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PRANIKEE

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The emblem of Pranikee

The emblem "***NABAGUNJARA***" is a chimeric animal and a common motif in Odishan art and literature. It literally means "nine forms". This form has been described by poet Sarala Das in the Odia version of the epic Mahabharata. Apparently, Lord Krishna appeared in Nabagunjara form consisting of the body of an elephant, a leg each of a horse, a deer and a tiger respectively; throat of a peacock, tail in the form of a serpent, waist of a lion, hump of a bull and head of a cock to fool his friend Arjuna. The chimera was holding a lotus flower in a human hand. Arjuna had never seen such a creature in his life and guessed that this could not be a real animal but a form assumed by Lord Krishna and immediately bowed down at his feet. It is said that the human hand with the lotus provided the clue. In the paintings and sculptures, however, the lotus is often replaced by a "Chakra" or the "stylized discuss" of Lord Krishna. Chimeric forms are encountered in literature and art all over the world. However, a chimera of nine animals is uniquely Odishan. Therefore, it was considered to be an appropriate emblem for the Journal of Zoological Society of Orissa.

Padma Shri Awardee Prof. Priyambada Mohanty-Hejmadi
Former Editor

From editor's desk

The Journal of the Zoological Society of Orissa, Pranikee was born in 1980. The first issue contained science articles and seminar papers of students and staff of the P.G. Department of Zoology, Utkal University. Subsequently, only research articles in Zoology adorned the journal. The present edition, the 25th issue, is the XXVth volume and is dedicated to Prof. B.K. Behura, the founder President of the society.

This volume has two parts. The first part contains a review article on "Biology in ancient India" contributed by Prof. B.K. Behura. The second part embodies research articles submitted by Zoologists of the state and outside.

I consider myself privileged to record an editorial note for a volume which is dedicated to a person who has played a pivotal role in the growth, development and ramification of Zoology in the state of Odisha.

(Pravati Kumari Mahapatra)
Editor

PRANIKEE

Journal of Zoological Society of Orissa

Abbreviation: Pranikee

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BIOLOGY IN ANCIENT INDIA

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INTRODUCTION

"The further backward you look the further forward you can see."

-Winston Churchill

For its survival, every animal tries to acquire as much knowledge as possible about its environment – the land, climate, plants, animals, water resources, enemies, predators, and the like. Ever since the ancestor(s) of humans evolved from their anthropoid ancestors to walk erect, they endeavored to acquire as much knowledge as possible about their surroundings, including plants and animals. However, how much of this knowledge about plant and animal life was able to fulfill his systematic requirements needs to be understood. In order to do so we must first preview the major ancient India civilizations. Most anthropologists agree that around 40,000 B.C., ancient man took to agriculture by cultivating wild shrubs, thereby slowly moving away from the practice of hunting and food gathering.

However, up till about 10,000 B.C., humans were still nomadic wandering in groups from place to place in search of food and shelter. Organized hunting of animals provided them with meat for food, but humans still collected grains from the wild. Later, they discovered that they could cultivate land and produce grains (paddy and wheat) at one place. Thus was born agriculture (Latin, *ager*- field; *culture*- cultivate) in *c* 8,000 B.C. People settled around their fields and established communities. They could now organize the growing of food in large quantities in a well-planned manner. This led to the increase in their knowledge about plants and animals. This further resulted in the employment of animals in agriculture and thus to the domestication of certain species of animals. Thus, prehistoric man gained practical

interest in plants and animals that provided him with food, clothing and other essentials and in the wild beasts that menaced him. The domestication of animals by early civilizations yielded practical information on their breeding, growth and nutrition. Most historians believe that the domestication of plants and animals had its beginning about 8,000 B.C., in the area of the Fertile Crescent, the arc of land stretching from the Western slopes of the Zargros mountains in Iran through southern Turkey, and southward to Israel and the Jordan valley.

EARLY MAN IN INDIA

During the early eighties of the twentieth century, the Geological Survey of India discovered on the banks of river Narmada, 200 metres west of village Hathnora, bound by Vindhyachal and Satpurea hill ranges in Sohore district of Madhya Pradesh, a fossilized partial human skull cap. This cranium was found amidst rich mammalian fauna that was strewn with lithic industry. This fossil belongs to the Lower Palaeolithic Age, from the Pleistocene geological epoch which lasted from about 2, 58,000 to 11,700 years ago. The human species to which the skull cap belonged has been described by schools of thought as *Homo erectus narmadensis* vis-à-vis *Homo sapiens-narmadensis*. During the nineties, the Anthropological Survey of India collected a fossilized collar bone, the clavicle and fossils of associated mammals, and stone tools from the vicinity of this site. These findings amply prove that tool-making ancestors of the modern *Homo sapiens* walked on the shores of river Narmada some 2.5 millions years ago.

THE INDUS VALLEY CIVILIZATION

Satellite photography (Landset imagery) has revealed that the now extinct Saraswati, mentioned time and again in the Rig Veda, as the “greatest among the great” and “ mightiest” river with “ limitless unbroken flow, swift-moving with a rapid rush and temptuous roar “,originated in the Siwalik hills at the foothills of the Himalayas in Himachal Pradesh, flowed through the Ghagger valley in Haryana and the Rajasthan desert, continued into Hakra in Cholistan desert of Sindh and

Pakistan, reached Rann of Kachchh in Gujarat through the Nara valley and drained into the Arabian Sea.

The Gaggar valley is eight to two kilometers wide in many places, indicating thereby that the extinct river Saraswati was a mighty one. The epic *Mahabharata* mentions that the river Saraswati was " disappearing into the desert "and was later "lost", and that the battlefield Kurukhetra was located to the south of the river. Scientific studies reveal that desertification of Rajasthan had begun by *Mahabharat* epic (composed *c* 3000-1000 B.C.) period and river Saraswati had dried up by 2000 B.C.

When the archaeological sites of the "Indus Valley Civilization ", one of the earliest civilizations of the world, were plotted on a map, an amazing discovery was made of the archaeological remains. Most were found to be clustered around the dried Ghaggar Hakra or Saraswati River. The Indus valley civilization was first discovered by Sir John Marshall in the 1920's at Mahenjodaro otherwise known as the " mound of the dead " in the Larkhana district in Sind, Pakistan in the valley formed by the river Indus. Since then, some 2,600 sites have been discovered in the region between Iran on the west, Turkemenia, Bactria and the Pamirs in the north, beyond Delhi into Western Uttar Pradesh in the east, until river Godavari in Maharashtra in the south. This area covers an area of some ten lac square kilometers. The Indus valley culture which dates back to 6500 B.C. reveals evidence of a strong agricultural economy with granaries for storing surplus grain at Mehrgarh in Pakistan.

Indian archaeologists have discovered nearly 700 sites relating to the early, mature and late phases of the Indus civilization, or Indus Valley civilization, or Indus-Saraswati valley civilization. Of the 700 sites, about two dozen sites have been excavated. Some of them are: Kalibagnan in Rajasthan; Bara and Rupnagar in the Punjab; Balu, Banamali, Bhagawanpura, Mitathal, Rupar and Siswal in Haryana; Alamgirpur and Hulas in Uttar Pradesh; Desalpur, Lothal, Rangpur, Rojai and

Sukotada in Gujarat; Manda and Burzahom in Jammu and Kashmir; and Dainabad and Nevasa in Maharashtra. Dholavara located in Kachchh, Gujarat, is the site of the 5,000 years old Harappan civilization. The excavations at Kalibagnan, on the now dry bank of the Ghaggar River, currently in the Ganganagar district of Rajasthan have brought to light, a settlement, which on the basis of Carbon-14 dating goes back to *c* B.C. 2, 900-700.

As we know, this civilization was not uniform. Mahenjo Daro was remarkable for urban planning, water supply and drainage, while Dholavira (Kachchh, Gujarat) had elaborate stone gateways and reservoirs, and the world's first script- the Harappan script. Lothal was a port with a dockyard and granaries. By 1900 B.C., people bagan abandoning the Indus-Saraswati cities and moved east.

THE ARYANS

The prehistoric people who had settled in Iran and northern India are known as Aryans. The *Rig Veda* was composed by the Aryans long before 2,000 B.C.. Many researchers claim, the Vedas were composed on the banks of the rivers Sindhu and Saraswati at the time that the Harappans were living there. The Vedic Aryans lived in the cities of the Indus-Saraswati valley. The distinguishing factors about the Vedic Aryans were their worship of fire, a major Vedic ritual, and yoga. The fire alter has been discovered in several Harappan buildings, and yogic meditation on the seals. A seal of Mahenjo Daro shows a central figure seated on a low throne in a yogic position, alongside which appear an elephant, a tiger, a rhinoceros, a buffalo and deer and two exceedingly stylized human figures. Strangely however, the horse and the cow do not appear on the seals, although we know that the cow was indigenous to that era and the horse was a major part of the Aryan culture.

As stated above, the *Rig Veda* was composed in the Indus-Saraswati basin. Then the "mighty Saraswati "as described in the *Rig Veda* dried up, and an eastward movement began. The Indus river pastoralists of the Vedic period became farmers, artisans, priests and warriors. Thereafter, the great Indian literature, science and

culture developed in the Indo-Gangetic plains, in Kashmir and then later further South.

Bharata-varsha or Aryavarta, the ancient name for north India, stretched from Afghanistan in the West to the Gangetic plains in the East. The people living here were known locally as Aryans and to the world as Hindus (from Sindhu), hence the name Hindustan (from the Sanskrit *stanum*=place). The religious connotation of Hindu came much later, when the followers of the older religion had to be distinguished from the followers of Islam. Whether Buddhist or Sanatanist, the people, were called Hindu.

The *Rig Veda* describes the region and its people as Bharatam janam (born in Bharata). Since time immemorial, all the land around and east of the Indus, was called India, after the river Sindhu by the people of the lands to the West of the River. The country was known not by one name, but by different names, such as, Jambudwipa, Bharatavarsha, Manavadwipa, Kumaridwipa, Hindustan, and India.

THE DRAVIDIANS

The Dravidians were commonly viewed to be the indigent natives of India. But they too were migrant inhabitants. The Dravidians, of the non-Aryan stock to which Tamil, Telugu, Canarese, and Malayalam speaking peoples of South India belong, were among the earliest known settlers in India. They came to the Indian Sub-continent from the Mediterranean region before 5,000 B.C. They originally settled in Northern and Western parts of India. But eventually, they moved to the South part around *c* 1,500 B.C., when the Indus valley civilization began to disintegrate. A second group of settlers from around the Caspian Sea in South Russia entered India around 1,500 B.C., and came to be known as the Aryans. For convenience of study, the history of mankind is divided into three periods: Ancient: *c* B.C. 4000–A.D. 600; Medieval: *c* A.D. 600–1700; Modern: *c* A.D. 1700– Present.

BIOLOGY IN ANCIENT INDIA

Botany

Dependence on plants for food, shelter and other purposes drew pre–Vedic Indians to the study of plant life. Plants were also intimately connected to trade and commerce. The Indus Valley civilization had trade links with West Asia, East Africa, and other centres of civilization. Most of the trade articles involved were plant products, including transport vessels were also made of wood. All this necessitated a scientific study of plants and plant life.

Agriculture was the primary occupation of the people during the Vedic period (c 1500–600 B.C.). There are indications that agriculture, medicine, and arbori-horticulture developed to a great extent during the Vedic period. Even at this early stage, knowledge of descriptive Botany and rudimentary plant physiology became necessary for the successful cultivation and propagation of plants. In Vedic literature a large number of terms are found which describe plants and their parts, external features and internal structures. An attempt to classify plants was also made. There is also evidence that indicates the practice of manuring and crop rotation for the improvement of soil fertility and plant nourishment. Some hymns in the *Rig-Veda* testify to the fact that people of the Vedic period had some knowledge of the process of preparation and absorption of food by plants through the action of light, and the storage of energy in their body (of plants).

The science of Botany and medicine derived from plant life, were called during the Vedic period as V<u>r</u>ksayurveda, the knowledge of tree life and Bhesajavidya. The systemic knowledge of medicine was derived from plant life, as the major portion of the medicinal substances came from plants. Both the terms occur in ancient Sanskrit texts like the *Agni Purana* and *Brhat-samhita*. In Kautilya's Arthasastra, we find the term *gulmavrk-sayurveda*, and in the *Dhanvantari-nighantu* the term *bhesajavidya*. The *gulmavrksayurvedajna* or applied botanist, according to the *Arthasastra*, *Agni Purana*, *Brhat-samhita*, and other Sanskrit texts, had to know the arts of seed collection and selection, soil selection, sowing mechanisms, seed germination,

propagation such as grafting and cutting, planting, nursing, manuring, crop rotation, cultivation under favourable meteorological conditions, plant treatment, plant classification, landscaping, and the like.

For example, a story associated to Jivaka, the physician, indicates ancient human's desire for systemic plant life knowledge. Jivaka, later physician of King Bimbisara, ruler of the Magadha Empire during B.C. 542–492, was a student of a celebrated teacher of medicine known as Bhisu Atreya at the University of Taxila. Records indicate that as parts of his eligibility examination process, he was asked to seek a yojana (8miles=12.87km.) on either side of Taxila and bring whatever plant he could see which did not have medicinal value. The story goes that Jivaka could not discover any plant which did not have medicinal properties. When he reported this to his teacher Bhiksu Atreya, he was, declared to have successfully passed his examination.

Thus, it appears, Botany in India has been a continually developing science since ancient times.

No systematic work on Vrksayurveda: knowledge of tree life (vriksha=tree; ayurveda=knowledge of life) or Bhesajavidya (the knowledge of medicine) (Bheshaja=medicine; vidya= science) belonging to the early period exists. However, there are scattered references on the subject throughout Vedic, Sanskrit and Pali literature from which it is possible to partially reconstruct an account of this science.

Specimens of wheat and barley recovered from the Harappan site prove that these crops were regularly cultivated. According to Sir John Marshall, the discoverer of Mahenjodaro, "The use of cotton was exclusively restricted at this period in India". The district of Koraput in Orissa, in the Eastern Ghats, once formed a part of the ancient Gondwanland, and, had a hot and humid climate. Before the Dravidians settled in this region, the "Nishada" civilization flourished here. According to the reputed linguist Sir George Abraham Grierson (1851–1941) the Soura and Gadaba tribes of that civilization, took to the cultivation of paddy (*Oryza*) in *c* 2300 B.C.

a) Pre-Vedic period (*c* B.C. 5,000-2350)

As stated earlier, Indus valley civilization had trade links with West Asia, East Africa and other centres of civilization. Most of the articles of commerce were plant products and the transport vessels as well. This necessitated scientific study of plants and plant life.

The process of photosynthesis demonstrated in 1727 A.D. by the British scientist Stephen Hales (1677-1761) is described in two stanzas of Mahabharat (composed *c* B.C. 3,000-1,000).

"Just as water may be drawn up by sucking through the lotus petiole applied to the mouth, so also plants (with roots) drink (absorb and draw up the stem) water (watery solution) with the help of air" (XII, 177, 16).

"With the help of *agni* (solar energy) and air this water (soil sap which is absorbed through the roots and conveyed to the leaves) is digested, i.e., prepared into food proper. And it is on account of the assimilation of this food that plants attain development and become graceful" (XII, 177, 18).

b) Vedic period (c B.C. 2,350-500)

Agriculture was the primary occupation of the people during this period. Agriculture, medicine and arbori-horticulture were well–developed. People had knowledge of descriptive botany and rudimentary plant physiology. There was an attempt to classify plants. The practice of manuring and crop rotation for improvement of soil fertility and plant nourishment was in vogue. Vedic literature records some 740 species of plants.

More complete and systematic accounts of parts of a plant are found in Taittsriya Samhita (*c* B.C. 2446) and *Vajarsaneyi* Samhita (B.C. 2446) and *Vajasaneyi Samhita.*

Brhadaranyaka Upanishad (*c* B.C. 900–600) enumerates ten cultivated grains (gramyani) : vrihi (paddy), yava (barley) ; tila (sesamum) ; masa (bean); anu (millet); priyangu (panic seed); masura (lentil); gohuma (wheat) ; khalva (pulse) and khalakula (vetch, pea family).

Classification and Morphology

The Rig-Veda (3,000–2,000 B.C.) divides plants into three broad classes: *vrksa* (tree), *osadhi* (herb useful to man) and *virudh* (minor herb). According to another classification based on their form of growth, plants are divided into *vrks*a and *druma* (tree), *visakha* (shrub with spreading branches), *sasa* (herb), *amsumalin* (spreading or deliquescent plant) (deliquescent–absorb enough water from air to melt away), *stambini* (bushy plant), *vratati* (climber), *pratanvati* (creeper), and *alasala* (creeper spreading on the ground).

The *Atharva-Veda* divides *sasa* further into prastrnati (expanding), ekasunga (one-sheathed or spathed) (apathe–large bract or pair of bracts enveloping spadix or flower cluster) (botainical), amsumati (having many stalks or branches) and kandini (jointed).

Different parts of a plant body are mentioned in many places in *Rig-Veda*.

The *Atharva-Veda* gives an almost complete enumeration of these parts in a hymn which repeats as follows.

> "Rich in sweets the roots,
> rich in sweets the tips of them,
> rich in sweets the middle of the plants (stem) ;
> rich in sweets the leaves,
> rich in sweets the flowers of them "

More complete and systematic accounts of the parts of a plant are found in the *Taittiriya Samhita* and *Vajasaneyi Samhita* according to which plants are comprised of *mula* (root), *tula* (shoot), *kanda* (stem), *valsa* (twig), *puspa* (flower) and *phala*

(fruit) while trees have in addition *skandha* (corona), (botany:inner side of corolla or appendage on top of the seed) *sakha* (branch), and *parna* (leaf).

Descriptive terms for the various parts of a tree or plant, its texture and colour, fruits and flowers etc., are also found in Vedic texts. For example: *valsa*: twig of plant; *satavalsa* to describe plants with many twigs *sahasravalsa kantakinah*: plants with thorns; *puspavati*; a blossoming plant: *prasuvari*: a plant with fragrant flowers.

Brhadaranyaka Upanishad (c B.C. 900–600) enumerates 10 cultivated grains (gramyani): *Vrihi* (rice), *yava* (barley), *tila* (sesamum), *masa* (bean), *anu* (millet), *priyangu* (panic seed), *masura* (lentil), *godhuma* (wheat), *khalva* (pulse), *knalakula* (vetch) (vetch- pea family).

