.6± 1.1°C	23.7± 2.9°C	22.7± 3.4°C

Figure 1. Mating process of M. cartusiana.

B. Oviposition

During the present study it was observed that unmated snails of *M. cartusiana* and *E. vermiculata* did not lay any eggs. Numbers of unmated M. cartusiana were 20 and 22 snail, while *E. vermiculata* were 24 and 20 snail for 2003/4 and 2004/5 respectively. Also, it was noticed that both mated snails 3 and 20 snail/pair of *M. cartusiana* and 8 and 6 snail/pair of *E. vermiculata* laid eggs during the two seasons respectively. On the other hand it was found that only one of both mated snails deposited eggs (N. = 5 and 0 snail/pair for *M. cartusiana* and 3 and 4 snail/pair for *E. vermiculata* during the same seasons respectively). Sometimes both mated snails did not lay any eggs and this might be due to their failed in copulation (N. = 2 and 0, 2 and 2 for both species during the same period respectively). (Tables 3 and 4). The pre-oviposition period (between mating and lying) differed according to species and season (Tables 1 and 2). It averaged 2.7 and 8.2 days from mating for *M. cartusiana* and 3.5 and 9.2 days for *E. vermiculata* for 2003/4 and 2004/5 respectively.

Table 3. Duration of *M. cartusiana* life span in two seasons

Season	Average life span period in days ± SD		
	Mated snails		Unmated
	Laid	Non laid	
2003 / 2004	497.7 ± 69.6	506.1 ± 122.7	541.1 ± 111.7
Range	376 – 708	305 - 652	335 - 761
No. of snails	11	9	20
2004 / 2005	529.4 ± 78.6	0.0	562.4 ± 106.9
Range	376 - 760	0.0	330 - 680
No. of snails	40	0	22

Table 4. Duration of *E. vermiculata* life span in two seasons

Season	Average life span period in days ± SD		
	Mated snails		Unmated snails
	Laid	Non laid	
2003 / 2004	920.0 ± 161.3	434.9 ± 106.6	605.6 ± 156.1
Range	631 – 1367	334 – 685	375 – 863
No. of snails	20	6	24
2004 / 2005	1532.4 ± 490	508.7 ± 97.5	702.8 ± 159.9
Range	603 – 2760	334 – 685	375 - 898
No. of snails	19	5	20

Snails preferred to deposit eggs in protected places; thus, *M. cartusiana* made small pits or holes one to three centimeters deep in the soil, while *E. vermiculata* dug a circular hole or chamber five to six centimeters deep; then each species laid its eggs singly at one time and adhere to one another in the same place forming an egg mass which so called batch, or cluster, or clutch, as well as sometimes laid its eggs singly scattered in the soil each one in small hole then covered with soil. *M. cartusiana* individuals spent an average time of 150.0 ± 5.4 minutes for depositing one clutch (which averaged 17.0 ± 1.2 eggs), while those of *E. vermiculata* took 362.5 ± 46.0 minutes on average depositing an average clutch of 55.3 ± 18.3 eggs (these observations from five individuals of each species and ± means SD).

M. cartusiana deposited an average of 5.0 ± 4.5 and 15.0 ± 7.1 clutches during its oviposition period which averaged 22.5 ± 23.8 and 64.1 ± 25.6 days in the two seasons 2003/4 and 2004/5. The rate of deposited individual clutches per day averaged 1.2 ± 0.7 and 1.2 ± 0.4 and the period elapsed between each deposited clutch to another averaged 5.6 ± 8.5 and 4.5 ± 4.7 days during the same seasons respectively. The clutch size averaged 11.4 ± 9.3 and 10.4 ± 8.3 eggs. Thus, the total number of deposited eggs per one individual of this species during its oviposition period averaged 63.0 ± 48.4 and 177.5 ± 103.5 eggs at the same seasons respectively. The eggs are white, with a smooth surface and spherical shape (mean diameter 1.6 ± 1.1 mm). 50 eggs weighed 0.08 gm.

E. vermiculata, during its oviposition period which averaged 15.2 ± 8.3 and 24.9 ± 23.3 days deposited an average of 2.2 ± 1.2 and 2.3 ± 1.4 clutches in the first and second seasons respectively. The rate of deposited clutches per day was one clutch. The period from each deposited clutch to another averaged 11.3 ± 4.6 and 16.3 ± 11.9 days during the same seasons respectively. The clutch size averaged 36.9 ± 20.0 and 44.9 ± 38.4 eggs. Thus the total number of deposited eggs by one individual of this species during its oviposition period averaged 89.6 ± 36.2 and 162.0 ± 30.1 eggs in the same two seasons respectively. Eggs of this species are white, with a smooth surface, and rounded to ovate in shape. Means of length and greatest width were 3.1 and 3.0 mm respectively and a clutch size of an average 40 eggs weighed 0.7 ± 0.5 gm.

C. Post-Oviposition

The post-oviposition period as calculated from the last deposited egg till snail individual death, followed the same trend as it differed according to species and season. This period averaged 142.4 and 72.0 days for *M. cartusiana* during the seasons of 2003/4 and 2004/5 respectively (Tables 1 and 2). While, for *E. vermiculata* it averaged 372.5 and 660.1 days during the same two seasons respectively.

D. Development

Length of incubation period differed according to species and seasons (Tables 1and 2) as it averaged 19.3 and 18.1 days for *M. cartusiana* during seasons 2003/4 and 2004/5 respectively, while it averaged 21.0 days in *E. vermiculata* during both seasons. Hatching success also differed according to species averaging 74.0 ± 27.8 and 77.8 ± 24.0 % for *M.*

cartusiana and 68.1 ± 31.0 and 71.2 ± 31.9 for *E. vermiculata* during the same two seasons respectively.

The maximum shell diameter of newly hatching juveniles was 1.7 ± 0.2 mm for *M. cartusiana* and 3.03 ± 0.1 mm for *E. vermiculata.* After 22 weeks (154 days) from hatching, *M. cartusiana* individuals had achieved a mean maximum shell diameter of 11.8 ± 0.4 mm, while those of *E. vermiculata* achieved a mean of 26.1 ± 0.0 mm after 30 weeks. Mean times from hatching to adulthood (juvenile's duration) were 185.8 and 141.3 days for *M. cartusiana* and 210.0 and 239.4 days for *E. vermiculata* during the two seasons respectively (Tables 1 and 2).

Thus the duration of life cycle from egg to adult (incubation period as well as juveniles) for *M. cartusiana* averaged 204.1 and 156.5 days, while it averaged 210.1 and 293.7 days in *E. vermiculata* during the same two seasons respectively (Tables 1 and 2). Life span (from egg till death) of both species differs according to mating, oviposition and season (Tables 3 and 4). In *M. cartusiana*, life span of unmated snails was longer (541.1 and 562.4 days) than mated in the two seasons. On the other hand, in mated snails this period differed according to oviposition; it averaged 497.7 days for laying (life cycle, sexual maturity, pre-oviposition, oviposition and post-oviposition period) and 506.1 days for non-laying snails (life cycle, sexual maturity till death) in season 2003/4. In the next season all mated snails deposited eggs, thus its life span averaged 529.4 days. For *E. vermiculata*, mating and oviposition prolonged its life span period in both seasons, followed by unmated snails while the lowest period was recorded in non-laying mated snails (Table 4).

E. Generation Period

Generation period (from egg to egg) was calculated only for laying snails, and it includes; life cycle, sexual maturity and pre-oviposition periods. Thus it averaged 369.8 ± 7.0 and 402.6 ± 30.2 days for *M. cartusiana* and 481.7 ± 140.7 and 868.7 ± 339.6 days for *E. vermiculata* during the seasons 2003/4 and 2004/5 respectively.

DISCUSSION

During the present study it was observed that copulation is essential for oviposition as unmated snails of both species *M. cartusiana* and *E. vermiculata* did not lay any eggs. Also, it was noticed that both mated snails or only one from both mated deposited eggs and sometimes both mated individuals could not lay any eggs and this may be due to their copulation failed. These results are in accordance with (Takeda, 1983), (Bride and Gomot, 1991) and (Bride *et al*, 1991), who mentioned that egg production in several species of stylommatophoran gastropods is stimulated by mating behavior. On the other hand, Capinera (2001) recorded that both mated individuals of *Helix aspersa* Müller deposited eggs.

Laboratory observations indicated that, before depositing eggs, the snail of both species required after mating a pre-oviposition period which differs from individual to another according to season which averaged 2.7 and 8.2 days and 3.5 and 9.2 days for *M. cartusiana* and *E. vermiculata* during two seasons respectively and these accepted with those mentioned

by (Heller, 1982) who recorded that *Thepa pisana* (Müller) laid its eggs from 9.0 to 14.0 days after copulation; (Baur and Baur, 1992) mentioned that *Arianta arbustorum* L. snails which had copulated deposited their first batch after 10.3 ± 1.5 days, and (Capinera, 2001) who stated that the brown garden snail *H. aspersa* deposited eggs beginning 3 – 6 days after mating. While, (Stringer *et al*., 2003) mentioned that this period reaches 140 days in *Paryphanta busbyi watti*.

Both individuals of *M. cartusiana* and *E. vermiculata* preferred to laid their eggs in protected places in batches or scattered in the soil each in separate hole and these accepted with those mentioned by (Baur, 1990) that *A. arbustorum* usually lays its eggs either in batches in small holes in the soil or singly on the soil surface, (South, 1992) who indicated that, most terrestrial gastropod species are oviparous and for oviposition some of them utilize natural holes or crevices in the soil or under stones and pieces of wood. On the other hand, (Baur, 1994) mentioned that *Zonitoides arboreus* (Say) lays single eggs scattered in forest litter.

Data indicated that, the total number of deposited eggs per individual differed according to species and season. Thus, the total number was 63.0 and 177.5 eggs during an oviposition period of 22.5 and 64.1 days for *M. cartusiana* during seasons 2003/4 and 2004/5 respectively, while those of *E. vermiculata* averaged 89.6 and 162.5 eggs during an average period of 15.2 and 24.9 days in the same seasons respectively. While, (Staikou and Lazaridou, 1990) mentioned that the mean number of eggs laid per snail of *M. cartusiana* was 32.67 ± 10.82. On the other hand, (Capinera, 2001) recorded that, the number of eggs deposited by *H. aspersa* at one time varies from about 30 – 120, averaging 86 and the total fecundity is estimated at 400 – 450 eggs annually.

Life cycle or maturity (including incubation period and juveniles) differs also according to species and season as it averaged 204.1 and 156.5 days for *M. cartusiana* and 210.0 and 293.7 days for *E. vermiculata* during the two seasons respectively. Staikou and Lazaridou (1990) decided that, 14% of *M. cartusiana* individuals reached maturity one year after hatching, while the majority reached maturity and laid eggs two years after hatching and all snails died after the reproductive period.

Sexual maturity period (including life cycle till mating) of both species averaged 190.3 and 228.6 days and 231.6 and 568.3 days after adulthood for *M. cartusiana* and *E. vermiculata* during the two seasons respectively; while, (Staikou and Lazaridou, 1990) found that, the sexual maturity of *M. cartusiana* was normally attained two years after hatching and (Stringer *et al*., 2003) mentioned that mating occurs at 406.2 ± 63.8 days after development from juvenile to adult in *Paryphanta busbyi watti*.

The life span period (from hatching till death) averaged 497.7 and 529.4 days and 920 and1532.7 days for *M. cartusiana* and *E. vermiculata* during the same two seasons respectively and this agree with those by (Heller, 1990) who mentioned that, life span is variable being annual in one habitat but stretching over several years in a different habitat with the range from several months to 19 years during his study on 75 species belonging to 57 genera and 30 families.

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IMMUNOTOXICITY OF AZADIRACHTIN IN FRESHWATER MUSSEL IN RELATION TO SURFACE ADHESION OF HEMOCYTES AND PHAGOCYTOSIS

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ABSTRACT

Lamellidens marginalis (Mollusca: Eulamellibranchiata) is a freshwater edible mussel distributed in the wetland of different districts of West Bengal of India. Hemocytes – the blood cells of this species perform diverse immunological functions including nonself recognition and phagocytosis of nonself particles. Assay of nonself surface (glass) adhesion of hemocytes was determined under the exposure of 0.006, 0.03, 0.06 and 0.09 ppm of a biopesticide azadirachtin in diverse span of exposure i.e. 1, 2, 3, 4 and 7 days in controlled laboratory condition. Shifts in percentage of adhesion were recorded against all the concentration screened. However, the patterns of shift were not uniform.