Anatomy

Although the Greek philosopher Theophrastus (c 370–287 B.C.) is regarded as the father of plant anatomy, we find a far more detailed description of plants in *Brhadaranyaka Upanisad* (*c* B.C. 900–600).

Physiology

It appears that the Vedic people had some knowledge of the preparation and absorption of food by plants and of the role played by light in this regard. They were aware of the phenomenon of storage of energy in the body of plants and also knew that plants draw nourishment from manure like cowdung (karisa, *sakrt*). According to *Taittiriya Samhita* (c 2446–B.C.), Vedic people practiced rotation of crops by fallowing the land and by sowing different crops alternatively in the same field. According to William Roxburgh, the author of the monumental work *Flora Indica* (1820), the Western world is indebted to India for the latter practice.

Evolution

Manusmruti (c 1200-900 B.C.) by the great sage Manu, presents ideas on creation of the universe, and orgin of life. Vedic thinkers believed that plants had preceded

animals, particularly man, in the process of evolution. This is indicated clearly in a hymn of the *Rig-Veda* (x.97.1). In the *Taittiriya Upanisad* (II.I) (c B.C. 900–600), this idea of evolution is suggested through the following passages.

"From that very Atman ether came to be; from ether air, from air fire, from fire water, from water the earth, from the earth herbs, from herbs food, and from food the person came into existence".

Similar ideas also occur in the *Chandogya Upanisad* (I.I.2) (*c* B.C. 900–600) and the *Brhadaranyaka Upanisad* (IV.I) (*c* B.C. 900–600).

Survey

It is of interest to note that the first Botanical survey of plants was undertaken by Jivaka, the court physician of King Bimbisara, ruler of Magadha (B.C. 542–494), under the instructions of his teachers.

(c) Post Vedic period (c B.C. 500–600 A.D.)

The study of botany made further progress in the post-Vedic period (c B.C. 500–600 A.D.). Indian literature of this period bears ample evidence of the post-Vedic people's knowledge of morphology (both external and internal), physiology, ecology, taxonomy, etc. of plants.

The famous Jain saint Ugraditya in his treatise on Ayurvedic medicine "Kalyanakarakam "(c 300 B.C.) describes some eighteen thousand flowers and their use for different ailments.

The *Caraka-Samhita* (I.1.122) of Caraka (*c* 100 A.D.) observes that only a person who is well-acquainted with the names and external features of plants and able to utilize them is to call an expert physician.

The *Amarakosa* (*c* 500 A.D.) has a chapter on plants which enumerates more than three hundred species of plants.

All methods of propagation–by seeds (*bijaruha*), roots (*mulaja*), cuttings (*skandhaja*), graftings or layerings (*skandha ropaniya*), apices (*agrabija*) and leaves (*parnayoni*) now known, were considered common knowledge to the people of Post-Vedic days. All these methods find mention in treatises like *Brhat–samhita* (c 600 A.D.), *Arthasastra* (c B.C. 300–300 A.D.), *Manu–samhita* (c 200 A.D.), *Abhidhana–chintamony* and *Sumangala–vilasini*.

Morphology

Post–Vedic Indians knew that air, warmth and water are necessary for germination. Post–Vedic literature contains detailed description of the parts of a plant as it grows. The *Mahabharata* refers to various parts of a tree. The *Visnu Purana* classifies different parts of a plant into mula or *pada* (the subterranean part) and *tula* or *vistara* (sub aerial part).

Terms used to describe the diverse kinds of plants, roots, stems, leaves, flowers, fruits etc., are suggestive of detailed knowledge of the morphology and functions of different kinds of plants. The body of a plant was distinguished into five regions: tvac (skin), mamsa (soft tissues or bast), asthi (wood or bone), *majja* (pith) and *snayau* (fibre in the bast).

Physiology

That plants absorb food from the soil in a state of soluble form was known, as the term padapa (drinker of rasa or fluid from the soil by roots) for plants suggests.

The greatest achievement of the ancient Indians in the field of botany was the discovery of the phenomenon of absorption, transportation, and preparation of food in the leaves in the presence of solar energy and air. This process of photosynthesis is described in two stanzas of the *Mahabharata* (XII, 177.16, 18) as follows:

"Just as water may be drawn up by sucking through the lotus petiole applied to the mouth, so also plants (with roots) drink (absorb and draw up the stem) water (watery solution) with the help of air."

"With the help of *agni* (solar energy) and air (CO_2) this water (soil sap which is absorbed through the roots and conveyed to the leaves) is digested, i.e., prepared into food proper. And it is on account of the assimilation of this food that plants attain development and become graceful inside."

Thus, what the British Stephen Hales (1677–1761) demonstrated in A.D. 1727, as photosynthesis was known to post–Vedic Indians in rudimentary form. As regards to circulation of sap, Kanada who was famous for his conception of atom has discussed it in his *Vaisesika–sutra* long before the Christian era.

Much later, Sankara Misra (c A.D. 1500) notes or queries it in his *Upaskara* which says as below.

"Water poured at the roots goes up in all directions through the interior of a tree. Neither impulse, nor impact, nor the sun's rays prevail there. How then is it caused?"

The exudation of sap (rasasruti) is clearly described in the *Rajanighanta*. The phenomenon of phosphorescence in plants is mentioned in Kalidasa's *Kumarasambhava.*

The importance of light, food, and water for the growth and sustenance of plants was well known. The maximum age of a tree is given as ten thousand years, and the causes of death are cited as unsuitable food, accident and disease.

Post–Vedic Indians also noticed that some plants close up their leaves at night as if sleeping, that plants are sensitive to tough, and that various kinds of flowers open their petals at different times of the day.

Plants have been regarded as living beings since Vedic times. A concise but clear discussion on the existence of life in plants is given in the *Mahabharata* (XII.184). Further evidence is to be found in Gunaratna's *Sukraniti*, Udayana's (c 10-11 Century A.D.) *Kiranavali*, Sankara Misra's *Upaskara* (*c* A.D. 1500) and the *Bhagavata - Purana*.

All the methods of propagation now known were common knowledge. Mention is made of propagation of seeds (*bijaruha*), roots (*mulaja*), cuttings (*skandhaja*), graftings or layerings (*skandhe ropaniya*), apices (*agrabija*), and leaves (*parnayoni*). All these methods are referred to in treatises like the *Brhatsamhita* (c 600 A.D.), *Arthasastra* (B.C. 300– A.D. 300), *Manusamhita*, *Abhidhana-cintamani*, and *Sumangala-vilasini*.

The idea of sexuality in plants seems to have been vaguely known, though there is a discussion in the *Sarirasthana* of the *Harita–samhita* as to how seeds are produced in plants. Only in one instance are a male and a female plant distinguished and that is in the case of *Ketaki* (Pandanus *odoratisasimus*). The male plant is called *sitaketaki viphala* or *dhulipuspika*, and the female, *avarnaketaki*.

Ecology

Caraka (c A.D. 100) and Susruta (original text believed to have been redacted by Nagarjuna between A.D. 300 and 400, classified lands according to the nature of the soil, climate, and vegetation into three categories namely Jangala, Anupa and Sadharana.

1. *Jangala*

A region of open spaces where a steady, dry wind blows. It is paraded by expansive mirages, has few rivers and rivulets, abounds in wells, and consists mainly of dry and rough sands. Plants common to this region are *Khadira* (*Acacia catechu*), *Asana* (*Terminalia tomentosa*), and *Badari* (*Zizyphus jujuba*) among others.

2. *Anupa*

A region of marshy tract bordered by seas. Swept by cold wind, it is impassable owing to its network of rivers (*nadimatruka*) and the sheets of accumulated rain water.

Some plants of this region are *Vanjula* (cane or reed), *Hintala* (a kind of palm) and *Narikela* (coconut).

The *Amarakosa* mentions the following plants growing in the waters of this region are *Saugandhika*, *kahlara*, *hallaka*, *indivara*, *kumuda*, *padmini* and *kokanada* (various varieties of lotuses and water lilies), *variparni* (*Pistia stratiotes*), *musika-parni* (*Salvania cucullata*), *jalaniti* (algao), and *saivala* (moss).

3. ***Sadharana*** or intermediate region

Have some features common to the other two regions. A few plants found in the regions are the *mandara* or *parijataka* (coral tree) and *satana* (kalpa tree). The rainfall in these regions is given in the *Arthasastra*.

TAXONOMY

In the naming of plants, a scientific and rational procedure was followed. For example, plants were named in accordance with their special association, medicinal and other properties, morphological characteristics, environmental association, and other noticeable peculiarities. Sir William Jones (1746–94), founder of the Asiatic Society of Bengal (1784) and the author of " A Treatise on the Plants of India" (1790), observed that, " Linnaeus himself would have adopted them had he known the learned and ancient language of this country ". Some plants derived their names from historical events. For example, the bodhidruma ('tree of enlightment') received its name from it being the tree under which Gautama (*c* 563-483 B.C.) sat when he attained *nirvana* and thus became the Buddha (*c* 528 B.C.). Medicinal properties were considered in naming plants like *dadrughna* ('curer of eczema') and *arsoghna* ('curer of piles'). Some examples of naming plants on the basis of their utility value

are the *danta-dhavana* (cleaning of teeth tree), and that *lekhana* (writing reed tree). Morphological features were embodied in *tripatra* (three-leafed), *panchangula* ('five-fingered') and satamuli (hundred-rooted). *Magadhi* (native of Magadha) and *Campeya* (native of Campa) were based on local association. The names *maruvaka* (desert crane 'i.e., a desert plant of which the flower resembles the shape of a crane') and *jalaja* (water plant) emphasized environmental association. Special features and other characteristics are reflected in the naming of plants such as *phenila* (lather-forming, 'i.e., soapberry) and *saradi* (autumnal).

Sometimes plants were given two names, one for their identification by the common people and the other to convey their medicinal and other properties. The former was called *paricayajnapika samjna* and the latter, *guna-prakasika samjna*. Thus the plant *sesbania* is called *vakra-puspa* (with papilionaceous flowers) and *vranari* (antidote to boil). Similarly, *Ricinus communis* is called *citra-bija* (with painted seeds) and *vatari* (antidote to rheumatism).

Classification of plants was based upon three distinct principles: botanical (udbhida), medicinal (virecanadi), and dietetic (annapanadi).

Botanical classification can be traced to the *Rig–Veda* (X.97) and *Atharva–Veda* (VIII. 7.4). Manu gives an elaborate classification, as do Caraka and Susruta. Their classifications include such divisions as: plant bearing fruit without flowers (*vanaspati*) (cryptogams of modern botany); plant bearing both flowers and fruits (*vanaspatya* or vrksa) (Phanerogams of modern botany); annual plant (*osadhi*); creeping plant (*virudh*); herb with succulent stem (*gulma*); grass including bamboo (*trna*). Plant families as such were not recognized. But allied plants or varieties, or even different species, were grouped together into what may be called a genus, based on floral characters. The specific characters were taken primarily from the colours of flowers. Thus the genus *Kovidara* includes the white–, yellow–, and red–flowered species. The first one is, again, divided into two varieties. Similarly, *bala* includes four species: *bala*, *atibala*, *mahabala* and nagabala.

Charaka divides plants of **medicinal value** into two main groups: purgatives (virecana) and astringents (anupana), the number of the former being 600 and that of the latter 500. The astringents, again, are divided into fifty groups under the *Vargas* or major heads. These include every item of therapeutics. Sushruta, however, classifies plants under 37 sections or *ganas.* All plants known to be of medicinal value upto his time are placed under one group or another.

Charaka classifies plants of **dietetic value** under 7 *vargas*: *sukadhanya* (cereal), *samidhanya* (pulse), *saka* (pot-herb), *phala* (fruit), *harita* (generally, green or yellowish vegetables or fruits), *sharayogin* (oil) and *iksu* (sugarcane). Caraka mentions more than 13 varieties of sugarcane.

Susruta classifies plants of dietetic value into 15 Vargas: *salidhanya*, *sasthika*, *vrihidhanya*, *kudhanya* (all cereals of different classes), *vaidala* (pulse), *tila* (sesamum), *yava* (barley), *simba* (bean and varieties), *phala* , *saka*, *puspa*, *odbhida* (mushroom), *kanda*, *taila* and *iksu*.

PATHOLOGY

Ancient Indian botanists made contributions to the study of plant pathology. The *Atharva–Veda* (VI. 50) refers to the destruction of corn by insect pests. Mention of blight and mildew occurs in Vinaya texts (of the Sarbastivada School – nearly 200 leaves in Brahmi and dated 5th Century A.D., discovered at Merv, in Soviet Central Asia).

The *Sukraniti* of Sukracharya (c 14th to 16th Century A.D.) speaks of grains which are likely to be attacked with poison, fire or snow, or eaten by insects. The *Arthasastra* (Kautilya, *c* B.C. 300 – A.D. 300), *Agni Purana* (c 800 A.D.) and *Brhat–samhita* (of Varahamihira, *c* A.D. 550) have each a chapter on Vrksayurveda. In the last named book, aetiology (study of causes of disease) diagnosis, and treatment of plants are given. According to Bhattotpala, Kasyapa (of unknown date, Kasyapiya–*krsisukti*) gives a prescription for diagnosing plant diseases. Among the

remedies suggested are the removal of affected parts and the taking of preventive measures against fresh infection through the wound. Barrenness of plants was also considered a disease, for which certain remedies were prescribed.

ZOOLOGY

Even when ancient men lived in caves in the early Stone Age (400,000– 200,000 B.C.), they had acquired considerable knowledge about different kinds of animals. As they gradually adopted a pastoral life in the late Stone Age, or early Bronze Age (5,000–1,000 B.C.), several common species of mammals and birds were domesticated for use in agriculture, transport and food. The maintenance of domesticated animals necessitated a more thorough knowledge of their habits and needs. Thus, through observation, acquaintance with animal life gradually became more systematic, leading to attempts at classification and formation of some basic concepts regarding the animal kingdom.

a) **Pre-Vedic Period** (c 5000–2350 B.C.)

Various articles such as seals, terracotas, clay figurines and potteries bearing engraved or painted representation of animals, unearthed in excavations in Baluchistan, Sind and the Punjab (all in Pakistan) and in various regions of India, depict different kinds of animals, such as humped bull (quite common); water buffalo, ibex (a type of wild goat) ; gaur, rhinoceros, lion, tiger, hare; some species of birds and fish and scorpion. These indicate familiarity of ancient Indians with a variety of domesticated and wild animals.

Animal remains discovered at various levels of these excavations of the Indus valley civilization sites reveal some 39 different species of animals: 26 species of vertebrates and 13 species of invertebrates. These can be categorized under different sections such as: (a) animals that were maintained in a state of domestication; (b) animals that remained in the vicinity of human habitations; (c) animals caught and

utilized as food, as evidenced by charred remains of animals; (d) animals probably imported for medicinal purposes, and (e) animals imported for ornamental purposes.

Domesticated animals include (1) The Indian domestic cattle or Zebu, *Bos indicus* Linnaeus, (2) The camel, *Camelus dromedarius* Linnaeus, (3) The domestic ass, *Equus asinus* Linn., (4) The dog*, Canis familiaris* Linn., (5) The Indian domestic pig, *Sus scrofa* Linnaeus, (6) The Water buffalo , *Bubalus bubalis* Linn. (7) The Indian domestic goat, *Capra hircus*, (8) The Indian domestic sheep, *Ovis orientalis* Gmelin, (9) The Indian elephant, *Elephas maximus* Linn., and (10) The domestic fowl, *Gallus gallus* (Linnaeus) .

The Indian jungle fowl, *Gallus gallus* of the Asian tropical forests and ancestor of all domestic breeds, appears to have been domesticated in India by 3200 B.C.. Cock fighting as a sport was known in ancient India as early as 1000 B.C..

Nath (1962) is of the opinion that the remains of the true horse, *Equus caballus* Linn., from Harappa (District Montgomery, Punjab, Western Pakistan), do not belong to the Harappan period (*c* 2500–1500 B.C.), but to the post-Harappan period (after 1500 B.C.), soon after the end of the final phase of the Harappan civilization. It is difficult to say whether the horse was there then, or was brought from outside by alien invaders who destroyed the prehistoric city.

There appears to be no evidence of the true horse in the Western Asiatic countries before 2000 B.C.. It was the Onager (Grey ass, *Equus anager*) and not the horse (Oriental Speed horse, *Equus przewalski*) that pulled the wheeled vehicles in Mesopotamia in *c* 3400 B.C. In Egypt, a horse as we know today is not found until 1500 B.C., despite the fact that war chariots are known to have existed between *c* 1500 B.C. and 1557 B.C. The true horse was introduced and domesticated only in the early Second millennium. The skeletal remains of the elephant found in Harappa suggest that the people of Harappa were well-acquainted with it.

Remains of the rhinoceros, *Rhinoceros unicornis* Linn., found in Harappa (Dist., Montgomery, Punjab, West Pakistan) (B.C. 2500–1500), Lothal (Dist., Ahmedabad, Gujarat) (*c* B.C. 2000–1200), and Kalibagnan (Dist., Ganganagar, Rajasthan) (*c* B.C. 2000–1500) suggests some interesting conclusions. This animal lives in marshy and humid forests. It is not found in the above regions now-a-days. The fossil findings indicate the extensive distribution of the rhinoceros in the earlier days. It strengthens the geological evidence that the desert conditions of the Rajasthan area is of recent origin and that Harappa, Lothal and Kalibagnan etc., had marshy and humid forests during 2500 B.C..

Remains of animals which lived in the vicinity of human habitation include (1) the monitor lizard, *Varanus* sp.; (2) the palm civet, *Paradoxurus* sp.; (3) the mongoose, *Herpestes* sp.; (4) the Large Bandicoot rat, *Bandicota indica* (Bechstein) and (5) the semi-domesticated house rat, *Rattus rattus* Linn. Remains of animals, probably caught and utilized as food include (91) several species of turtles and tortoises; (2) the Gharial, *Gavialis gangeticus*; (3) and several species of fish, as for example, *Rita rita* Ham., *Wallago* sp. and *Arius* sp. Remains of animals probably imported for ornamental purposes include species of freshwater and marine *molluscs.*

The *Ramayana* (composed *c* B.C.3000–1000 B.C.) enumerates more than 100 mammals, birds, reptiles, fish, insects etc. The *Mahabharat* (composed *c* B.C. 3000–1000 B.C.) contains descriptions of seven wild and seven domesticated animals. Natural habits and diseases plaguing some animals are also mentioned.