Azadirachtin induced shift in surface adhesion of hemocytes indicated a state of immunological toxicity and stress in the species. Phagocytosis of nonself particulates is considered as a classical immunological response in bivalve. We have examined phagocytosis of yeast (*Saccharomyces cerevisiae*) by hemocytes of mussel exposed to 0.006, 0.03, 0.06 and 0.09 ppm of azadirachtin for 1, 2, 3, 4 and 7 days *in vitro*.

Alteration in phagocytic response and adhesion of hemocytes is suggestive of cellular stress which may lead to decline in population of *L. maginalis* in xenobiotic affected districts of West Bengal of India.

Keywords: Azadirachtin, *Lamellidens marginalis*, hemocytes, biopesticide.

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INTRODUCTION

Lamellidens marginalis, an economically important edible bivalve is distributed in the freshwater ecosystem of West Bengal of India. Hemocytes are the circulating blood cells of bivalve that perform various immunological functions and discriminate the nonself surface through adhesion (Armstrong, 1985; Lackie, 1988; Johansson and Soderhall, 1992; Johansson *et al*., 1995; Saha and Ray, 2006). Azadirachtin, a new generation biopesticide is recently being introduced in the agricultural sector of India. Azadirachtin is a limonoid compound obtained from a neem plant *Azadirachta indica*. During monsoon season, the agricultural runoff containing azadirachtin residues contaminate the fresh habitat of mussel. In this present study, the immunotoxicity of azadirachtin is reported in *L .marginalis* in relation to phagocytosis and surface adhesion. Nonself surface adhesion of hemocyte was estimated under the exposure of azadirachtin in controlled laboratory condition. A discrete population of hemocyte of invertebrates is capable of recognition and adhesion to various nonself surfaces and considered as immune response (Armstrong, 1980; Johansson and Soderhall, 1989a; Hose *et al.*, 1990; Martin *et al.* 1999). Aquatic invertebrates distributed in polluted environment engulf invading microorganisms and parasites (Shapiro *et al*., 1977; Bayne, 1990; Factor and Beekman, 1990; Hose *et al.*, 1990; Tyson, 1995; Martin *et al.*, 1999) as innate immune response. Phagocytic responses of hemocytes *in vitro* were screened under the sublethal exposure of azadirachtin in bivalve (Fontaine and Lightner, 1974; Paterson and Steward, 1974; Armstrong and Levin, 1979; Ehlers *et al*., 1992).

MATERIALS AND METHODS

Adult *L. marginalis* of 9± 0.5 cm of length weighing 70 ± 10 gm was collected from selected habitat of district of south 24 Parganas of West Bengal, India and transported to the laboratory. Animals were maintained in standard glass aquaria in batches and fed with chopped *Hydrilla*. The water of the static water environment was replenished in every 24 hours and animals were acclimatized for one week (Heasman and Fielder,1983). LC_{50} of azadirachtin was determined in *L. marginalis* as 1.8 ppm / 96 hours in static water environment (Krishnaja *et al.*, 1987; Shibu Vardhanan and Radhakrishnan, 2002). The temperature of water kept constant at 28 ± 2^0C. *L. marginalis* was exposed to 0.006, 0.03, 0.06 and 0.09ppm of azadirachtin in batches along with the control for a span of 1, 2, 3, 4 and 7 days duration. During treatment animal received uniform ration of daylight and checked for mortality and morbidity. Blood was collected aseptically from posterior adductor at a volume not exceeding 1ml per bleed per day. Entire bleeding and collection procedure was carried out at 4°C to prevent cell aggregation. Cell viability was estimated with 2% trypan blue following the principle of dye exclusion. Experiments were carried out with cell suspension with greater than 95 % viable cell. For study of surface adhesion, fixed number of control and treated hemocytes of *L. marginalis* were placed over grease free sterile glass surface and was incubated for a period upto 150 mins at 37º C in humid chamber. Percentage of adherent hemocytes were microscopically determined after a period of cell incubation i.e.150 mins (Guria *et al*., 2002). After 150 mins glass surfaces were subjected to gentle jetting of sterile snail saline for collection of nonadherent hemocyte. Ratio of adherent to nonadherent

hemocyte were determined by Neubeur hemocytometer (Armstrong, 1980). For assay of phagocytosis, fixed numbers of viable hemocytes were challenged with freshly cultured yeast at an optimal phagocytic ratio of 1:10. Both cell types were maintained in short term culture system for 6 hours to complete the phagocytosis *in vitro*. Cells were subsequently processed fixed and stained for microscopic observation. Phagocytic index (PI) was determined microscopically (Ehlers *et al.*, 1992; Adamowicz and Wojtaszek, 2001).

$$PI = \frac{\text{Total number of phagocytosed cells}}{\text{Total number of cells}} \times 100 \times \frac{\text{Total number of yeast cells engulfed}}{\text{Total number of phagocytosed Cells}}$$

PI = % of phagocytosis X Average number of yeast cells engulfed

RESULTS

In the controlled population, 91.2 ± 0.3 % of hemocyte adhered over glass surface after incubation for 150 minutes. Hemocytes collected from *L.marginalis* exposed to 0.09 ppm of azadirachtin for 7 days expressed lowest percentage of adherent cell as 50± 0.7% (Figure 2). Hemocytes collected from *L. marginalis* exposed to 0.006 ppm of azadirachtin for 1,2,3,4and 7 days expressed intermediate values of adherent cell percentage. Animal exposed to 0.03 ppm of azadirachtin for 1,2,3,4and 7 days of exposure resulted depletion in percentage of adherent hemocyte and highest inhibition was recorded against 0.09 ppm for 7 days exposure (Figure 2). A sharp decrease of cell adherence was recorded against 0.06 ppm of exposure for 1,2,3,4and 7 days as 81.30± 0.2, 72.54±1.2, 62± 0.8, 54±0.3 and 49±0.2 % at 150 mins interval of incubation. Exposure of the animal in 0.09 ppm for 1,2,3,4 and 7 days resulted a decrease in distribution of adherent hemocytes at each interval of incubation. At 0.09 ppm of exposure, a steady decrease of adherent cells were recorded against 1,2,3,4 and 7 days of exposure respectively. Pattern of cell adherence were identical in all the experiment. A steady decrease in the percentage of adherent hemocytes were recorded against time scale for all the concentration used. A steady depletion of efficacy of hemocytes for surface adhesion against all the concentrations of biopesticide indicated an alteration in immunoefficacy of hemocyte under pesticide exposure. Impairment of phagocytic response were recorded against all concentration of azadirachtin tested. Highest and lowest inhibition of phagocytic response were determined as PI 30 ± 2.1 against 0.09ppm / 7days and 335 ± 1.9 against 0.006 ppm / 1day respectively against the control value of 350 ± 2.5 (Figure 1). Both experiments were repeated for 5 times and the data were interpreted by mean ± standard deviation and compared in paired t-test at $P < 0.05$ (Bailey, 1959). In our present observation, sublethal concentration of 0.006, 0.03, 0.06 and 0.09 ppm of azadirachtin resulted disruption of recognition of nonself surface by hemocytes of *L. marginalis*. Phagocytic response expressed a decreasing trend (Figure 1) under azadirachtin exposure as evident from PI determined in control and treated sets.

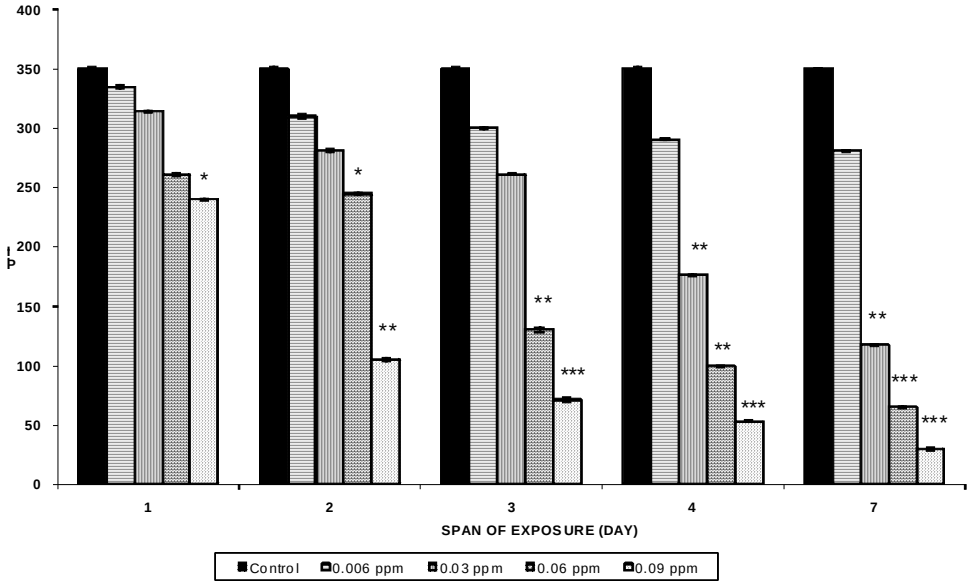

Figure 1. Phagocytic response of hemocytes of azadirachtin exposed L.marginalis challenged with yeast. Data is represented as Mean ± S.D. Statistical significance is shown at $P<0.05^{*}$, $P<0.01^{**}$, $P<0.001^{***}$.

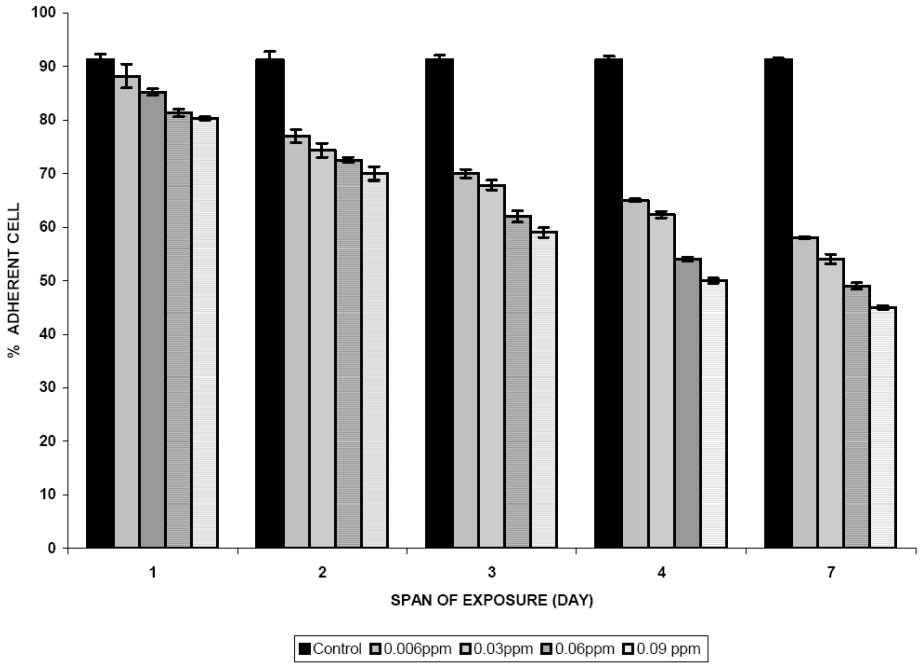

Figure 2. Non self surface adhesion of hemocytes of L. marginalis exposed to azadirachtin in vivo. Data is represented as Mean ± S.D.

DISCUSSION

Hemocytes – the chief effector cells of immunological response of *L.. marginalis* are composed of subpopulations namely adherent and nonadherent types (Guria and Ray, 2002). Efficacy of hemocytes for discrimination and adherence over nonself surface was modulated by sublethal concentrations of azadirachtin. Hemocytes of invertebrates phagocytose invading microorganism and pathogen under proper elicitation (Goldenberg *et al.*, 1984; Bayne, 1990). Azadirachtin induced impairment of phagocytic response (Figure 1) indicated a physiological state of immune suppression. Freshwater of West Bengal receives diverse xenobiotics. Alteration of nonself surface adhesion and phagocytic response of hemocyte of *L. marginalis* is indicative of possible immunological impairment which may lead to decline of this economically important species in its natural habitat.