Taittiriya Samhita (c B.C. 2446) classifies animals as follows.

Type – I

1. Those supported by bones (vertebrates)
2. Those supported by flesh (invertebrates).

Type – II

1. Those having incisors on one side
2. Those having incisors on both sides.

Type – III (based on the colour and number of limbs).

About 80 anatomical structures of the horse have been enumerated in *Taittiriya Samhita* (*c* B.C.2446).

(b) VEDIC PERIOD (c B.C. 2350–500 B.C.)

Rig Veda (c B.C. 2350) mentions many kinds of mammals, a few species of birds, reptiles, fishes, worms and insects, horses and deer of different colours. It states that a horse possesses 34 ribs. According to "*Cyclopedia Anatomicae* "by Gyorgy Fahr (1996), a horse has 18 pairs, i.e., 36 ribs. The number of ribs fluctuates between the species. The Arabian breed of the modern horse possesses 17 pairs of ribs. It therefore appears, the record of 34 ribs of the horse in the Rg Veda is not inaccurate. In the *manduka sukta* (vii-7-103) of this veda occurs the passage "sangobatsanga sashayanah". In this is described how frogs remain inactive for months, and when the rains come, they become active and start croaking.

The famous frog hymn in the *Rig Veda* (VII.103) in translation runs as follows.

1. Hibernating throughout the year like Brahmins observing a vow. Animated by the divine Parjanya the frogs are croaking loudly.
2. When the heavenly showers fall on them lying in a pond like shrivelled skin, like the lowing of cows for their calves, sound the voices of frogs in unison.
3. When showers fall on longing creatures, and slake their thirst at the start of the rainy season.

   With rapture in the voice, like the son his father they greet each other without cessation.
4. One repeats the word of another, like students echoing the voice of the teacher, Together they form a chorus,

"When at rainfall loudly they croak."

(Behera, K.S. 1979. Reflections on the kingdom- 'Animalia' Symposium on Recent Trends in Aphido-logical Studies, Bhubaneswar, June 9-12, 1979, Pt. IV. Pp. 17-22)

In the Rig Veda, the flow of blood in the body has been compared with the flow of underground water. *Atharva–Veda* (c B.C. 1500–500 B.C.) mentions observations such as impotence of bulls due to castration, croaking of frogs during rains, the four-footed structure of frogs; the devouring of foetal membrance by cows after the delivery of calves; the stinging with claws and tail by scorpions; the injection of poison through bites by insects and the presence of twenty nails in the paws of a tiger. Snakes and worms have been classified mostly on the basis of colour, form and anatomical structures. Snakes are stated to possess two pairs of teeth, a pair of jaws and a pair of tongues (VI. 56.3). Twenty one kinds of vipers have been distinguished (I.27.4). Anatomical features of the ox and the horse are provided. More than 50 anatomical structures of the ox are given.

Chandogya Upanishad (c B.C. 900–600 B.C.) provides a broad classification of animals on the basis of mode of origin and anatomical characteristics, etc. All living creatures, on the basis of their seed (bija*)*, in a general sense their mode of origin, are classified into 3 main groups:*Jivaja* (viviparous) all mammals; *Andaja* (oviparous) all birds, reptiles, insects and worms; and *Udbhijja* (of vegetable origin) it was believed that all udbhijja animals arose from vegetable matter.

As members of a pastoral society, Vedic Indians were particularly interested in animal life and in the taming, training and breeding of livestock (domestic animals). Cows were highly valued for giving milk, and oxen for farm work. Special attention was paid to the cow, bull, goat and sheep; and horse stallions were sometimes gelded, while mares were exclusively used in driving war chariots.

In Vedic literature, we find mention of some 250 animals.

Lac is obtained from the lac insect native to India and Southeast Asia. Lac is the only resin of animal origin. Even thousands of years ago, people in India used to collect and grow lac for diverse uses. In Vedic period, there are references to the labours of this insect for producing lac. *Atharva Veda* provided a brief account of lac, the lac insect, and the medicinal uses of lac. In the epic *Mahabharata*, there is

reference to *Laksha Griha*–a beautiful palace made of lac, a very inflammable material that the Kauravas built for the destruction of the Pandavas.

Bee-keeping has been practiced in India since time immemorial. The earliest references date back to Vedas and Ramayana.

There is mention of tasar silk (*Antheraea*) as early as 1590 B.C. Export of raw silk from India dates back to 58 B.C. (Shamitha, 2007).

The great sage surgeon, philosopher and teacher of medicine Susruta (c B.C. 800–600), is renowned all over the world for his contributions to surgery in general and plastic surgery in particular, especially rhinoplasty (plastic surgery of the nose). He is the first surgeon to describe as many as 101 blunt and 20 sharp surgical instruments (yantra). In his *Susruta Samhita* (Sanskrit: 'samhita'=a collection of systematically arranged verses or, 'a text') compiled in verse, a full chapter (IX of Section I) is devoted to the principles of experimental surgery (Natarajan, 2008). This implies intimate knowledge of human anatomy and physiology by the physicians of the Vedic period which must have of necessity been gained through dissection of animals and intimate knowledge of the anatomy and physiology of experimental animals.

(c) POST–VEDIC PERIOD (*c* 500– 600 A.D.)

Post-vedic Indians acquired a much more comprehensive and detailed knowledge of animals, particularly in connection with the study of medicine.

As per the account of the Greek traveller Megasthenes (c B.C. 400), Kalinga exported elephants to Singhala (Sri Lanka) by boat.

CLASSIFICATION

Charaka samhita (*c* 100 A.D.) of Charaka; *Susruta-samhita* of Susruta (*c* A.D. 300-400); *Mahabhasya* of Patanjali (*c* 150 B.C.) ; *Tattvarthadhigama-sutra* of Umasvamin (c 40 A.D.) and *Padartha-dharma-sanghita* of Prasastapada (*c* 500

A.D.) provide various types of animal classification based on food habits and habitats (Charaka), on the number of senses (Um-asvamin) etc..

The *Charaka-samhita* of Charaka (*c* 100 A.D.) classifies all animals into 4 divisions: *jarauja*–born from the uterus (viviparous); *andaja*–born of an ovum or egg (oviparous); *svedaja* or *usmaja*–born of moisture and heat spontaneously or asexually generated; and *udbhijja*–born of vegetable organisms. An almost identical classification occurs in the *Susruta samhita* (original text believed to have been redacted by Nagarjuna between *c* A.D. 300 and A.D. 400).

Charaka has also classified creatures according to their characteristics as given below: *krmi*–parasites found in living creatures; *kita*–wingless insects; *patanga*–flying insects ; ekasapha–solid-hoofed ungulate animal (solid– hoofed, horse family, equines) ; *dvisapha*–cloven–hoofed animals (hoof divided: ruminant quadrupeds, e.g., ox, sheep); *mrga*–herbivorous animals; *kravyada*–carnivorous animals; svapada–dangerous beasts of prey; *vyala*–beasts of prey; *gomayu*–creature with poisonous fangs or stingers; and *sarpa*–snakes.

Charaka has also made a classification of animals according to their food habits and habitats.

(1) *Prasaha*–Creatures who grab and tear off their food, carnivorous and non-carnivorous land quadrupeds and birds, lion, bear, horse, mule, ass, cow, camel, dog, fox, cat, vulture, hawk, etc., 29 species.

(2) Bhumisaya–or *bilesaya*– burrowing animals (mammals and reptiles): hedgehog, porcupine, small mongoose, python, lizard, frog, etc., 13 species.

(3) *Anupa*–Marshy and wetland dwelling creatures. Mammals: elephant, yak, rhinoceros, buffalo, pig, etc. Nine species.

(4) Varisay– aquatic animals (mammals, reptiles, fish, Mollusca, Crustacea), whale, dolphin, crocodile, tortoise, crab, oyster, etc. 10 species.

(5) *Jalacara* or *ambucarin*- creatures that live around or on the surface of water. Crane, swan flamingo, pelican, etc. 29 species.

(6) *Jangala*–herbivorous animals–mostly deer, living in dry and hilly jungle lands and forests. 17 species.

(7) *Viskira*–gallinaceous birds that scatter their food in the process of eating. Peacock, pheasant, partridge, sparrow, quail, etc. 19 species.

(8) *Pratuda*–birds that pierce or tear their food (worms and fruits) with their beaks. Bulbul, pigeon, Indian koel, kingfisher, mynah, wood-pecker, green parakeet, etc. 30 species.

Su'sruta on the basis of their food habits and habitats classifies animals as follows:

Jangala–Wild, herbivorous quadrupeds, strong–legged and quick-footed: various species of deer and antelope; *Viskira*–Birds which scatter their food while feeding; *Pratuda*–Birds that pierce or tear their food with their beak; *Guhasaya*–Carnivorous quadrupeds living in natural caves or hollows: lion, tiger, wolf, hyena, bear, panther, cat, jackal and others; *Prasaha*– Birds of prey: vulture, kite, hawk, owl etc; *Parnamrga*–Arboreal animals e.g., ape, squirrel, some reptiles, some Carnivora; *Bilesaya*–Animals that live in holes or burrows: Some species of rodents, Insectivora and reptiles and *Gramya*–Domesticated quadrupeds like horse, mule, ass, camel, goat, cow and sheep.

Table 1. Different types of animals

Animals				
Anupa				Jangala
Kulachara	Plava	Ko'sastha	Padin	Matsya
(herbivorous quadrupeds, frequenting banks of rivers and ponds, elephant, Rhinoceros, buffalo, etc.)	(amphibious birds goose,duck Crane etc.)	(Molluscs-Conch, pearl oyster, Snail etc.)	(aquatic mammals with pedal appendages; tortoise, turtle, Crocodile, crab, etc.)	(fresh water and marine fish)

Susruta-samhita (*c* A.D. 300–400 A.D.) classifies snakes into 5 groups (V.4.2-17):-

Venomous

1. *Darvikara*- Hooded, swift in movement, diurnal, bear on their hoods or bodies marks of chariot wheel, ploughs, umbrellas, cross-bands, goads, etc. Most deadly when young. 26 species.
2. *Mandalin*- Vipers. Thick, without hood, slow-moving, nocturnal, bear circle or rings on the body. Most deadly when middle-aged. Two groups.
3. *Rajimat*– Without hood, nocturnal, often of variegated colours on the upper part and sides, bear series of dots or marks. Most delay when aged. Different varieties noted.
4. *Vaikaranja*- Hybrid snakes. Ten species named.

Non-venomous

1. *Nirvisa*- Non-venomous. Though without venom, can kill by strangulation or crushing of bones. Twelve kinds noted.

According to *Agni Purana,* a snake possesses 32 teeth in all, of which 4 are fangs, two on either side.

Patanjali (c 150 B.C.) in his *Mahabhasya* speaks of *Ksudrajantus* (small animals), defines them variously and groups those under 5 categories.

(1) animals without bones
(2) animals without any blood of their own
(3) animals so minute in size as to number more than a thousand in a palmful
(4) animals not easily crushed
(5) All animals upto the ichneumon (insects which lay aggs on the larvae of other insects) in the animal series.

A more comprehensive classification of creatures is found in the ancient Jaina work Tattvartha-*dhigama-sutra* of Umasvamin (*c* 40 A.D.). This classification is based on the number of senses- two, three, four or five-possessed by animals.

1. *Creatures with two senses: touch* (as evidenced by contractability of tissues) and *taste* (as indicated by their selection and rejection of food).

(1) *Ap–adika* (Vermes without lateral appendages)
(2) *Nupuraka* (ringlike creatures with pendants, i.e., *vermes* with un-segmented lateral appendages)
(3) *Gandupada* (knotty–legged Arthropoda including Crustacea, Myriapoda, and others).
(4) *Sankha* (Conchifera) and suktika (pearl oyster): some forms of mollusc. (Conchifera–Term applied by Lamarck to bivalve molluscs and to very different branchiopods)
(5) *Jalauka* (leech).

II. Insects with three sense : Smell, touch and taste

(1) Pipilika (ants)
(2) Rohinika (red ants)
(3) Upachika , Kunta and tupuraka (bug and flea)
(4) *Trapusabija* and *karpasasthika* (cucumber and cotton weevil and louse)
(5) *Trnapatra* (plant louse)

(6) *Kastha-haraka* (termites).

III. *Creatures with four well-developed and active senses*: Sight, smell, taste and touch

(1) *Bhramara, varta and saranga* (bees, wasps and hornets)

(2) *Matsika, puttika, damsa and masaka* (fly , gnat, gad-fly and mosquito)

(3) *Vr'scika and nandyavarta* (scorpion and spider)

(4) *Kita* (butterfly and moth)

(5) *Patanga* (Grasshopper, cockroach and locust).

IV. *Animals (besides humans*) with five well- developed and active senses, besides man (sight, smell, taste, touch and sound)

1) *Matsya* (fishes)

2) *Uraga* (apodal reptiles including snake)

3) *Bhujanga* (limbed reptiles and frog)

4) *Paksin* (birds)

5) *Catuspada* (quadrupeds).

Vertebrates, on the basis of their mode of reproduction have been grouped under

1. *Andaja* (oviparous): Snake, lizard, chameleon, fish, crocodile and bird.
2. *Jarayuja* (viviparous): mammals born with a placenta, e.g., man, cow, buffalo goat, sheep, horse, tiger, bear, dog and cat.
3. *Potaja* (mammals with deciduate=in which the villi of the placenta are so intimately united with the uterine mucous membrane that the latter comes away with the foetus at birth), e.g., porcupine, elephant, hedgehog, hare, squirrel, ichneumon (Egyptian mongoose, *Herpestes*, *ichneumon*), mouse, bat and Insectivora.

Prasastapada (c 500 A.D.) in his *Padartha-dharma-sanghita* classifies animals into two broad divisions: *Ayonija* (asexually generated animals) (corresponds to svedaja group of Charaka) and *Yonija* (sexually generated animals) which included *Jarayuja* (viviparous) and *Andaja* (oviparous).

In *Ramayana* more than 120 animals–birds, snakes, other reptiles, insects and fish and the like are enumerated.

In Mahabharata, as in *Chandogya Upanishad*, Mahabharata classifies mobile, living creatures into 3 divisions: *Jarayuja* (viviparous); *Andaja* (oviparous) and *Svedaja* (born of moisture). *Mahabharata* also describes 14 different types of animals: 7 domesticated; 7 wild. These animals, on the basis of anatomical features are divided into: those with many legs and those having two legs. Interesting observations on diseases, and, certain natural habits of some of the animals are found in this epic.

Charaka and *Susruta* lay special stress on the use of fish as a valuable article of food.

Susruta classifies fish into (i) saltwater and (ii) freshwater fishes.

Ashokan Pillar Edict v (c 246 B.C.)

Five kinds of fish and other aquatic animals have been referred to:

1. *Anathikamacche*: (literal meaning : cartilaginous or boneless fish like prawn, shrimp, jelly fish, starfish): shark (dogfish–a cartilaginous fish)*
2. *Vedveyake* : eel or eel-life fish *
3. *Gangapuputake* : freshwater porpoise *
4. *Samkujamacche* : skate and ray fish *
5. *Kaphatasyake* : globe fish *

(* As identified by S.L. Hora. J. *Asiatic Soc. Bengal.* 16 (1950): 43-56).

In Kautilya's *Arthasastra* (c 300 B.C.), there are several references to fish and fisheries, rearing of animals such as cow, buffalo, goat, sheep, horse and elephant. There is a chapter on the Superintendent of cows: his duties, classification of cattle and the like. In another Chapter, Kautilya discusses the breed, age, colour, marks,

group or class and the like of *horses.* In a separate chapter on elephant, he classifies them into four categories based on the type of training they received: *damya* (tameable); *sannahya* (trained for war); aupavahya (trained for riding) and *vyala* (rogue elephant). Each of these is again subdivided into several groups. He has also described how war elephants were trained in rising, bending, crossing fences and pits, charging straight or in a zigzagging manner and trampling under foot horses, chariots and infantry.

HEREDITY AND GENETICS

The concept of heredity was known to the ancient Indians. In the Brahmanas (*c* 800 B.C.), an explanation for the phenomenon of hereditary transmission was sought. The great medical practitioners Susruta (*c* 800 – 600 B.C.) and Charaka (*c* 100 B.C.) following Dhanvantari (period undetermined) hold that all the organs are potentially present at the same time in the fertilized ovum and unfold in a certain order. Charaka knew the factors that determine the sex of a child. The scientific principles which govern the principles of inheritance, however, were unknown until the historic experiments of the Austrian monk Gregor Johann Mendel (1822–1884 A.D.)

Ancient treatises of uncertain dates

Hastyayurveda or Gajayurveda by sage Palakapya (who lived in Eastern India, *c* B.C. 600–500 B.C.); *Asvayurveda* by Gana; *Asvachikitsa* and *Asvasastra* by Nakula (deal with the treatment of diseases of elephants and horses).

The *Asvasastra* deals with the anatomy, life, characteristics and training of horses. These are based on the observations of Salihotra, the author of an earlier work of the same name, who is believed to be the founder of Veterinary Science in ancient India.

According to *Susruta sanghita** (200 A.D.) there were twelve kinds of life destroying and terrifying insects *(kitah*) such as “Tunginasa, Vichilika, Talaka,

Wahaka, Koshtagara, Krimikara, Mandala puchchka, Tunganabha, Sarshapika, Avalguli, Shambuka and Agnikita". " Their bite is as painful as that of serpents, and causes diseases resulting from the three humours joined together; the bite, which feels as if the incision has been with caustic or fire, is red, yellow, white and pink colour accompanied by fever, pain of limbs, hair standing on end, pain, vomitting, diarrohea, thirst, heat, giddiness, yawning, shivering, hiccup, burning sensation, intense cold, vesicles or pustules increasing, swellings, knots under the skin, circles, etc.." Susruta named five kinds of mosquitoes (mashakah) viz., Samudrah Marine or coastal mosquito; Parimandelah "Regional" or "circular" mosquito; Hastimasakah or Elephant mosquito, perhaps because of large size; Krishnah or Black or dark mosquito and Parwatiya or Hill mosquito.

* The author of the *samhita* (compendium or treatise) was Susruta the First, who lived in the Fifth Century B.C. His work was named "*Salyatantr"* and was mainly concerned with surgical matters. Another Susruta (anonymously it appears) living in the Second Century A.D. wrote another " *Susruta samhita"* revising and complementing the original work of Susruta the First and added much new material. Perhaps the second Su'sruta was the great Nagarjuna himself of the Second Century A.D. (Rao, 1981). The learned physician (Susruta) had suspected the bite of the mosquioto as being the cause of malaria (Wheeler, 1946).

We are told in Kautilya's *Arthasastra* (*c* B.C. 300-300 A.D.) that to check the spreading of plague a rat cess was imposed requiring citizens to kill at least a certain number of rats, failing which, they had to pay a rat race. Cats were let loose and people were warned not to catch them. This shows people were conscious about the role of some animal species in the transmission of diseases.

NATURAL HISTORY

Kalidasa, the immortal poet and playwright of Sanskrit literature, lived between *c* B.C. 200 and A.D. 415. He produced two plays *Abhijanana Sankutalam*, *Vikramorvasiyam* and Mahavikanimitra and four epics and short lyrical verses, *Raghuvamsa*, *Meghaduta: Purva Megha and Uttara Megha, Kumarsambhava and*

Rutusamhara. In these literatures are recorded observations on about 50 animals, seventy plants, 14 topics of general zoology, and 7 topics of general botany.and about a dozen topics of general biology. There is mention of about 18 species of birds.

In 34 verses, Kalidasa has used the word *hamsa* referring to the types: (i) hamsa, (ii) rajahamsa, (iii) kalahamsa, (iv) kadamba and hiranya hamsa. According to Gupta (1962), they refer to different types of the goose (Anser*)*. Kalidasa's observations about their aquatic habitats, gregarious habits, flight in definite formations tally with the accounts of our modern ornithologists on our winter visiting gooses.

In Kaslidasa's work, there is mention of two different types of pigeons, the *paravata and kapota.* It appears, domestication of the pigeon was practiced in those days, but colours and varieties as a result of such domestication were not too many. However, the exactness with which the semi-domestic variety of the Rock Pigeon has been described as ashy mottled with black and white bespeaks of the poet's acute observation. Paravata or the Wild Blue Rock Pigeon (*Columba livia* Gmelin) has been rightly described as being of a colour resembling smoke. 'Valabhi' or a silent undisturbed highest place in a building has been correctly described as the roosting place of the Wild Blue Rock Pigeon.

Kalidasa in his description of the peacock, correctly observes that the train of the male bird is large, gorgeous, provided with shining ocelli, and rises high and spreads like a fan during dancing. The birds have crest. Outer lateral corner of the eye is white, shines in the moonlight and the neck is blue. He records correctly that in the wild state, they inhabit dense forests abounding in mountains, rivers, streams and lakes. That they are diurnal, gregarious and roost in the evening and night on tree tops. That they are very sensitive to danger and approach of tigers, panthers and other wild cats. They are enemies of snakes especially cobras. During the rainy season, they are active, sex intoxicated and the males, dance, sing and enter into courtship. The song is sweet. The 'cry' of the peacock is divided into two folds and

appears to sound in or on the head or forehead. The renowned ornithologist Salim Ali (1979) records the sound of the peacock thus: "Call: a loud harsh, screaming *may-awe*, and short gasping shrieks *ka-aan*, *ka-aan* repeated rapidly 6 to 8 times with a pumping action of the head and neck." It appears from the accounts of Kalidasa on the peacock that during his time, in ancient India, the peacock was common, was domesticated and kept in private houses and high posts erected for the purpose, and valued for recreation and as a house pet.

MEDICAL SCIENCES

The period *c* 500 B.C.–A.D.500, witnessed the compilation of the works of ancient teachers who were the founder-writers of different aspects of Ayurveda. These aspects or eight parts of Ayurveda are: Kayachikitsa (therapeutics), Salya- tantra (major surgery), Salakyatantra (minor surgery), Bhutavidya (demonology:mental disorders), Kaumarabhrtya-tantra (paediatrics) and Agada-tantra (toxicology): Agada-tantra discusses methods of diagnosis and treatment of the bites of poisonous snakes, insects, and the like and of herbal or other poison cases; works on this branch of Ayurveda mentioned in the commentaries on Sushruta and Charaka include the *Kasyapa, Alambayana, Usana, Sanaka or Saunaka,* and *Latyayana-samhitas*; the originals of these are lost, Rasayanatantra (geriatrics) (preservation and increase of vigour, improvement of memory, prevention of diseases and the like) and Vajikarana-tantra (virilification) (increasing of virile powers). This indicates highly advanced knowledge of human anatomy and physiology. One has to assume these were gained through intimate knowledge of vertebrate (mammalian) anatomy and physiology and experiments conducted on them.

TAMIL SANGAM LITERATURE (c 400-800 A.D.)

The Tamil Sangam literature of South India abounds in references to a large number and variety of animals, birds, reptiles, fish and insects. The descriptive accounts of animal life reveal attempts at a serious study in natural history, habits, modes of life and ecological distribution of many animals and birds. Some notable recordings are

Several varieties of parrots are noted for imitative speech, message carriers and ornamental pets. The parrots are noted to be especially fond of the fruit of the nimba tree. Soaring kites and vultures have the power of sighting their prey from a great height, swooping down on them, picking them up in their sharp beaks, and then soaring back to the sky. Monkeys share food with their mates. Elephants of the desert region peel the bark of the tree called *yam*, squeeze out its water for the female to drink.

From the foregoing account, it is clear that ancient Indians had considerable knowledge of animal life.

Table 2 Estimated dates, period, ages and important events

Paleolithic time, Early Stone age	*c* 200,000 B.C.
Recorded history (Egypt)	*c* 5,000 B.C.
Earliest human settlement in India	*c* 5,000 B.C.
Mahabharata War	*c* 3102 B.C. (or 3000 B.C.)
Indus valley civilization	*c* 2500 B.C.–1500 B.C.
a) Mohenjo Daro (Dist. Larkana, Sind, Pakistan)	*c* 2500 B.C.–1500 B.C.
b) Harappa (Dist. Montogomery, Punjab,Pakistan) (14 year drought led to) Drying of river Saraswati and collapse of Indus Valley Civilization c B.C. 1500	*c* 2500 B.C.–1700 B.C.
European history	*c* B.C. 1000–1000 A.D.
Pre-Vedic Age	*c* B.C. 4000–1500 B.C.
Vedic Age (Late Pleistocene)	*c* B.C. 1500– 500 B.C
Post Vedic Age	*c*.B.C.500–600A.D.
Rig veda	*c* B.C. 1300–1200 B.C.
Vedas	*c*B.C. 2500–2000 B.C. to

	B.C. 750–500 B.C.
Ramayana-composed	*c* B.C. 3000–1000 B.C.
Mahabharata-composed	*c* B.C. 500–200 A.D.
Entry of Aryans into India	*c* B.C. 1500
Kurukhetra War	*c* B.C. 1429 (mid-February) or c B.C. 2156
Taittriya samhita	*c* B.C. 2446
Manusmruti	*c* B.C. 1200–900 B.C.
Epic Mahabharata (composed)	*c* B.C. 900
Upanishad	*c* B.C. 900–600 B.C
Susruta	*c* B.C. 800–600 B.C.
Gajashastra	*c* B.C.600–500 B.C.
Saraswati River dried up by	*c* B.C. 1700
Brahmana Literature	*c* B.C. 800
Taittiriya Brahmana	*c* B.C. 1700
Gautama Buddha	*c* B.C. 563–483 B.C.
Visit of Megasthenes (Greek) to India	*c* B.C. 400
Visit of Trautman to India	*c* B.C.300
Ashoka	*c* B.C. 300
Kautilya, (Chanakya Bishnugupta) (Arthasastra)	*c* B.C. 321–296 B.C.
Ashokan Pillar Edict	*c* B.C. 246
Kalidasa	*c* B.C. 200–A.D.415
Kharavela (Emperor of Kalinga)	*c* B.C. 182
Patanjali (Mahabhasya)	*c* B.C. 150

Umasvamin (Tattvartha-dhigama – sutra)	*c* A.D. 40
Caraka (reduction of Caraka samhita on the identification of Caraka with one having the same name who happened to be the court physician of king Kaniska) (74-101 A.D)	*c* A.D. 100
Manu samhita or Manu Smriti	*c* A.D. 200
Susruta (original text believed to have been redacted by Nagarjuna)	*c* A.D. 300–400 A.D
Vikramaditya and Kalidas	*c* A.D. 300–650 A.D
Tamil Sangam Literature (South India)	*c* A.D. 400–800 A.D.
Prasastapada (Padartha-dharma-sangraha)	*c* A.D. 500
Amarakosa	*c* A.D.500
Vishnusharma (Panchatantra)	*c* A.D. 500
Varahamihira (Brhat-samhita)	*c* A.D. 550
Brahmasphuta-siddhanta (compiled by Brahmagupta, born A.D. 598)	*c* A.D. 628
Visit of Yuan Chang (Chinese pilgrim) to India	*c* A.D. 630–645 A.D.
Brhat-samhita	*c* 6th Century A.D.
Bhavisya Purana	*c* 7th Century A.D.
Agni Purana	*c* 8th Century A.D.
Dallana	*c* 10th Century A.D.
Ancient India	*c* B.C. 4000– A.D. 600
Medieval India	*c* A.D. 600- A.D. 1700
Modern India	*c* A.D.1700–Present

Table 3 Agriculture down the ages in India

B.C. 40,000	Ancient man takes to agriculture cultivating wild shrubs and moving away from hunting and gathering.
B.C. 8,000	Farmers stay put at one place and grow certain plants as crops– creating agriculture and civilization in that order.
B.C. 6,000	Oranges cultivated in India.
B.C. 3,500	Cotton cultivation in India in a big way.
B.C. 3,000	Harappan civilization: builds dams, evidence of terrace farming. They produce wheat, barley, sesame and dates.
B.C. 2,300	Cultivation of paddy.
B.C. 2,000	Tea and bananas grown in India.
B.C. 1,800	Ragi (Finger millet, *Eleusine coranana*; Odia-Mandia) found in the Hallur archaeological site, on the Tungabhadra river.
B.C. 460	Brinjal (Egg plant) grown and harvested in India.

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ANURAN FAUNA OF RIAT LABAN RESERVE FOREST, MEGHALAYA WITH A NOTE ON THE BREEDING HABITAT OF SOME SPECIES.

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ABSTRACT:

The state of Meghalaya is represented by a total of 49 species of amphibians. The present study carried out from Riat Laban Reserve Forest, Shillong, Meghalaya, India, reveals the presence of 16 species of anurans belonging to 5 families and 10 genera. The family Ranidae has the highest number of species with a total of 6 species. The frog *Pterorana khare* is a new record for the state of Meghalaya and has been reported from this Reserve Forest. Riat Laban Reserve Forest covers a total area of 204.66 hectares and the vegetation cover of this forest comprises of sub-tropical pine and broad leaf vegetation. This reserve forest has diverse habitat types and therefore serves as perfect breeding grounds for many anurans.

Key words: Anura, Reserve Forest, habitat.

INTRODUCTION

Meghalaya is one of the eight sister states of the North East India which is recognized as part of the Himalaya and Indo-Burma global biodiversity hotspots. The state comprises of an area of 22,549 sq. Km and lies between 23º 47’ and 26º 10’N latitude and 89º 0’ and 92º 47’ E longitude. Shillong, the capital city of Meghalaya lies on 91°53'E and 25°34'N at an altitude of 1450 m above the sea level with the highest point being Shillong Peak at 6,449 feet (1,966 m) (Fig.1). The average maximum and minimum temperature of Shillong is around 17˚C and 7.5˚C respectively. Shillong experiences a prolonged rainy seasons with intermittent rain for almost throughout the year. The average annual rainfall in Shillong is about 2100 mm and is spread over eight months from March to October where two thirds of the rainfall occurs from June to September from southwest monsoons. The forests

of Meghalaya can be broadly grouped into tropical, subtropical and temperate types. The total recorded forest area in the State is 9,506 Sq. Km. which includes Reserved Forests, Protected Forests, National Park and Unclassed Forests. There are 24 reserve forests and 5 protected forests in the state which are controlled and managed by the government (Tiwari et al., 1996).

Below the Shillong Peak is a wide range of forest area called as the Riat Laban Reserve Forest (25º35'N; 91º55'E; elevation: 1588m asl) which covers a total area of 204.66 hectares. Owned and controlled by the State Forest Department of Meghalaya, this forest is located in southern part of Shillong city. The vegetation cover of this forest comprises of sub-tropical pine and broad leaf vegetation. The pine forest vegetation comprises of the Khasi Pine (*Pinus khasiana*). Among broad leafed trees, a few flowering trees such as *Rhododendron formosum*, *Rhododendron arborea* and *Pyrus pashia* are also found. Various types of habitat are found within this Reserve Forest, some of which include fast flowing streams, cascade waterfalls, ponds, water tanks, temporary rainfed pools, etc., all of which serve as potential breeding habitats for the anurans.

Earlier accounts of Anurans from Meghalaya in general and the Khasi Hills in particular, have been reported and described by Boulenger (1890,1920), Roonwal and Kripalani (1961), Yazdani and Chanda (1971), Pillai and Yazdani (1973), Pillai and Chanda (1979), Roy et al. (1995), Hooroo et al. (2002), Khongwir (2003), Iangrai (2004), Rangad et al. (2007), Das et al., (2010), etc. However, if we consider the reports on amphibian carried out earlier, it presents the description of anurans from different areas of Khasi Hills and Garo Hills. Recently, Sen (2004) reported that the state of Meghalaya is represented by a total of 49 species of amphibians. It is therefore evident that there is practically no comprehensive record and information about the composition of the amphibians from Reserve and Protected Forests of Meghalaya. The lack of information on the species composition of these Reserve and Protected Forests initiated the present study, which deals with the

composition of the Anuran fauna of Riat Laban Reserve Forest with a note on the breeding habitat of some selected species.

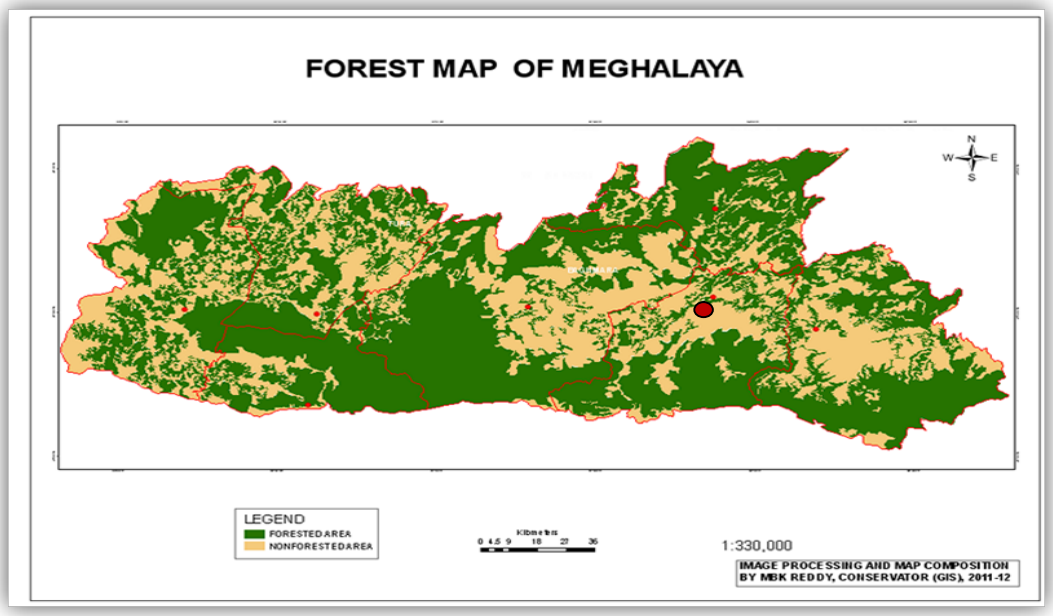

Fig. 1 Forest Map of Meghalaya showing Riat Laban Reserve Forest (

MATERIALS AND METHODS

To study the Anuran Fauna of Riat Laban Reserve Forest, Shillong, field survey work was carried out throughout the year. The survey was carried out from the year 2006 till 2011. Survey of the anurans was carried out employing visual encounter survey, sampling of breeding sites, egg mass survey as well as sampling by acoustic sounds produced by the anurans. Day and night opportunistic surveys were carried out and night time surveys were conducted using torches as well as head lamps. The equipments that were used for collection include hand nets, fish nets and specimen bottles or plastic bags for keeping the collected specimens for the purpose of identification and photography. The anurans encountered were identified in the field. Morphometric measurements were taken by the help of Mitutoyo dial calliper (to the nearest 0.1mm). The anurans after photography were released back to their natural environment. Voucher specimens were deposited at the Museum in the

Department of Zoology, North Eastern Hill University, Shillong and Zoological Survey of India, Shillong and Zoological Survey of India, Kolkata. Survey work was conducted in all types of habitats ranging from, forest streams, water tanks within the forest area, grasslands, temporary rainfed pools, cascades, etc. and these habitats served as perfect breeding grounds for many species of anurans.