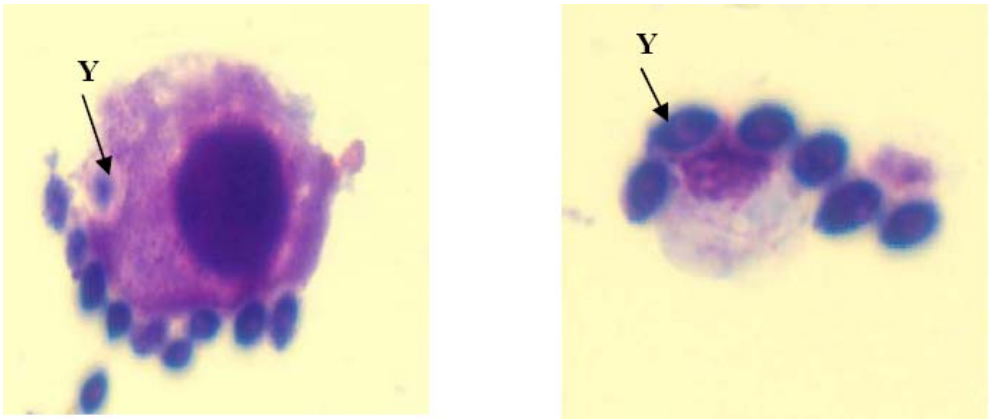

Figure 3. Phagocytosis of yeast (Y) by hemocytes of *L. marginalis*.Control.

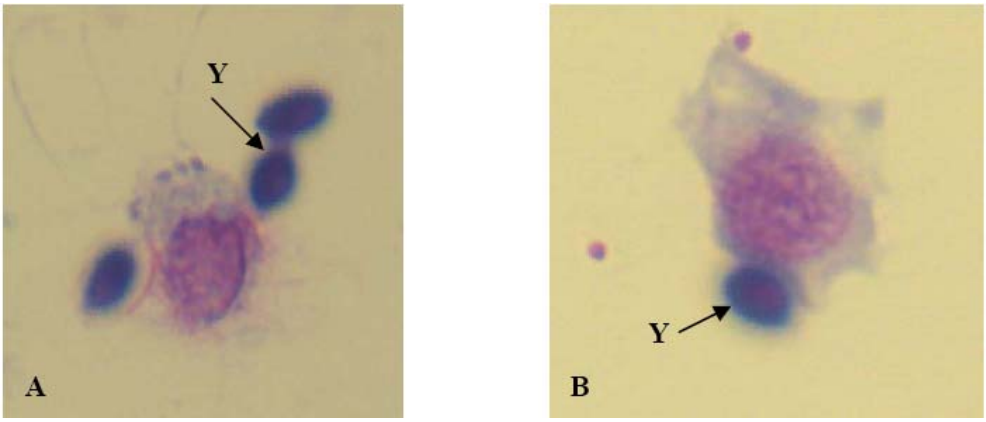

Figure 4. Phagocytosis of yeast (Y) by azadirachtin exposed hemocytes of *L.marginalis*. [(A). 0.006ppm / 7 days, (B). 0.09ppm / 7 days].

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COMPARISON OF NEIGHBOR-JOINING AND MAXIMUM-PARSIMONY METHODS FOR MOLECULAR PHYLOGENY OF ORYX SPECIES USING 12S RRNA AND 16S RRNA GENE SEQUENCES

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ABSTRACT

The molecular phylogeny using mitochondrial DNA sequences provides valuable insights for wildlife conservation. However, the inference of phylogenies may be affected by the marker type as well as by the statistical model applied for data analysis. This study reports a comparative evaluation of neighbor-joining (NJ) and maximum-parsimony (MP) methods for phylogenetic inference among three Oryx species (*Oryx leucoryx*, *Oryx dammah* and *Oryx gazella*) as well as two closely related (same subfamily but different genera) outgroups including Addax and Roan, using 12S rRNA and 16S rRNA genes. We observed that the 12S rRNA based phylogenetic inference is unable to differentiate between the genus Oryx and Addax, regardless of the method used. However, the 16S rRNA gene segment accurately grouped all the five taxa using MP but not the NJ suggesting the possible superiority of the former method for phylogenetic analysis using similar data structure. This report also describes a stepwise protocol that can easily be followed for constructing the trees using PHYLIP software.

Keywords: Mitochondrial markers; 12S rRNA; 16S rRNA; Phylogenetic trees; Oryx; PHYLIP software.

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INTRODUCTION

Both 12S rRNA and 16S rRNA genes of mitochondrial DNA are commonly used for understanding the phylogenetic relationship among various taxa. The nucleotide sequences of 12S rRNA have been applied for wildlife forensic biology [1] and molecular phylogeny of endangered species [2,3]. Pandey et al [4] have utilized 12S rRNA for molecular identification of Indian leopard, which is an endangered species except in Central Africa and India. Mitochondrial 16S rRNA has been used to elucidate the pattern of relationships and systematic status of 4 genera, including 9 species of skates living in the Mediterranean and Black Seas [5]. A heminested PCR assay based on species-specific polymorphism at the mitochondrial 16S rRNA gene has been designed for the identification of seven pecora species including Blackbuck, Goral, Nilgai, Hog deer, Chital, Sambar and Thamin deer [6]. Molecular studies on endangered Pecoran have shown lower sequence diversity in 16S rRNA gene as compared to cytochrome b gene, both between and within species however the 16S rRNA gene harbored a larger number of species-specific mutation sites than cytochrome b gene, suggesting that it could be more useful for species identification [7]. NaNakorn et al [8] have assessed the level of genetic diversity of critically endangered Mekong giant catfish species using sequences of 16S rRNA and detected 4 haplotypes among 16 samples from natural populations. A single base in the 16S rDNA sequences from the endangered species *Pinna nobilis* was found to be different in all analyzed individuals from a single population sample differentiating it from the others [9]. Recently, mtDNA sequence data have been used to determine genetic diversity and phylogenetic relationship in Oryx species [10,11]. Owing to application of various molecular markers and statistical methodologies it is intriguing to compare the topologies of phylogenic trees resulting from different computational approaches. In this investigation, we have performed a comparative evaluation of neighbor-joining (NJ) and maximum-parsimony (MP) methods for phylogenetic inference among the five antelope taxa including three Oryx species and two closely related outgroups, using 12S rRNA and 16S rRNA genes.