RESULTS AND DISCUSSION

The survey work that has been carried out revealed that Riat Laban Reserve Forest harbours a rich composition of anurans which comprises of 16 species. These anuran species belonged to 5 families and 10 genera. The anuran families recorded include, Bufonidae, Dicroglossidae, Megophryidae, Ranidae and Rhacophoridae. These anurans were recorded from various habitat i.e. forest streams, grassland, cascades, rainfed pools, ponds, water tanks etc. located in Riat Laban Reserve Forest, Meghalaya. With our present survey and documentation, the number of species composition in this region has definitely increased. Given below is an account of the anurans of Riat Laban Reserve Forest with the breeding habitats of some of the recorded species. (Table 1)

Table1 List of anurans recorded from Riat Laban Reserve Forest, Shillong, Meghalaya, India

FAMILY	SPECIES	COMMON NAME	BREEDING PERIOD	BREEDING HABITAT	GAA STATUS
Bufonidae	*Duttaphrynus melanostictus*	Common Indian Toad	Throughout the year except winters.	Breeds in any kind of water bodies.	LC
Dicroglossidae	*Fejervarya nepalensis*	Nepal Cricket Frog	Breeds during Monsoon	Grassland pools, marshes, rainfed pools and temporary water bodies.	LC
	Fejervarya pierrie	Pierre's Cricket Frog	Breeds during Monsoon	Grassland pools, rainfed pools and temporary water bodies.	LC

					Cont.
	Fejervarya syhadrensis	Syhadra Cricket Frog	Breeds during Monsoon	Grassland pools, rainfed pools and temporary water bodies	LC
	Fejervarya teraiensis	Terai Cricket Frog	Breeds during Monsoon	Grassland pools, rainfed pools and temporary water bodies	LC
Megophryidae	*Xenophrys sp.*	-	Monsoon breeder (Mid March – August)	Leaf litters and mossy rocks on banks of forest streams.	LC
Ranidae	*Pterorana khare*	Khare's stream frog	Post monsoon breeder (October – November)	Not Known	NE
	Amolops sp.	-	Not known	Not known	NE
	Hylarana nicobariensis	Nicobarese Frog	Winter breeder (October to February)	Breeds in slow moving streams and lays eggs attached to rocks and substratum.	NE
	Sylvirana danieli	Daniel's Frog	Monsoon breeder (April to July)	Not known	NE
	Odorrana mawphlangensis	Mawphlang Odorous Frog	Monsoon breeder (May – August)	Breeds inside the crevices of the boulders and stones in the forest streams where the water current is low.	DD
	Odorrana livida	Large Odorous Frog	Monsoon breeder (mid-June– August)	Breeds inside the crevices of the boulders and stones in the forest streams where the water current is low.	NE
Rhacophoridae	*Polypedates teraiensis*	Common Tree Frog	March to early September once the monsoon sets in.	Breeds in rainfed pools, temporary water bodies and also ponds.	LC
	Rhacophorus bipunctatus	Twin Spotted Tree Frog	During Monsoon. April– August	Lay eggs in small foam nest near or above small pools, ponds or even on tree holes.	LC
	Philautus shillongensis	Shillong bush frog	April - August	Lays its eggs on leaves of trees, shrubs, bushes and bamboos.	LC
	Philautus andersoni	Anderson's bush frog	Monsoon period	Lays its eggs on leaves of trees, shrubs, bushes and bamboos	LC

Bufonidae

1. ***Duttaphrynus melanostictus*** (Schneider, 1799)

   Common name: Common Asian Toad.

   SVL: 96 mm–165 mm

Duttaphrynus melanostictus is the most common species among the Indian toads and is found in almost all biotopes and even at altitudes of about 3000 m. It is widely distributed in Meghalaya and is commonly found in the forest floor of Riat Laban Reserve Forest, Shillong. It is nocturnal and is found in and around human habitation as well. It breeds throughout the year except during winters. Its breeding habitat includes all kinds of stagnant water bodies and pools (Plates 1a and 1b).

Dicroglossidae

Genus: ***Fejervarya***

Four species of *Fejervarya* namely *Fejervarya nepalensis* (SVL: 21.4 mm–31.2 mm), *Fejervarya pierrie* (SVL: 23.1 mm–29.4 mm), *Fejervarya syhadrensis* (SVL: 27.1 mm–37.2 mm) and *Fejervarya teraiensis* (SVL: 35.7 mm–52.3 mm) are found to be present in Riat Laban Reserve Forest. All these four species of *Fejervarya* share a common type of habitat, i.e., marshes, grassland pools, rainfed pools, temporary water bodies and ponds. They breed in these types of habitats, and breeding period is throughout the Monsoon season (Plates 2a, 2b, 2c, 2d, 2e and 2f).

Megophryidae

1. ***Xenophrys*** sp.

   SVL: 52 mm

This species of *Xenophrys* was collected from a forest stream in Riat Laban Reserve Forest. It is found in forest areas covered with thick vegetation mostly dominated by ferns. Males of this species are found calling from twigs and branches of vegetation about 1 m–1.5 m above the ground and also from banks of streams that are covered with leaf litter and stones. Sometimes adult males are also found perching on mossy rocks. The breeding period of this *Xenophrys sp.* is Monsoon (Mid March–August).

They lay their eggs under leaf litter and pebbles, both adjacent to the stream and also amongst damp vegetation (Plate 3).

Ranidae

1. ***Pterorana khare*** Kiyasetuo and Khare, 1986

   Common name: Khare's stream frog

   SVL: 41.41 mm–53.81 mm

Pterorana khare (Plate 5) was first described and reported by Kiyasetuo and Khare (1986) from Nagaland. This species was later reported by Dutta (1997) from Manipur and in 2003, Dey and Ramanujam reported the occurrence of this species from the banks of the river Tlawng, 21 Km from Aizawl in north-west of Mizoram. *Pterorana khare* was also recorded as a new record for the state from Riat Laban Reserve Forest by Rangad et al., (2007). This frog is found to inhabit tropical forest streams, sometimes found hiding underneath boulders of rocks during day time and is also found in swift waterfalls among rocks (Plate 4). Not much is known about the breeding biology of this species but the breeding period is from October–November (post monsoon breeder).

2. ***Hylarana nicobariensis*** (Stoliczka, 1870)

   Common name: Nicobarese Frog

   SVL: 34 mm – 55.5 mm

Sylvirana nicobariensis, commonly known as Nicobar Island frog or Nicobarese frog is a small slender frog which is grayish to reddish brown in color. *Sylvirana nicobariensis* has been reported from Nicobar Island, Assam and Tripura (Dutta, 1997). This is a nocturnal stream frog which is found in tropical and subtropical forest streams. This species is a stream breeder that breeds during the winter months (October – February) when the level of water is low in the rivers and streams. It lays its eggs attached to the substratum or rocks in the stream (Plates 6a and 6b).

3. ***Amolops*** sp.

   SVL: 60.43 mm - 90.80 mm

This is a cascade frog which is found in lotic water bodies, fast flowing streams and cascades. During the day, this species can be found sheltering inside the huge boulders of rocks in the fast flowing streams. They come out of their hiding places at night and with the help of their large discs, they stick to the wet rocky walls and mossy boulders along the streams. Tadpoles have suckers on their mouth which help them to adhere to the wet surfaces of rocks and can therefore withstand the strong water currents. Nothing much is known about the breeding period of this species of *Amolops* but it is believed to breed during the monsoon season in the fast flowing streams of the forest (Plates 7a, 7b and 7c).

4. ***Sylvirana danieli*** Pillai & Chanda, 1977

   Common name: Daniel's Frog

   SVL: 60.34 mm

This species was originally recorded from Mawphlang, Khasi Hills, Meghalaya by Pillai and Chanda, 1977. During the current survey work, this species of Ranid was also recorded from Riat Laban Reserve Forest, Shillong. *Sylvirana danieli* is a semi-aquatic and nocturnal stream frog that is found to inhabit the edges of small hill streams covered with dense vegetation. They are found hiding under stone boulders and pebbles along the stream during the day time. In addition they are also found in leaf litters and leaf fall in the forest (Plates 8a and 8b). The breeding of this species is during the monsoon (April–July) but not much is known about the breeding biology of this species.

5. ***Odorrana mawphlangensis*** (Pillai and Chanda, 1977)

   Common name: Mawphlang Odorous Frog

   SVL: 72.8 mm–95.6 mm

Originally described from Mawphlang by Pillai and Chanda, 1977, this Odorous frog inhabits sub-tropical moist forests, fast flowing forest streams and cascades. Often they are found perching on the mossy rocks beside the streams, and, sometimes they are also found near the banks of the fast flowing streams of the

forest. The breeding habitat of this species is inside the crevices of the boulders and stones in the forest streams where the water current is low. This species is a monsoon breeder, and breeding period is from May–August (Plates 9 and 4).

6. ***Odorrana livida*** (Blyth, 1856)

   Common name: Large Odorous Frog

   SVL: 47.5 mm–110 mm

Originally described from Burma (Blyth, 1855), this species was also recorded from Meghalaya by Pillai and Chanda, 1967. This is a cascade Ranid found in swift, rocky forest streams of sub tropical moist forests. It is found mostly in forest areas having an altitude range of 1500 – 1800 m asl. They are often observed on stream banks with rocks and gravel and sometimes found sticking to the wet walls of the rocks. *Odorrana livida* secrete substances from glandular back emitting an odour when disturbed or handled. It breeds during the monsoon, and breeding starts from mid-June–August. The Breeding habitat is similar to that of *Odorrana mawphlangensis*. (Plates 10 and 4).

Rhacophoridae

1. ***Polypedates teraiensis*** (Gravenhorst), 1829

   Common Name - Common tree frog

   SVL: 42 mm–78 mm

Polypedates teraiensis is an arboreal nocturnal tree frog that is commonly found to inhabit tropical and subtropical forests and urban landscapes (40–1800m asl.) It prefers moist places, temporary water bodies and pools, and sometimes it is also found in human habitations. This tree frog starts breeding from the month of March to early September once the monsoon sets in. Breeding habitat of *Polypedates teraiensis* includes temporary water bodies, rainfed pools, water tanks and ponds (Plates 11a and 11b).

2. ***Rhacophorus bipunctatus*** Ahl, 1927

Common name: Twin spotted tree frog

SVL: 55 mm - 65 mm

This arboreal nocturnal tree frog is widely distributed throughout Meghalaya and Northeast India. It is very common in Cherrapunjee. *Rhacophorus bipunctatus* is usually found hiding underneath the leaves of trees and also tree holes during the day time. During the night, they come out of their hiding places to the vicinity of the water body for breeding. Breeding period is from April to August. Adult females lay eggs in small foam nest near or above small pools, cemented tanks, and temporary rainfed pools in overhanging vegetation, under stones or even on tree holes (Plate 12 a and 12 b).

3. ***Philautus shillongensis*** Pillai and Chanda, 1973

Common Name: Shillong Bush Frog

SVL: 14.64 mm - 21.40 mm

Philautus shillongensis was first reported by Pillai and Chanda (1971) from Malki forest, Shillong. This species is endemic to North East India and is commonly found in and around Shillong. It inhabits mostly thick bushes and bamboos and often hides underneath leaf covers. Males often call from twigs and branches of shrubs, bushes and bamboos. The breeding period of *Philautus shillongensis* is during the monsoon season i.e. from April to August. *Philautus shillongensis* displays direct development without any tadpole stage. It lays its eggs on leaves of trees, shrubs, bushes and bamboos requiring little or no water for its development (Plate 13a and 13 b).

1. ***Philautus andersoni*** (Ahl 1927)

Common name: Anderson's bush frog.

SVL: 25 mm – 30 mm

Philautus andersoni is an arboreal and nocturnal bush frog which produces high pitched calls from branches of trees and vegetation. They also exhibit a unique mode of development which is direct without any larval stages. It inhabits

subtropical broadleaf forest vegetation, thickets, bushes, shrubs and other vegetation cover on forest edges. The breeding period of *Philautus andersoni* is not known but probably it breeds during the monsoon period (Plate 14).

Riat Laban Reserve Forest is a forest area which is under the control of the State Forest Department. In this forest human settlement and felling of trees or cutting of branches are strictly prohibited. Therefore this pristine undisturbed forest harbors a rich variety of anuran fauna with a record of 16 species out of the total 49 known species (Sen, 2004) from Meghalaya. This forest represents about 33 % of the total amphibian species of Meghalaya. It can also be stated here that the frog *Pterorana khare,* which is a new record for the state has been reported from this Reserve Forest and, therefore, this adds up to the increase in the number of amphibians from Meghalaya. The different types of habitat found in Riat Laban Reserve Forest, Shillong provides a congenial breeding ground for these 16 species of anurans. Riat Laban Reserve Forest which is adjacent to Shillong city where urbanization, human settlement and development are on the rise has a high diversity of habitats and this is responsible for the anuran diversity in this part of the Shillong city. However, habitat destruction due to the expansion of Shillong city has deprived various amphibians of their feeding, breeding and hiding places. The thick canopy vegetation in Riat Laban Reserve Forest as well as the climatic conditions prevailing in Shillong where rainfall is seen to spread from March to October attributes to the occurrence of the anurans in this Reserve Forest.

From this study it was found that the Common Indian Toad, *Duttaphrynus melanostictus* breeds throughout the year except during the winters and it breeds in almost all types of terrestrial habitats. This toad shows a great adaptability to urbanization and other changes in their natural habitat. The four different species of Dicroglossid i.e. *Fejervarya nepalensis, Fejervarya pierrie, Fejervarya syhadrensis and Fejervarya teraiensis* share a similar kind of breeding habitats such as temporary pools, grasslands, marshes, rainfed pools and ponds and their breeding period is during the monsoon season. They are also found to be present in marshes

and small temporary pools in the city as well. This kind of adaptability is not present among the species, which are now rare or absent. There is one species of *Xenophrys* (identification still being worked out) which is found in thick fern bushes and canopy vegetation in the forest and it prefers to breed in moist leaf litters, mossy rocks and damp vegetation close to the streams of the Reserve Forest.

The family Ranidae which has the highest representation of anuran species from this Reserve Forest has a total of 6 species. Out of these, one species is a winter breeder (*Hylarana nicobariensis*), four species are monsoon breeders (*Amolops sp., Sylvirana danieli, Odorrana mawphlangensis and Odorrana livida*) and one species is a post monsoon breeder (*Pterorana khare*). This unique ecosystem has thick vegetation cover as well as perennial streams which have water in them throughout the year. Therefore this allows the frog like *Hylarana nicobariensis* belonging to the family Ranidae to breed during the winter months when the water level is low in the streams of this Reserve Forest. However, the breeding habitat of *Amolops sp., Odorrana mawphlangensis and Odorrana livida* is found to be in the fast flowing streams and cascades of this forest while for *Sylvirana danieli* the breeding habitat is under the rocks found along the streams during the monsoon period. Members of the family Ranidae show high susceptibility to habitat destruction, urbanisation and pollution. Thus the Riat Laban Reserve Forest serves as a perfect home for such species of frogs whose populations are seen to be affected by changes in habitat due to human activities.

The Family Rhacophoridae includes frogs that are more or less arboreal, hence presence of vegetation is essential for them. Among the family Rhacophoridae, *Polypedates teraiensis* and *Rhacophorus bipunctatus* share a similar type of breeding habitat i.e. temporary water bodies, rainfed pools, water tanks and ponds, and in addition *Rhacophorus bipunctatus* is also found to lay its eggs in tree holes where water as well as overhanging vegetation near the water body are available. These two species of Rhacophorids are commonly found in Riat Laban Reserve Forest. *Polypedates teraiensis* starts breeding during the pre-monsoon period i.e.

March and this lasts till about early September. *Rhacophorus bipunctatus* on the other hand breeds from the Month of April – August during the monsoon. Two species of *Philautus* namely *Philautus shillongensis* and *Philautus andersoni* have been recorded from Riat Laban Reserve Forest and they are found in bushes, shrubs and thick canopy vegetation in this forest. They exhibit direct development and they breed during the monsoon from April – August. These frogs however do not require any water for their breeding and therefore they lay their eggs in leaves of vegetation. It appears that these Rhacophorids are very sensitive to changes occurring due to urbanisation and changes in habitat.

The list of amphibians from Meghalaya has definitely increased from the total recorded species list by Sen (2004). This increase in the species composition can be attributed to the presence of Reserve and Protected Forest in the state of Meghalaya which are taken care of by the State Forest Department, Government of Meghalaya. Such forests where human activities like deforestation and human settlement are strictly prohibited provide a perfect home for the occurrence and abundance of many species of anurans. Preservation of such forest is very important as they increase the biodiversity of the state and the region as a whole.

ACKNOWLEDGEMENTS

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Plate 1a *Duttaphrynus melanostictus* at the breeding site;

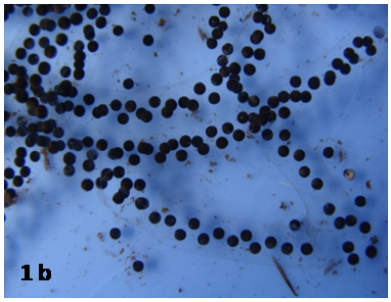

Plate 1b Eggs of *Duttaphrynus melanostictus.*

Plate 2a *Fejervarya nepalensis*

Plate 2b *Fejervarya pierrei*

Plate 2c *Fejervarya syhadrensis*

Plate 2d Amplecting pairs of *Fejervarya teraiensis*

Plate 2e and 2f Breeding habitat (temporary rainfed pools) of the four different species of *Fejervarya.*

Plate 3 *Xenophrys* sp. with eggs;

Plate 4 Typical breeding habitat of *Pterorana khare*, *Odorrana mawphlangensis* and *Odorrana livida.*

Plate 5 *Pterorana khare* .

Plate 6a *Hylarana nicobariensis*

Plate 6b Breeding habitat of *ylarana nicobariensis.*

Plate 7a *Amolops sp.*

Plate 7b: Breeding habitat of *Amolops sp*.

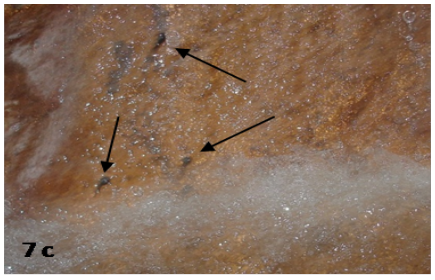

Plate 7c: Tadpoles of *Amolops sp* with suckers

Plate 8a *Sylvirana danieli.*

Plate 8b Breeding habitat of *Sylvirana danieli.*

Plate 9*Odorrana mawphlangensis.*

Plate 10 *Odorrana livida.*

Plate 11a *Polypedates teraiensis.*

Plate 11b Breeding habitat of *Polypedates teraiensis.*

Plate 12a Amplecting pair of *Rhacophorus bipunctatus.*

Plate 12b Foam nest of *Rhacophorus bipunctatus* in overhanging vegetation.

Plate 13 a*: Philautus shillongensis*

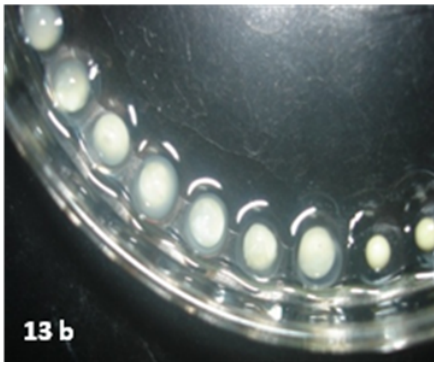

Plate 13 b: Eggs (magnified) of *Philautus shillongensis*

Plate 13 c Breeding habitat of *Philautus shillongensis*.

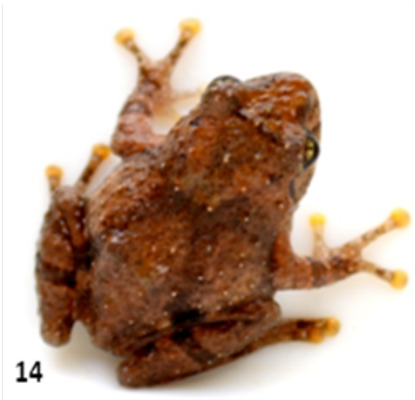

Plate 14 *Philautus andersoni.*

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COMPARATIVE KARYOMORPHOMETRICAL ANALYSIS OF TWO COMMERCIAL GIANT PRAWNS OF ODISHA

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ABSTRACT

The present study focuses on the karyological analyses of two commercially important prawns namely, *Penaeus monodon* (Fabricius, 1798) and *Macrobrachium rosenbergii* (de Man, 1879). The number and characteristics of chromosomes of these two giant prawns were studied from spermatogonial metaphase of testes tissues. A total of 50 mitotic metaphases were examined in each case. The diploid chromosome ranged from 84 to 88 with a mode at 88(2n=88), representing 60% of the metaphases in *Penaeus monodon*. For *Macrobrachium rosenbergii* the diploid chromosome ranged from 114 to 118 with a mode at 118(2n=118) representing 70% of the metaphases observed. Here sex chromosomes were not detected.

Key words: prawn, karyomorphometry, *Penaeus monodon*, *Macrobrachium rosenbergii.*

INTRODUCTION

Two commercially available giant prawns of Odisha are *Penaeus monodon* (Fabricius, 1798), the largest Indian marine prawn (Fig.1a) and *Macrobrachium rosenbergii* (de Man, 1879), the giant freshwater prawn (Fig. 1b). Prawn is one of the key aquaculture invertebrate species. According to nutritionists, prawns are an excellent source of protein (Athiyaman and Rajendran, 2012) and a good source of omega 3 fatty acids. They have high levels of vitamin B_{12}; zinc, iodine, phosphorus, potassium, selenium, iron, calcium, magnesium and sodium. They are also low in saturated fats. Omega 3 fatty acids reduce the risk of wide range of health problems including asthma, pulmonary disease, rheumatoid arthritis, multiple sclerosis, psoriasis and inflammatory bowel disease (Annonymous, 2010).

Cytogenetic study has immense importance in characterising, classifying and drawing evolutionary relationship among the species (Biswal et al., 2010).

Chromosomal studies in fishes become a priority area of research in recent years (Barat et al., 2002; Sahoo et al., 2007). In general, cytogenetic studies of crustaceans are relatively few and very difficult to perform because their chromosome numbers are large (Niiyama, 1962; Mittal and Dhall, 1971; Vishnoi, 1972; Nayak and Ahmed, 1986; Campos-Ramos, 1997; Dumas and Campos-Ramos, 1999; Zhang et al., 2003; Lee et al., 2004) and they are of small dot and rod shaped.

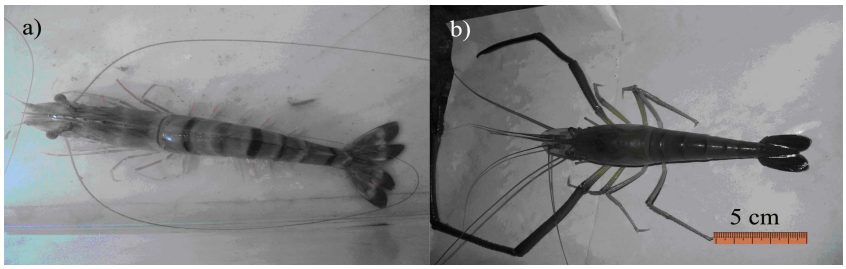

Fig.1 (a) *Penaeus monodon* and (b) *Macrobrachium rosenbergii*

Their shapes are very variable including metacentric, sub metacentric and acrocentric chromosomes (Salema and Heino, 1990; Damrongphol et al., 1991; Tan et al., 2004). Karyological studies were carried out in an attempt to differenciate the two species of mud crabs namely *Scylla serrata* and *Scylla tranquebarica* (Gopikrishna and Shashi Shekhar, 2003.) By understanding the basic genetic profile of these prawns, genes of commercial importance can be identified and product quality can be improved in terms of consumer preference and human nutrition such as formation of long chain omega 3 fatty acids. The objective of this study is to investigate the karyomorphometrical data of these two giant edible prawns as karyotype analysis is a key step towards the gene manipulation and related genetic engineering.

MATERIALS AND METHODS

Five adult males, each of *Penaeus monodon* and *Macrobrachium rosenbergii* were collected from Chilika lagoon (19º 48′ N, 85º 38′E) and Daya river (20º 13′N, 85º 52′E) of Odisha respectively for the present study. After capture, they were

transported from the collection sites in live condition in oxygen filled polythene bags and buckets to the laboratory and maintained in aquaria. Testes tissues were dissected out and pretreated with 1% KCl solution and then fixed in freshly prepared Carnoy's 1:3 acetoalcohol fixatives. The fixed material was kept in refrigerator for 2 days. Then the tissues were centrifuged for thrice at 3,000rpm for 10 minutes. The fixative was changed for thrice with an interval of 15 minutes. Slides containing chromosome spreads were prepared from the fixed tissues following Kligerman and Bloom (1977). The tissues were stained in 10% Giemsa (Lillie, 1977) in Sorenson's phosphate buffer (pH 7) for 25 minutes and differentiated in distilled water. The well spread metaphase plates were observed, analysed and photomicrographed at 100X magnification with an Olympus research microscope coupled with digital camera. The karyotyping was done according to Novitski (1977).

RESULTS

50 well spread metaphase chromosome plates were observed from 5 individuals of each species. The observation revealed a distinct peak at 88 in *Penaeus monodon* and 118 in *Macrobrachium rosenbergii* (Fig. 2). Out of 50 metaphasic plates of *Penaeus monodon*, 30 plates showed 88 chromosomes while six had 87, seven had 86, five had 85 and rest two had 84 chromosomes each (Table 1). Hence, the diploid chromosome number was determined to be 88 (2n=88). In *Macrobrachium rosenbergii*, out of 50 metaphasic plates, 35 showed 118 chromosomes while five had 117, four had 116, two had 115 and the rest four had 114 chromosomes each. Hence, the diploid chromosome number was determined to be 118 (2n=118). The chromosomes observed in both the species are of small rod like and also dot like. The position of centromere is indistinct which may be due to its diffusion.

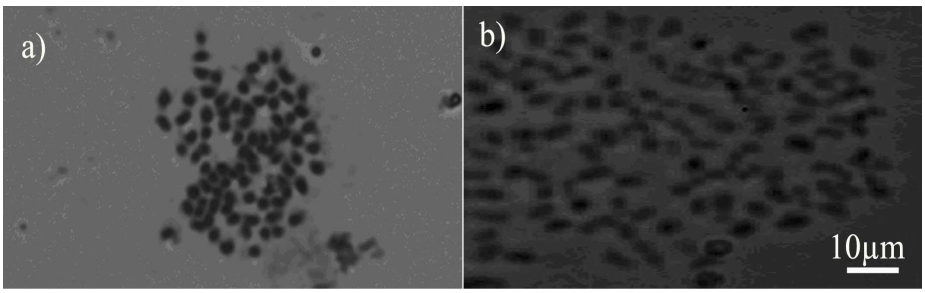

Fig.2 Metaphasic plate of (a) *Penaeus monodon* and (b) *Macrobrachium rosenbergii.*

DISCUSSION

The present comparative karyotypic analysis on *Penaeus monodon* and *Macrobrachium rosenbergii* report the chromosomes to be almost rod and dot (oval to round) shaped. To establish diploid chromosome number in case of large number of small chromosomes, the numbers observed in maximum metaphasic plates is to be considered (Ahmed, 1984). Accordingly, the diploid chromosome number in the marine giant tiger prawn, *Penaeus monodon* (Faricius) is 88 while that of giant freshwater prawn, *Macrobrachium rosenbergii* (de Man) is 118 (Fig. 3).

There was no evident difference in the degree of chromosome condensation using different concentrations of colchicine. However, longer incubation led to a greater chromosome condensation. This is a study where good metaphasic plates were observed without treatment with chemicals like colchicine. Since the present paper is restricted exclusively to aspects of chromosome morphology, the results of crossing experiments, which do suggest the existence of sex chromosomes in Penaeidae and Palaemonidae are not considered here.

The metaphase chromosome counts obtained from testes of *Penaeus monodon* and *Macrobrachium rosenbergii,* have diploid chromosome numbers 88 (n=44) and 118 (n=59) respectively with specific formula (Nayak and Ahmed, 1989) of their shape (Table 2 and Fig.3). The chromosomes of these two species of decapod crustaceans were studied from the male germ cells. Till now, there is no positive proof on the reduction of the chromosome number as a result of chromosome fusion. To attempt to formulate any rule for general application, it is necessary to accumulate cytological data on a very large number of species.

Table 1 Number of metaphase plates observed

Sl No	Species	No of cells observed	No of chromosomes obtained
1	*Macrobrachium rosenbergii*	35	118
		5	117
		4	116
		2	115
		4	114
2	*Penaeus monodon*	30	88
		6	87
		7	86
		5	85
		2	84

Table 2 Number and formula of chromosomes for each species

Sl No	Species	2n	N
1	*Penaeus monodon*	88 =8V+80R	44
2	*Macrobrachium rosenbergii*	118 = 8V+110R	59

V= Metacentric chromosome, R= Rod or dot shaped acrocentric chromosome.

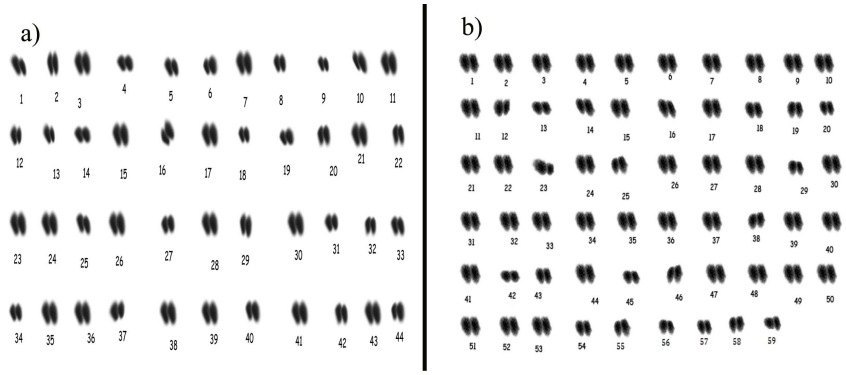

Fig.3 Karyotype of (a) *Penaeus monodon* and (b) *Macrobrachium rosenbergii*.

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AGE DETERMINATION BY SKELETOCHRONOLOGY IN THE INDIAN BULL FROG *HOPLOBATRACHUS TIGERINUS* DAUDIN, 1802 (ANURA: RANIDAE)

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ABSTRACT

Skeletochronology is a standard technique for the assessment of individual growth rates in amphibians and reptiles. Estimation of age, longevity, age at sexual maturity and breeding of the bull frog *Hoplobatrachus tigerinus* Daudin, 1802 by skeletochronology have been described in the present study. Frogs were collected from their natural habitat. They were anesthetized prior to the snout - vent length measurement and clipping of fourth toe of left hind limb. These procedures are standard operating procedures followed for amphibians. Bones were preserved in 70% alcohol, decalcified with 10% EDTA and processed for histology. Sections of 10 µm were stained with hematoxylin and eosin stain. Cross sections of bones exhibited growth rings, each consisting of a broader growth zone, and a chromophilic line of arrested growth (LAG). The first growth ring appears in the second year of life. The results suggest that this frog can attain an age of fifteen years in nature.

Key words: *Hoplobatrachus tigerinus,* Skeletochronology*,* line of arrested growth.

INTRODUCTION

Age determination and rate of growth are two important parameters in ecological, demographical and gerentological studies. Information concerning age is very important in understanding life history traits of animals and evaluating the status of populations (Alcobendas and Castanet, 2000).Several methods had been used to access the age of individual animal among vertebrates. The method of size frequency distribution in the population was commonly used for fish, amphibia and reptiles. However, this method was not reliable because of overlapping of body size in a

population (Smirina, 1994). Eye lens weight was also considered as a parameter for age estimation among the amphibians. The testis lobation was yet another method used for age estimation for both amphibians and reptiles (Misawa and Matsui, 1999). The two models are not ideal because animal sacrifice is essential. The morphological parameters like body weight and body length were also used for age estimation among all vertebrates. This method is not practicable because of overlapping of body size and weight of different age groups (Hamilton, 1934; Gelder and Van Oomen, 1970). The dental criteria i.e. eruption or wearing of teeth is also common method of age determination among mammals (Klevezal and Kleinenberg, 1967). Another popular method includes mark-release and recapture by tagging larger animals (Castanet, 1994; Smirina, 1994). But, this method is not effective in studying amphibians from metamorphosis till old age. The major drawbacks of this method are migration and high mortality of the young ones and difficulty to tag amphibians just after metamorphosis due to their small size. Moreover, it is rather difficult to organize repeated catching and this method can be used in long term studies. In order to estimate age of an individual a more reliable method of age determination among vertebrates was developed which is based on studying the pattern of growth layers in the osseous tissues and counting the lines of arrested growths (LAGs) laid down in the midshaft diaphysis of bones. This method is known as skeletochronology (Hemelaar and Van Geldar, 1980; Castanet, 1994; Smirina, 1994). This is fundamentally different from other techniques as it uses biological marks recorded in the hard tissues and provides the authenticity of results.

Amphibians reflect distinct annual rings consisting of broader growth zones of growth rings (GR), corresponding to fast growing period in warmer months in the year and lines of arrested growth (LAGs), corresponding to arrested bone growth in colder months in both the long bones and phalanges (Hemelaar, 1981,1988; Castenet and Smirina,1990; Smirina, 1994). By studing the number and pattern of annual layer deposition, the rate of growth, age at sexual maturity and longevity of individuals in a population can be determined. There are various reports on

temperate and tropical anurans describing the formation of the growth marks to be annual i.e. *Rana bedriages* (Erismis et al., 2002); *Rana latastei* (Guarino et al., 2003); *Polypedates maculatus* (Kumbar and Pancharatna, 2001a; Mahapatra et al., 2008); B*ufo melanostictus* (Kumbar and Pancharatna, 2001a, 2004; Nayak et al., 2007) and *Euphlyctis hexadactylus* (Nayak et al., 2008). Age estimation of *Hoplobatrachus tigerinus* inhabiting southern India have been studied by Pancharatna and Kumbar (2005). The aim of the present study was to estimate age in the Indian bull frog *Hoplobatrachus tigerinus* inhabiting eastern India.

MATERIALS AND METHODS

Eight dead specimens of the Indian bull frog *Hoplobatrachus tigerinus* were collected from Bhubaneswar (28º18´N, 85º50E´), Odisha, India. For each specimen, total snout-vent length (SVL) to the nearest mm was recorded using digital calipers to the nearest 0.01mm. The limb bones were investigated for growth rings.

The bones were washed in water and decalcified in 10% EDTA solution for 24 to 48 hours depending on the size of the bones. After decalcification, the bones were washed in running tap water to remove excess of EDTA. Following washing the bones were dehydrated through a series of upgrade alcohol and processed for paraffin block preparation. The paraffin blocks prepared were sectioned (8-10µm) with the help of Weswox rotary microtome. The sections were then stained in Delafield's haematoxylin and eosin and examined under a compound microscope. Growth rings and lines of arrested growth were clearly visible in the cross sections. The LAGs and GR were counted starting from the bone marrow cavity to the outer margin of the bone. Photographs were obtained from appropriate sections using Canon EOS 450 12.2 Mega pixel camera (EF-S 18-55 1S Kit) connected to Hund H500 WETZLAR microscope.

The relationship between age and body size (SVL) was assessed by drawing scatter plots. The correlation coefficient 'r' was calculated by Karl Pearson's method (Steel and Torrie, 1980).

RESULTS

The histology of femur revealed a central marrow cavity (MC) and an outer cortical layer in the cross section. The cortical layer was lined by an endosteal layer (E) in the inner side and a periosteal layer (P) towards the outer side. Distinct matris (M) was present between these two layers. Above the endosteal layer in the matrix thin and darkly stained chromophillic lines known as lines of arrested growth (LAGs) were observed. The LAGs appeared as concentric rings. The intermediate zone between two LAGs was lighter in colour and referred to as annual growth ring. In the cortical matrix osteocytes were evenly distributed. But the LAGs were devoid of the osteocytes. Since, a single LAG appears in one year, age of frogs was estimated by counting the number of LAGs.

Skeletochronological analysis of eight specimens was done in the present study (Fig. 1, Table 1). All the specimens were matured adult. Three specimens were female showing 1, 7 and 14 LAGs (Figs. 1 A, F, H). Five specimens were males showing 2,3,5,6 and 8 LAGs (Figs.1B, C, D, E, G).