METHODS

We obtained the sequences of the three Oryx species including *Oryx leucoryx* (Arabian Oryx), *Oryx dammah* (Scimitar Horned Oryx) and *Oryx gazella* (Gemstok or Plains Oryx) from GenBank. The sequences of Addax (*Addax nasomaculatus*) and Roan (*Hippotragus equines*) were used as outgroups owing to their close relationship to Oryx yet representing two separate sister taxa [11,12]. The GeneBank accession numbers for 12S rRNA and 16S rRNA of the five taxa analyzed are: *Oryx leucoryx* (U86971; U87021), *Oryx dammah* (U86970; U87020), *Oryx gazella* (U86972; U87022), Addax (U86973; U87023) and Roan (U86975; U87025) respectively. There were a total of 241 and 342 positions in the partial sequence datasets of 12S rRNA and 16S rRNA respectively. The taxonomic classification of Oryx species is as follows: Kingdom (Animalia) - Phylum (Chordata) - Subphylum (Vertebrata) - Class (Mammalia) - Order (Artiodactyla) - Family (Bovidae) - Subfamily (Hippotraginae) - Genus (Oryx) - Species (*Oryx leucooryx, Oryx dammah, Oryx gazell*a). The two outgroups differed at the genus level with the following classification: Genus (Addax)-

Species (*Addax nasomaculatus*) and Genus (Hippotragus)-Species (*Hippotragus equines*) respectively.

The sequences were aligned by ClustalW [13] and the generated alignment file was converted to PHYLIP file using BIOEDIT software [14]. The PHYLIP software was used for all phylogenetic analyses [15]. Various menus of the PHYLIP package including Seqboot (a general bootstrapping and data set translation tool; we used 1000 replicates throughout), Dnadist (a program that computes distance matrix from nucleotide sequences), Neighbor (a program that implements the neighbor-joining method), Dnapars (a program that performs unrooted parsimony) and Consense (computes consensus tree by the majority-rule consensus tree method) were used to construct phylogenies using both NJ and MP methods. The outtree file was opened with Treeview program [16] to evaluate the topologies of various tree types including radial, clad and phylogenic trees. The entire protocol is summarized in Figure 1.

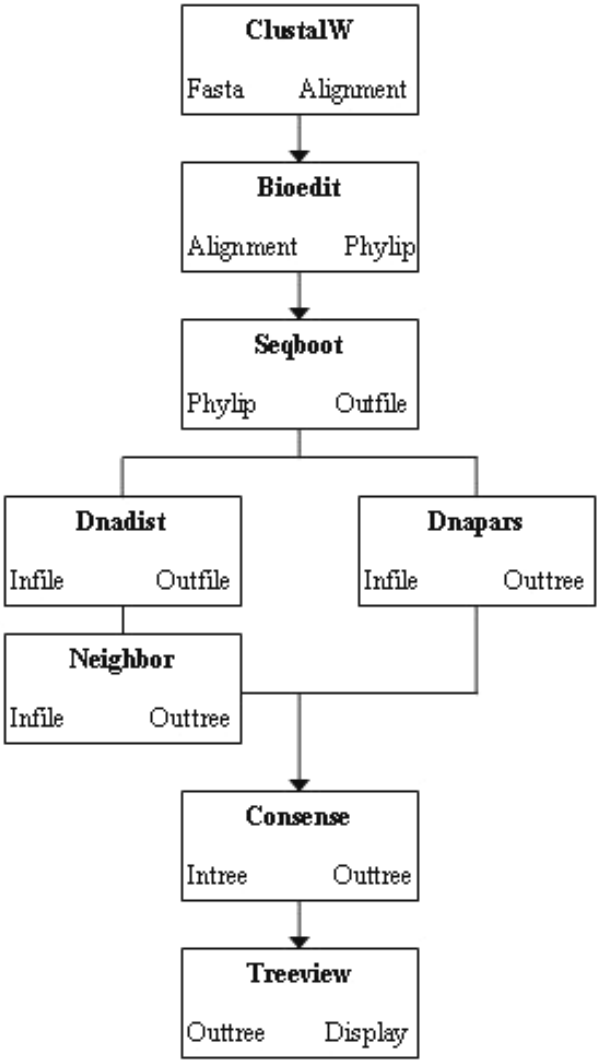

Figure 1. Schematic presentation of the protocol used. The top entry in each box indicates the software tool and the bottom entries indicate the input and the output file types.

RESULTS AND DISCUSSION

The aligned sequences of all the taxa are shown in Figure 2. The frequency of the polymorphic sites in 12S rRNA was found to be comparatively less than 16S rRNA (5.8% versus 9.1%) and so as their respective average evolutionary divergences (0.028 versus 0.043). Lei et al [17] have examined the mitochondrial rRNA genes of Chinese antelopes and observed that average sequence divergence values for 12S and 16S rRNA genes were 6.3% and 9.9% respectively. The results of phylogenetic analysis clearly showed the failure of 12S rRNA to differentiate between the genus Oryx and Addax, irrespective of the method used (Figure 3). Although mtDNA evolves faster than the nuclear genome, the rate of evolution is different for different regions of mtDNA and has been used to examine various phylogenetic relationships. Since the 12S rRNA gene is highly conserved and therefore failed to accurately differentiate taxa at genus level in our study but could be applied to illustrate phylogeny of higher categorical levels such as in phyla or subphyla. With regard to 16S rRNA gene sequences, the NJ method did not produce satisfactory trees (Figure 4, left panel); however, the use of MP method efficiently differentiated various taxa and demonstrated the anticipated phylogenies (Figure 4, right panel). The16S rRNA gene is regarded as a highly useful segment of mitochondrial genome for phylogenetic studies at mid-categorical levels such as families or genera [18]. The phylogenetic tree resulting from MP analysis of 16S rRNA sequences is supported by an earlier report based on using approximately 1250 base pairs long sequences of mitochondrial control region of Oryx species [11]. MP has been regarded as the only method that can easily tackle the insertions/deletion of nucleotides, which may provide important phylogenetic information [19]. Although MP is quite efficient in obtaining the correct topology [20], it may also result incorrect trees even if the rate of nucleotide substitution is fairly constant among the taxa [21,22].

```
Oryx Leucoryx
CGGCAACGGC CCAAAACTCA AAGGACTTGG CGGTGCTTTA TACCCTTCTA GAGGAGCCTG   60
TTCTATAATC GATAAACCCC GATAAACCCC ACCAATCCTT GCTAATGCAG TCTATATACC  120
GCCATCTTCA GCAAACCCTA AAAAGGAATA AAAGTAAGCA TAATCATCAT ACATAAAAAC  180
GTTAGGTCAA GGTGTAACCT ATGGAATGGA AAGAAATGGG CTACATTTTC TACTTTAAGAA 241

Oryx Dammah
.......... .......... .......... .......... .......... ..........   60
....G..... .......... .......... .......... .......... ..........  120
.......... .......... .........C. .......... .......T.C ..........  180
.......... .......... .......... .......... .......... ........... 241

Oryx Gazella
.......... .......... .G........ .......... .......... ..........   60
.......... .......... .......... .......... .......... ..........  120
.......... .......... .........C. .......... .......T.C ..........  180
.......... .......... .......... .......... .......... ........... 241

Addax
.......... .......... .......... .......... .......... ..........   60
.......... .......... .......... .......... ......A... ..........  120
.......... .......... .........C. .......... .......T.C ..........  180
.......... .......... .......... .......... .......... .....C..... 241

Roan
.......... .T........ .......... .........C. .....C.... ..........   60
.......... .......... ........T. .......... ......A... ..........  120
.......... .......... .......... .........G C......T.. ..........  180
.......... .......... .........G .......... .......... .....A..... 241
```