Table 1 Body size (SVL) and number of LAGs in males and females of *Hoplobatrachus tigerinus*

Frog No	SVL (cm)	No of LAGs	Age (years of life)
1	5.9	2	3rd
2	6.5	3	4th
3	6.9	5	6th
4	10.2	8	9th
5	14.3	6	7th
Female			
1	6.5	1	2nd
2	10.3	7	8th
3	15.1	14	15th

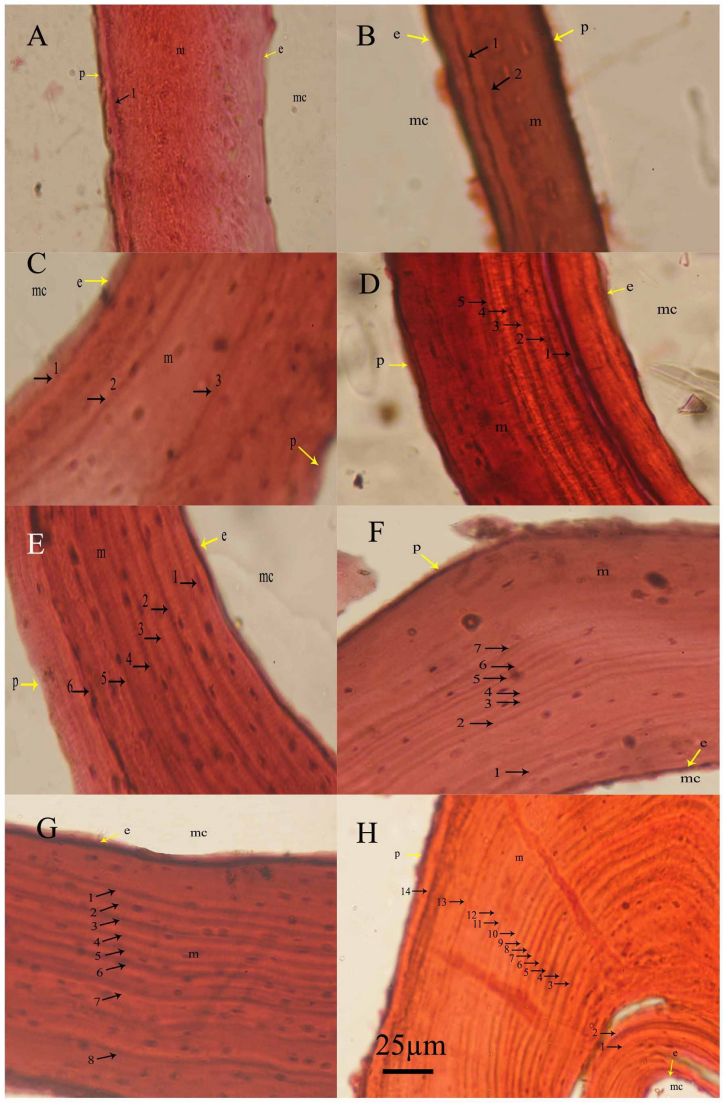

Fig.1 Cross section of femur of adult *Hoplobatrachus tigerinus* showing growth rings. MC: central marrow cavity; C: cortex; E: inner endosteal layer; P:outer periosteal layer; 1-14: No of Lines of arrested growth(LAGs).

There exist a positive correlation between SVL and LAGs for both males and females (Figs 2 and 3). The correlation coefficient 'r' was 0.683 for males and 0.999 for females.

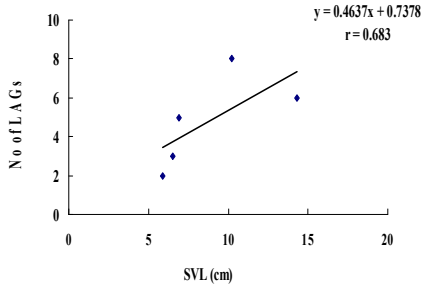

Fig. 2 Correlation between SVL (cm) and No of LAGs in male *Hoplobatrachus tigerinus.*

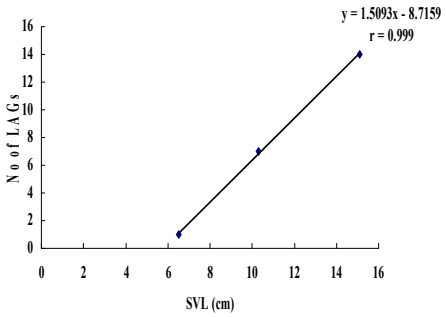

Fig. 3 Correlation between SVL (cm) and No of LAGs in female *Hoplobatrachus tigerinus.*

DISCUSSION

The present study based on skeletochronology has proved to be one of the best methods for estimation of age in *Hoplobatrachus tigerinus*. In anurans it has been reported that the first LAG is formed in winter and periosteal growth begins anew each spring (Hemelaar, 1981; Kalb and Zug, 1990; Marnell, 1998). Since the hatching of tadpoles are accepted at age '0' (Marnell, 1998), the frogs without LAG

are considered to be in one year of growth. In the present study, males frogs showed 2-8 LAGs and female frogs showed 1-14 LAGs.

Longivity of *H. tigerinus* inhabiting South India has been reported to be 7 years (Kumbar and Pancharatna, 2005). Present study shows longevity of *H. tigerinus* to be 15 years which is 8 years more than the finding of Kumbar and Pancharatna (2005). There are also reports (Smirina, 1994) on the longevity of some temperate aquatic species i.e., *Rana ridibunda* (twelve years), *Rana esculenta* (twelve years) and *Rana temporaria* (fourteen years). Other temperate species reported by Smirina (1994) are *Bufo bufo* (twelve years), *Bufo americanus* (five years), *Bufo arenarum* (eight years) and *Bufo pentoni* (six years). Ashkavandi (2012) have reported maximum years of male *Rana ridibunda* to be 11 years in nature. Reports on longevity of tropical anurans such as *Rana cyanophlytectis* (Seven years: Kulkarni and Pancharatna, 1996; six years: Dutta et al., 2011)), *Limnonectes limnocharis* (four years: Pancharatna and Deshpande, 2003), *Microhyla ornata* (five years: Kumbar and Pancharatna, 2001b; four years: Dutta et al., 2011), *Chirixalus simus* (four years: Dutta et al., 2011), *Polypedates teraiensis* (five years: Dutta et al., 2011), *Fejervarya* sp. (four years: Dutta et al., 2011), *Polypedates maculatus* (seven years: Mahapatra et al., 2008), *Euphlyctis hexadactylus* (fourteen years: Nayak et al., 2008), and *Duttaphrynus melanostictus* (twelve years : Nayak et al., 2007).

Present study has shown that the females live longer than the male frogs. In the temperate species *Bufo bufo*, females living longer than the males have also been described (Smirina, 1983; Hemelaar, 1988).

The mature females were comparatively larger in size (SVL) than the amles (Table 1). Larger females have been observed in different anuran species, i.e., *Bufo pardalis* (Cherry and Francillon, 1992), *Bufo americanus* (Acker et al., 1986; Kalb and Zug, 1990; Howard et al., 1994), *Rana cyanophylyctis* and *Microhyla ornata* (Pacharatna and Deshpande, 2003).

There exist a positive correlation between SVL and LAGs for both males and females (Figs 2 and 3). The correlation coefficient 'r' was 0.683 for males and 0.999 for females. But there was overlapping of SVL and LAGs. A comparatively larger frog (SVL= 14.3 cm) showed less number of LAGs than a frog with SVL 10.2 cm (Table 1). Frogs of equal size also showed different number of LAGs (Male frog No 2 and Female frog No 1, Table 1). So, it is concluded that body size should not be considered as a parameter for age estimation in *H. tigerinus*.

The present investigation suggests that females are larger in size than the males, longevity of females is more than males, and maximum age is fifteen years in natural population, there exists a positive correlation between body size and LAGs for both male and female frogs.

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BIOCHEMICAL, MICROBIOLOGICAL AND NUTRITIONAL QUALITY CHARACTERIZATION OF ACID SILAGE PRODUCED FROM CARP WASTE WITH AND WITHOUT THE ADDITION OF ANTIOXIDANTS

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ABSTRACT

Fish silage is a liquid product made from whole fish or parts of fish that are liquefied by the action of natural enzymes in the fish, in the presence of an added acid. It can be used as a feed supplement for fish, livestock and poultry or also as a fertilizer. Acid silage was prepared from the visceral waste of carps with (Formic acid+Hydrochloric acid+Butyl Hydroxy Toluene (FHB)) and without (Formic acid +Hydrochloric acid (FH)) the addition of synthetic antioxidant Butyl Hydroxy Toluene (BHT). The Biochemical, microbiological and nutritional quality of the silages was compared. The addition of antioxidant did not significantly ($p>0.05$) alter the proximate composition of the silage (FHB). The addition of BHT significantly ($p<0.05$) reduced the rate of oxidation in FHB. The synthetic antioxidant was not found to significantly ($p>0.05$) reduce the production of volatile bases and also the microbial load.

Key words: Acid silage, Indian major carps, synthetic antioxidant.

INTRODUCTION

In India, the contribution of aquaculture to the total inland fish production has increased sharply from 46% in 1985-86 to 84% in 2002-03 (Katiha and Bhatta, 2002; Dehadrai, 2003). Of the total world carp production, 95 per cent of the total world carp production is from Asia and 12% is from India. Low-income people favor carps because of their low price and good taste. In many areas of Asia, carps are the major

source of animal protein for the poor. Carps dominate aquaculture production in freshwater ponds, cages, pens and recirculating systems and production in inland fisheries. The Indian major carps, *Catla catla*, *Labeo rohita* and *Cirrhinus mrigala* accounts for 25 to 27 per cent each of the total aquaculture production in the country (Dey et al., 2003). These species together constitute about 85 per cent of the total freshwater aquaculture production. Traditionally fish has played a dominant role in the lives of the people of Odisha. Along with West Bengal and Kerala, Odisha is considered to have the highest per capita fish consumption. Like west Bengal and unlike Kerala, the preference of Odia people is for the fresh water fish. Majority of the fish markets are dominated by the presence of Indian major carps. Carp muscle contributes 70% of its total body weight. The rest of the parts usually go as waste. These extremely perishable wastes are high in moisture and range from 30 to 40% crude protein (CP) on a dry matter (DM) basis. Excluding fish discards and skeletons, the largest part of this waste consists of viscera; however, visceral waste has great potential to be used as a protein supplement in animal feeds (Fai et al., 1997). The best alternative solution is to utilize the waste material for the production of by – products. In most cases, they are upgraded into fishmeal, but the silage process has been reported to be a feasible, simple and lower-cost alternative (Vidotti et al., 2003). During the last two decades, fish silage has been successfully used as a low cost ingredient in aquaculture diets (Espe et al., 1992).

Fish silage can be defined as a liquid-pasty product generated from fish (dead fish, unused species, marine fishing by-products, commercial fish waste and industrial residues or residual fractions from marine peptone processing) in an acidic medium (Raa and Gildberg, 1982). Formic acid (organic acid) is the best choice for the preparation of chemical silage, the silages made using formic acid are not excessively acidic and therefore do not require neutralization before being used (Oetterer, 2002). Since organic acids are costly, a combination of organic and inorganic acid can be used in the preparation of silage. Acid silages if prepared with only inorganic acids will have a very low pH (around 2) which requires nuetralisation before it can be used

in feed (Vizcarra-Magaña et al., 1999). Fish visceral waste is an excellent source of proteolytic enzymes and marine peptones for supporting bacteriocin production (Vázquez et al., 2005). The low pH created by the addition of the acid helps in the activation of these enzymes favouring the liquefaction of the waste. Potassium sorbate and butyl hydroxyl toluene are also added to prevent mould, yeast growth and oxidation development, respectively.

Lipid oxidation is an important parameter in evaluation of the quality of silages especially if made from visceral parts. In the present study, the effect of addition of antioxidant Butyl Hydroxy Toluene on the biochemical, microbiological and nutritional quality of acid silage prepared from the viscera of carps is investigated, analysed and interpreted.

MATERIALS AND METHODS

Preparation of acid silage

Visceral wastes of carps were collected from a local fish market in Bhubaneswar, Odisha. The visceral wastes were washed in potable water, chopped and ground using meat grinder into paste for silage preparation. The paste was divided into 2 lots. To one lot, 1.5% Formic acid+1.5% Hydrochloric acid+200ppm Butyl Hydroxy Toluene (BHT) +0.1% Potassium sorbate was added (Formic acid+Hydrochloric acid+BHT (FHB)). To the other lot, only 1.5% Formic acid+1.5% Hydrochloric acid+0.1% Potassium Sorbate was added (Formic acid+Hydrochloric acid (FH)), only 0.1% Potassium sorbate was added. Ensilation process was aided by incubating the materials in air tight plastic containers at room temperature of 28 ± 2^0 C. The silage was stirred twice daily to ensure the uniform distribution of acid. Once the pH had stabilized, samples of known volume were drawn from both the lots to determine the proximate composition. Samples of known volume were also drawn at 0, 15, 30 and 60 days to study the biochemical and microbiological quality.

Determination of proximate composition

Moisture, Crude protein, crude fat and ash content were estimated using the Association of Official Analytical Chemists (AOAC) (2000) procedure. In brief, the dry matter content was determined by drying the homogenate in an oven at 105 ^{0}C until a constant weight was obtained. The crude protein content was calculated by multiplying the total nitrogen concentration with 6.25. Crude fat was determined using the Soxhlet extraction system. Ash content was measured by dry ashing in Muffle furnace at 550 ^{0}C for 6 hours.

Biochemical and microbiological analysis for quality assessment

The pH of the silage in distilled water (1: 5 W/V) was determined by using a glass electrode digital pH meter (Cyberscan 510, Eutech instruments, Singapore). Total volatile base nitrogen (TVB-N) was estimated by the microdiffusion method (Conway, 1950). Oxidation stability of the sample was assessed by measuring Thiobarbituric acid (TBA) value (Tarladgis et al., 1960). Microbiological analysis of aerobic plate count was determined using standard culture medium. Twenty-five grams of silage was aseptically weighed and homogenized with sterile mortar and pestle with 225 ml sterile normal saline for one minute. The homogenized sample was serially diluted using 9 ml sterile normal saline. Further, serial dilutions were made and 0.5 ml of each dilution was pipetted onto the surface of the plate count agar (Himedia), in triplicates, after which they were incubated for 48hours at 37°C.

Statistical analysis

All the treatments were triplicated. The data were analyzed using parametric t-test to find the effect of addition of antioxidant on the proximate composition of acid silage prepared from visceral mass of carps. One way analysis of variance (ANOVA) was performed to find the effect of treatments on pH, Total Volatile Base Nitrogen values (TVBN), Thiobarbituric Acid values (TBA) and Total bacterial load. All the statistical analysis was carried out using SAS 9.2.

RESULTS AND DISCUSSION

Nutritional quality of acid silages

Proximate composition

Proximate composition of the carp viscera showed 24.14(±2.63) % dry matter, 37.7(±0.42)% protein, 40.60%(±0.32) crude fat and 4.25% (±0.44)ash and a pH of 6.67(±0.02). Vidotti et al. (2003) have reported that the acid silages prepared from freshwater fishes have a crude protein content of 44.3%. The addition of antioxidant does not significantly ($p>0.05$) alter the proximate composition of silage (Table 1). The silage had a balanced composition in minerals (ash), protein and lipid fractions which make it an interesting product in animal feeding. The results also show that during the ensiling process only minor variations were observed in the dry matter, the protein, lipid and minerals fractions. Vast variation can be found in the proximate composition of fresh water carp viscera as reported by different authors (Ahmed and Mahendrarkar, 1996: Bhaskar and Mahendrarkar, 2007). The difference in composition could be due to age, sex, body weight, season or feeding aspects (Sikorski and Kolakowski, 2000).

Table 1 Proximate composition of ensiled visceral waste of carps prepared with and without the addition of BHT

Parameters	**FH**	**FHB**	**t value**	**P**
Dry matter (%)	25.53±0.33[a]	25.39±0.29[a]	0.55	0.6143
Crude protein (%)	38.2±0.88[a]	38.97±1.46[a]	-0.78	0.4794
Crude fat (%)	39.19±0.53[a]	38.75±1.06[a]	0.65	0.5538
Ash (%)	4.44±0.47[a]	4.56±0.23[a]	-0.4	0.7111

FH- Acid silage prepared without the addition of BHT

FHB- Acid silage prepared by adding 200ppm BHT

Values represent Mean±SD of 3 replications

Treatment means with same superscripts does not differ significantly ($p>0.05$)

Physico chemical parameters of the acid silages

pH

Maintenance the acidity in fish silage is important in keeping the product more hygienic and safe by inhibiting the growth of pathogenic organisms. The pH of FH and FHB significantly ($p<0.05$) dropped to 3. 3 and 3.12, respectively on the second day for obvious reasons (Fig. 1). Both the lots showed fluctuations in pH till the 60th day of storage. The pH of silage FHB remained significantly ($p<0.05$) lower than silage FH till the 60th day of storage indicating a possible effect that the synthetic antioxidant, Butyl Hydroxy Toluene(BHT) may have on reducing the pH of silage. The pH at the end of 60th day of storage, 3.4 and 3.15 of FH and FHB, respectively are within the acceptable range that should be maintained for silages. The pKa of the preservative acid determines the final pH that silages reach (Raa and Gildberg, 1982). According to Haaland and Njaa (1989) and Vizcarra-Magana et al. (1999), deamination reactions probably caused slight changes in pH during storage. The silage at pH 4.5 and above is always susceptible to spoilage caused by *Clostridium botulinum*, *Staphylococcus aureas* and fungus (Anonymous, 1971).

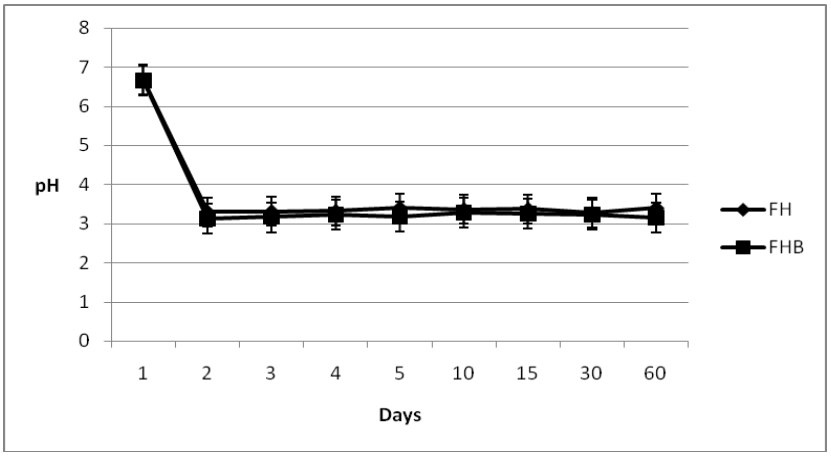

Fig. 1 Changes in pH of ensiled visceral waste of carps prepared with and without the addition of BHT stored at 28±2^{0}C.
FH- Acid silage prepared without the addition of BHT
FHB- Acid silage prepared by adding 200ppm BHT
N=3

Total Volatile Base Nitrogen (TVBN)

The total volatile base nitrogen (TVBN) values of both the FH and FBH decreased during the 2nd day (Fig. 2). Thereafter, it increased significantly ($p<0.05$) till the 7th day for both FH and FHB. The TVBN values showed a declining trend till the 60 days of storage although insignificant ($p>0.05$). This could be attributed to the escape of volatiles like ammonia during the storage. The limit of acceptability for TVBN in fresh fish is 34-40mg%. In the present study, the TVBN levels in both the treatments were well below the limit of acceptability indicating the non- spoilage of silage during storage. This could be attributed to the bacteriostatic action induced by the low pH maintained in the silage.