(A) 12S rRNA

```
Oryx Leucoryx
CATTTGTTCT CTAAATAAGG ACTTGTATGA ACGGCCACAC GAGGGTTTTA CTGTCTCTTA  60
CTTCCAATCA GTGAAATTGA CCTCCCCGTG AAGAGGCGGG GATGAACCAA CAAGACGAGA 120
AGACCCTATG GAGCTTCAAC TAACTAACTC AAAGAGAACA AACTTAATCA CCAAGAGATA 180
ACAGCACTCT GTATGAGTTA GCAGTTTTGG TTGGGGTGAC CTCGGAGAAT AAAAAATCCT 240
CCGAGCGATT TTAAAGACTA GACCCACAAG TCAAACCAAA TTATCGCTTA TTGACCCAAA 300
TATTTGATCA ACGGAATAAG TTACCCTAGG GATAACAGCG CA                     342

Oryx Dammah
.......... .......... .......... .T........ .......... ..........  60
.......... .......... .......... .......... .......... .......... 120
.......... .......... .......... .......... .......... .......... 180
.T.A...... .......... .......... .......... .......... .......... 240
.......... .......T.. .......... .......... .......... .......... 300
.......... .......... .......... .......... ..                     342

Oryx Gazella
.......... .......... .......... .T........ .......C.. ..........  60
.......... .......... .......... .......... .......... .......... 120
.......... .......... ......G... .......... .......... .......... 180
.......... .......... .......... .......... .......... ......C... 240
.......... .......... .......... .......... .......... .......... 300
.......... .......... .......... .......... ..                     342

Addax
.......... .......... .......... .......... .......... ..........  60
.......... .......... .......... .......... .......... .......... 120
.......... ......T... ..G....... .....A.... .......... .......... 180
.......... T.....A... .......... .......... .....G.... .........C 240
.......... .......... ....T..... .......... .......... ....T..... 300
C......... .......... .......... .......... ..                     342

Roan
.......... .......... .......... .T........ .......C.. ..........  60
.....G.... .......... .......... .......... ...AG..T.. .......... 120
.......... .......... ........C. .....A..T. .......... .......... 180
...A...... T....G.... A......C.. .......... .........C .......... 240
.......... .......... ...TT..... .....T.... C......... ....T..... 300
.......... ......C... .......... .......... ..                     342
```

(B) 16S rRNA

Figure 2. Alignment of 12S rRNA and 16S rRNA genes of three oryx species including the Oryx leucoryx, Oryx dammah and Oryx gazella and the two outgoups including Addax and Roan. The identical sites are represented by dots.

NJ method has been reported to give consistent trees provided the unbiased distance measures are used [23]. However, NJ method is criticized as producing only one final tree instead to generating several trees with possible correct topologies [24]. Tateno et al [25] have shown the similar efficiencies of MP and NJ methods for obtaining the correct topology of trees when the extent of sequence divergence is ≤5% and ≥1000 nucleotides are used for analysis.

In conclusion, the results of this preliminary study indicate that MP appears to be a more reliable method for phylogenetic inference among the five taxa using 16S rRNA gene sequences. However, additional studies with typical sequence data sets including different marker types, sequence lengths and number of taxa are necessary to ascertain the pros and cons of MP and NJ methods in a broader perspective.

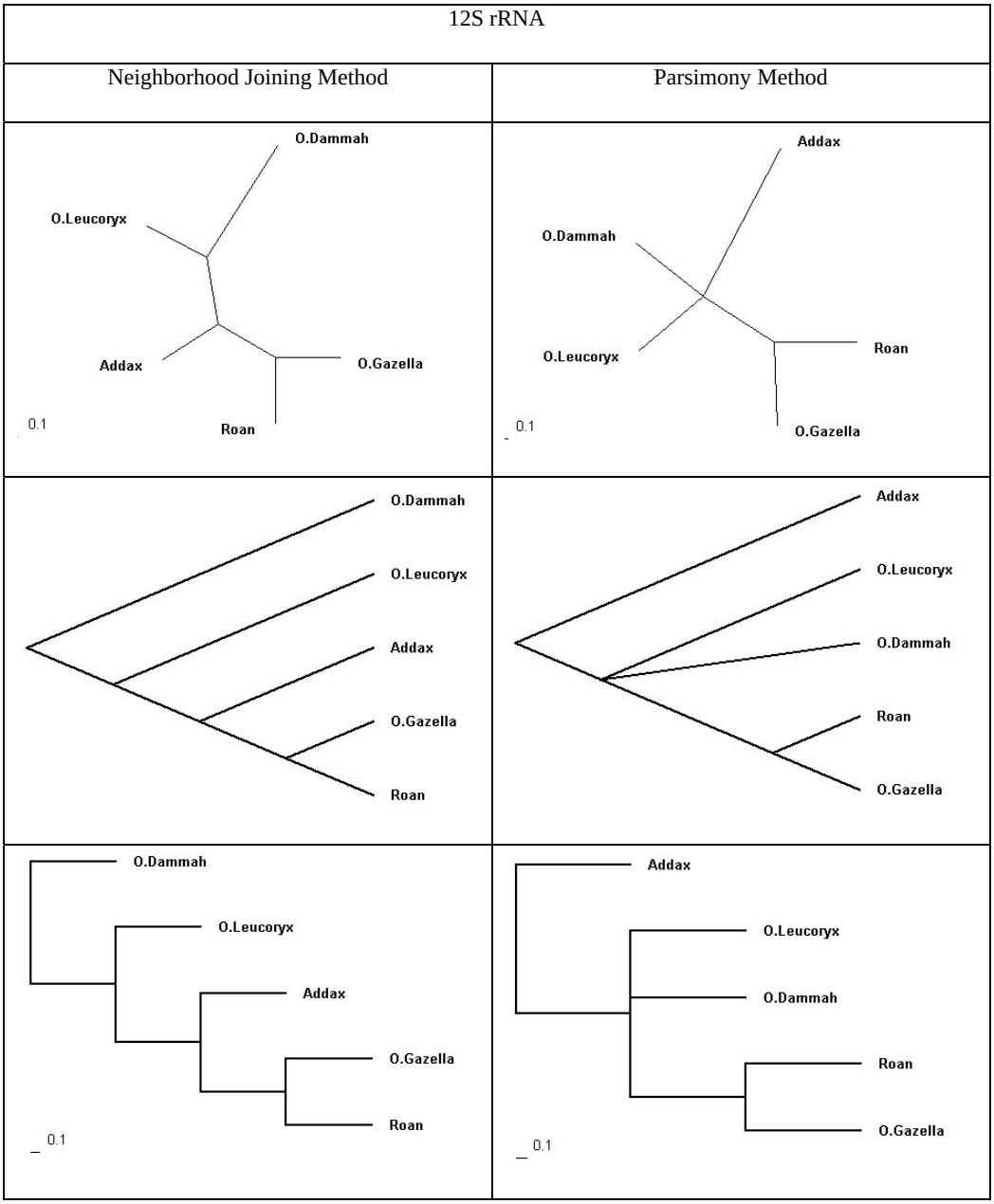

Figure 3. Bootstrap consensus trees for 12S rRNA gene using neighborhood joining (left panel) and parsimony (right panel) methods.

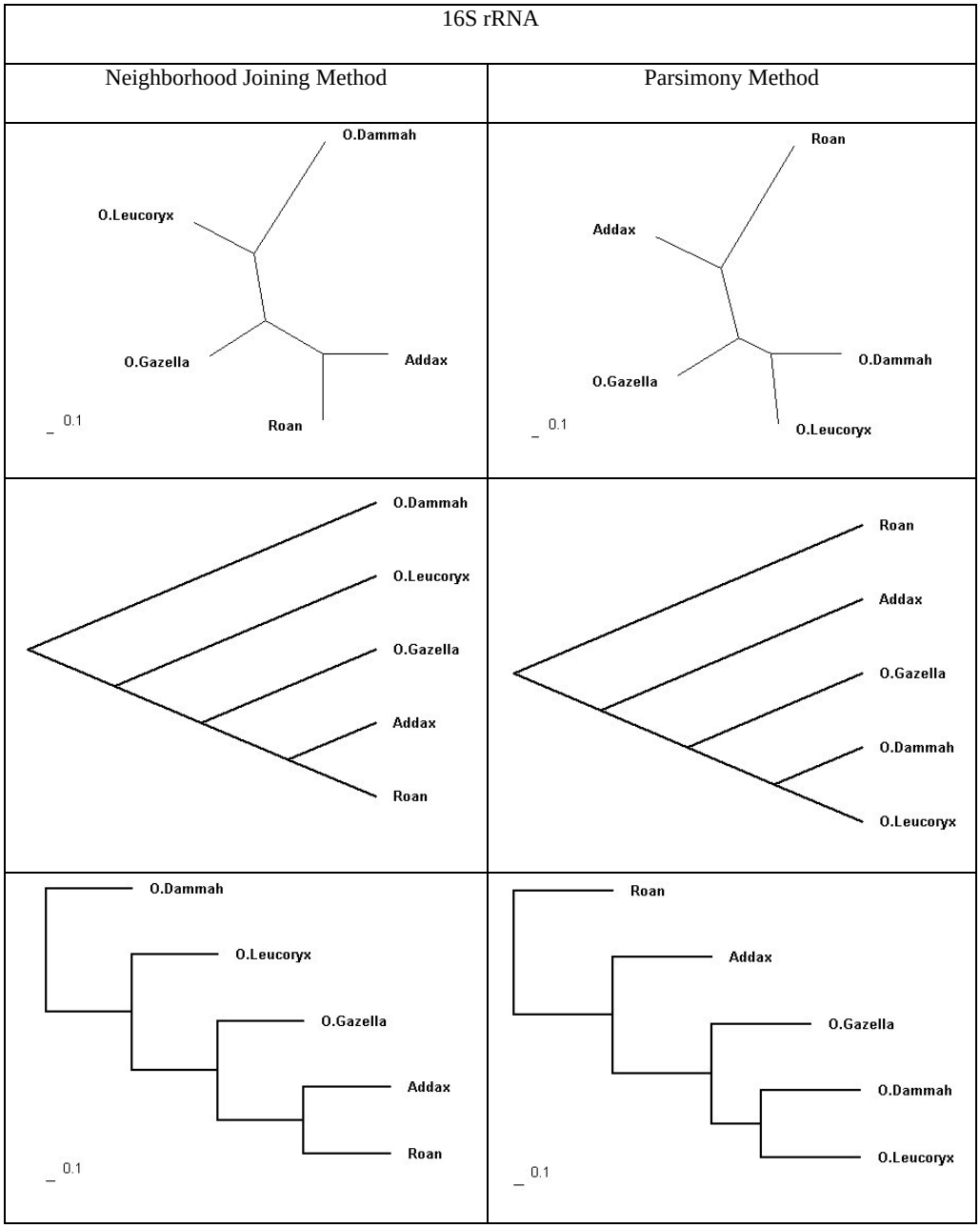

Figure 4. Bootstrap consensus trees for 16S rRNA gene using neighborhood joining (left panel) and parsimony (right panel) methods.

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