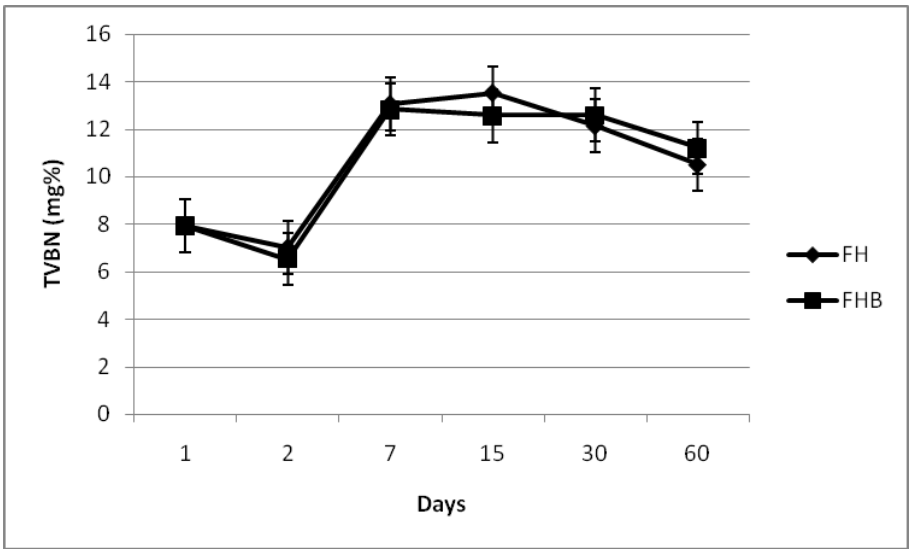

Fig. 2 Changes in TVBN content of visceral silage of carps prepared with and without the addition of BHT during storage at 28±2^{0}C.
FH- Acid silage prepared without the addition of BHT
FHB- Acid silage prepared by adding 200ppm BHT

N=3

Haaland and Njaa (1989) recorded TVBN value in the form of NH_3 as 112 mg/ 100g on the 14th day of ensiling the capelin fish using 1.4% formic acid. Ali and Sahu (2002) have reported a TVBN value of 79.8mg % for acid silage prepared from marine fishes. The major portion of TVBN value is contributed by trimethyl amine which is absent or found in very limited amount in freshwater fishes. This could be another reason for the low TVBN values found in the carp viscera silage. Ahmed and

Mahendrarkar (1996) have also reported a low TVBN value of 9mg % for fermented carp visceral silage. According to Connel (1980) TVBN more than 100 to200 mg·100 g-1 on dry weight basis of salted dried fish could indicate spoilage. There was no significant difference ($p>0.05$) in TVBN values between FH and FHB all through the 60 days of storage indicating the insignificant effect the antioxidant has on formation of volatile bases.

Thiobarbituric Acid (TBA) values

Fresh carp viscera had a thiobarbituric acid (TBA) value of 0.47±0.01mg malonaldehyde/kg. The addition of antioxidant to FHB decreased the TBA value to 0.45 mg malonaldehyde/kg on the 2nd day although the change was insignificant (Fig. 3).

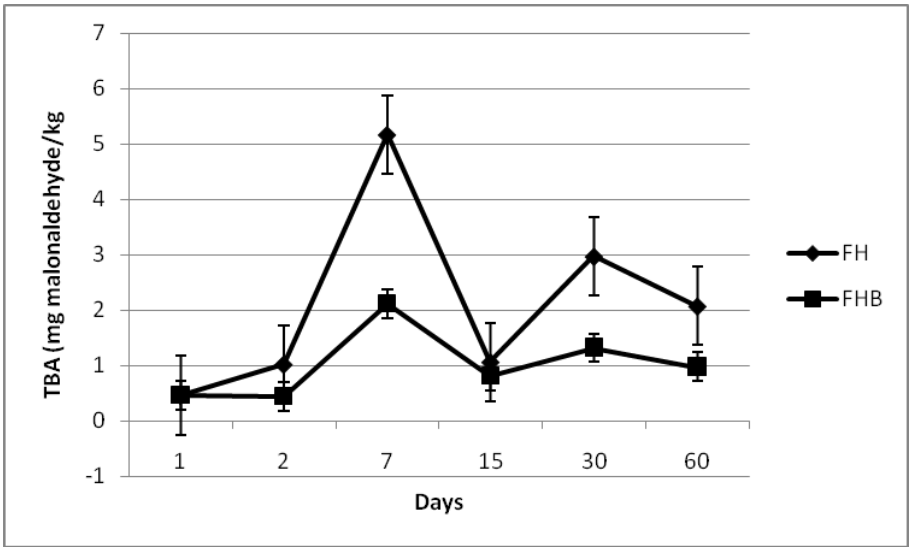

Fig. 3 Changes in malonaldehyde content of visceral silage of carps prepared with and without the addition of BHT during storage at 28±2^{0}C.
FH- Acid silage prepared without the addition of BHT
FHB- Acid silage prepared by adding 200ppm BHT
N=3

Delgado *et al* (2008) have proved the protective effect of antioxidant on the Spanish mackerel silage. The TBA values of FH significantly ($p<0.05$) increased by the 2nd day of storage and reached a peak of 5.17(±0.01) mg malonaldehyde/kg by the 7th

day of storage. The TBA value of FHB increased significantly ($p<0.05$) only after the 2nd day of storage, but the highest TBA value attained was only 2.12($\pm$0.02) in the treatment. All through the storage period the TBA values of the FHB remained significanly lower ($p<0.05$) than the FH which asserts the importance of addition of antioxidants to silage especially if made from fatty fish or body parts like viscera. Ahmed and Mahendrarkar (1996) have also reported that addition of antioxidants to silage results in slowing down the auto oxidation of lipids in fish viscera.

Microbiological quality of acid silages

Total Bacterial Load

Fresh visceral mince had a high bacterial load. Zahar et al. (2002) has reported a total bacterial load as high as $4.5x10^5$ for fresh sardine waste. Fig 4 depicts the total bacterial load in the FH and FHB till 60 days of storage. In the FH and FHB, the there was a highly significant reduction in the total bacterial load on the 2nd day. This could be due to the reduction in pH by the acid which induces a bacteriostatic action. Thereafter a significant reduction in bacterial load was found on the 30th day of storage. No significant reduction in bacterial load was found for the next 30 days. Bhaskar and Mahendrarkar (2007) have also reported a significant reduction in Total bacterial count of fish viscera till 4 weeks of storage. Reduction in total bacterial load soon after acidification of fish viscera and during storage of silage has also been observed in earlier studies by Mahendrakar et al. (1991). According to Delgado etal (2008) a reduction of aerobic mesophiles and coliforms was observed in Spanish mackerel silage, due to low pH maintained during the process. No significant difference in bacterial load has been found in both FH and FHB throughout the storage period indicating the absence or insignificant effect of BHT on bacterial growth.

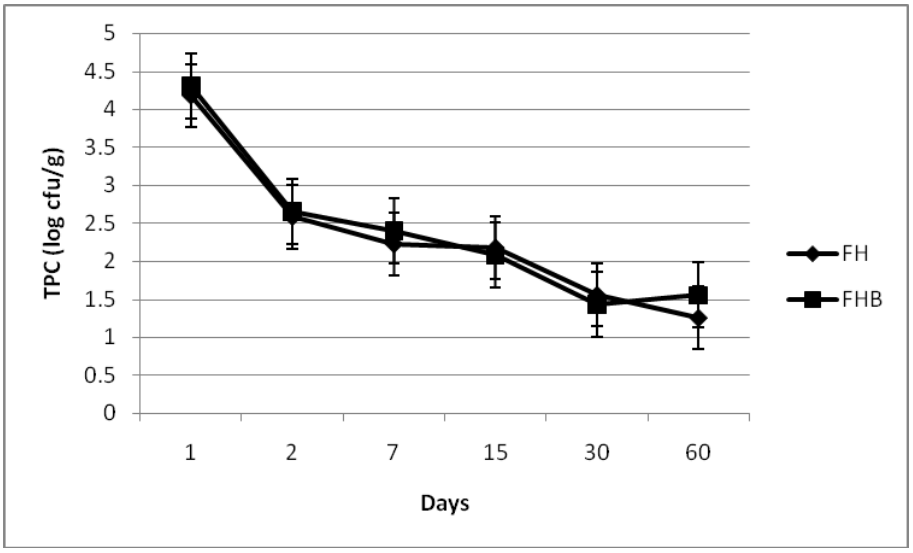

Fig. 4 Changes in total bacterial load of visceral silage of carps prepared with and without the addition of BHT during storage at 28±2^{0}C.
FH- Acid silage prepared without the addition of BHT
FHB- Acid silage prepared by adding 200ppm BHT
N=3

CONCLUSION

The processing of carps results in considerable quantities of processing discards of which visceral waste is the major one. Ensilation using acid could be a viable alternative to convert these wastes into useful by products. During the acid ensiling process only slight variations occurred to the dry matter, the protein, lipid and mineral fractions which prove the applicability of the process. The balanced protein, fat and mineral content of the silages could be made use of in preparation of poultry, fish and livestock feed. The addition of BHT has slowed down the process of auto oxidation in acid silages prepared from carp fish viscera and the low pH has prevented the proliferation of microorganisms.

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OUTER MEMBRANE PROTEINS OF *AEROMONAS HYDROPHILA* CAHH14 AND ITS IMMUNE POTENTIAL IN ROHU, *LABEO ROHITA*

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ABSTRACT

The present work was to study the outer membrane proteins (OMP) of CAHH14 strain of *Aeromonas hydrophila* (Gene bank accession number JF330411) and its immune potential in rohu, *Labeo rohita*. The immune potential in rohu was studied by intraperitoneal injection of OMP antigen with and without Freund's complete adjuvant. After post immunization for two months the fish were challenged with present strain and the relative percentage survival (RPS) ranged from 79-88%, which reflects the protective immunogenicity nature of OMP in rohu. The antibodies titre was found to be higher in immunized group of fish as compared to control fish. Further, polyclonal antibodies raised in rabbit against the OMP of CAHH14 strain detected three immunogenic bands of molecular weight 114.2, 68.4, 33.3 kDa by western blotting which can be used as potential candidates for the development of subunit vaccines.

Key words: *Aeromonas hydrophila,* OMP, RPS, Western blotting.

INTRODUCTION

Aeromonas hydrophila, a Gram negative motile rod of the family Aeromonadaceae has been recognized as a ubiquitous and heterogeneous emerging aquatic pathogen for over a century. The bacterium is mainly responsible for causing fatal hemorrhagic septicemia and epizootic ulcerative syndrome in fishes and results in million dollar losses annually to commercial aquaculture (Karunasagar et al., 1989, Poobalane et al., 2008, Sahu et al., 2012). Different form of infection due to *A. hydrophila* have been recorded in the Indian Major carps *Catla catla*, *Labeo rohita* and *Cirrhinus mrigala* (Karunasagar et al., 1989, Sahu et al., 2012) which are most important cultured species of freshwater fishes in India. A variety of virulence factors contribute to the overall virulence of this bacterium. These includes mostly lipopolysaccharides (LPS), a type III secretion system (T3SS) acting as adhesion structures, outer-membrane proteins (OMPs), extracellular products such as enzymes and toxins (Rahman and Kawai, 2000, Janda and Abbott, 2010, Sahu et al., 2011).

Control of diseases through various antibiotics and chemotherapeutic agents increases the risk of development of antibiotic resistant strains (Radu et al., 2003). Development of *A. hydrophila* vaccine is therefore vital to aquaculture industry. Researchers suggest that a template for the development of successful vaccine may be achieved by the study of prospective antigens *in vivo* (Brown et al., 1988). As the OMP of Gram negative bacteria is found to be associated with pathogenecity and protective antigenecity, they have been described as one of the most important antigen in vaccine formulation (Majhi et al., 2006, Gochhayat, 2010). In the present study we have focused on OMP, due to their exposed epitopes on their cell surface and conserved nature they seem to be highly immunogenic (Rahman and Kawai, 2000). The aim of the present study was to express the OMP *in vivo* and to evaluate its level of protection in *L. rohita*.

MATERIALS AND METHODS

Fish

Advance fingerlings of rohu, *L. rohita* weighing 20-30 g were obtained from fish farm of Central Institute of Freshwater Aquaculture (CIFA), Bhubaneswar, Odisha, for the present study. They were then acclimatized in 500 l FRP (Fiberglass Reinforced Plastic) tanks for two weeks prior to the start of experiments. Fish were fed with commercial pellet during the experimental period.

Bacterial strain

CAHH14 strain of *A. hydrophila* (Accession No. JF330411) isolated from ulcerative lesions of *L. rohita,* maintained in the Fish Health Management Division, CIFA, was used in the present study. The strain was identified upto species level using cultured, morphological, biochemical characteristics as per the scheme given by Popoff and Veron (1976) and at molecular level through 16S rRNA profiling study (Sahu et al., 2012). The bacterium was subcultured on the tryptone soya agar (Himedia, India) before preparation of antigen.

Extraction of OMP antigen

Outer membrane protein (OMP) of the bacterial cell was obtained as per the method given by Austin and Rodgers (1981). Broth culture (24 h old) was prepared by inoculating *A. hydrophila* CAHH14 strain aseptically into nutrient broth and incubated at 37^{o}C. Then it was centrifuged at 8000xg for 45 min. The supernatant was discarded and the pellet was washed and resuspended in the Tris buffer containing EDTA (10mM). This bacterial cell suspension

was sonicated at 50 hz for 10 min. After sonication the unbroken cells were sedimented by centrifugation at 8000xg and the supernatant collected was further subjected to ultracentrifugation at a speed of 45,000xg. The supernatant was discarded and the sediment was resuspended in Tris buffer containing 0.5% sarcosyl. It was further centrifuge at 45,000xg in the ultracentrifuge for about 45 min and finally the pellets collected after ultracentrifugation was OMP.

SDS PAGE analysis of OMP antigen

The OMP protein profiles of CAHH14 strain of *A. hydrophila* was analyzed by using 1 dimensional Sodium dodecyl sulphate polyacrylamide gel electrophoresis (1D SDS-PAGE) as reported by Laemmli (1970). The electrophoresis was carried out using Mini Protien tetra electrophoresis cell (Bio Rad, USA). Electrophoresis was performed at 120 V until the bromophenol dye font reaches the bottom of the gel. The gel was then stained with Coomassie Brilliant Blue R-250 followed by destaining. The molecular weights of the proteins were determined with standard molecular weight marker (Banglore Genei, India) using Alpha Inotec Software.

***In vivo* virulence study of pathogen**

The pathogenecity test as well as determination of LD_{50} of the present strain was conducted by intraperitoneal injection of 0.1 ml of live bacterial cell suspension at varied concentration of 1.7×10^3, 1.7×10^4, 1.7×10^5, 1.7×10^6 and 1.7×10^7 cfu/fish to each group of fish (10 Nos. of rohu) acclimatized previously in different tanks of 50 l capacity. Fish were monitored for 5 days and mortality was recorded. In order to fulfill Koch postulate, dead and moribund fish were sampled for the presence of challenge bacterium. LD_{50} value was calculated as per the method given by Reed and Munch (1938).

Raising of polyclonal antibody in rabbit

New Zealand white rabbit weighing 1.5 kg was used to raised polyclonal antibodies against the OMP of CAHH14 strain of *A. hydrophila***.** The first dose was given intramuscularly (on the hind leg) with emulsion of Freund's complete adjuvant and 150 µg of OMP antigen while the subsequent booster doses were given on 14^{th} and 28^{th} days of immunization with emulsion containing same dose of OMP antigen and Freund's incomplete adjuvant.

Determination of antibody titer

Blood from rabbit was collected by ear vein puncture on 1^{st} and 42^{nd} days of post immunization and serum separated was used for antibody titer determination. The antibody

titration was performed in 96 well flat bottom microtitre plates as per the method given by Karunasagar et al. (1997). Briefly a serial two fold dilutions of serum was made with PBS (pH-7.2). An equal volume of OMP antigen was then added to each of the serum dilutions in the wells. The plate was slowly tilted back and forth to ensure proper mixing of contents and then incubated at 35^{o}C for 12 h in a moist chamber. Antibody titre was recorded as $\log_2$ of the reciprocal of the highest dilutions giving visible agglutination. The control sample consisted of serum from the unimmunized rabbit.

Western blot analysis

Western blotting was performed as per the method given by Towbin et al. (1979) with the serum of immunized rabbit collected on 42nd day of post immunization. Briefly, the unstained gel containing the resolved OMP was subjected for electrotranfer onto a nitrocellulose membrane. After the transfer, the membrane was washed in deionized water and blocked with 5% defattened milk overnight at 4^{o}C. Membrane was then incubated with the primary antibody at 1:20,000 dilution followed by addition of secondary antibody i.e. goat-anti-rabbit horse radish peroxidase (HRP) conjugate (Jackson, USA) each for 1 h at 37^{o}C. Final colour was developed using diaminobenzidine- hydrogenperoxide (DAB-H_2O_2).

Immunization protocol for fish

This study consisted of three experimental groups, each comprising of 25 no. of Indian major carps, rohu. Group I fish were injected intraperitoneally with emulsion of OMP antigen and PBS at a dose of 100 µl corresponding to 90 µg protein. Group II fish were injected intraperitoneally with emulsion of OMP antigen, PBS and Freund's complete adjuvant (FCA) at same dose of 100 µl corresponding to 90 µg protein. Group III fish were the control fish, injected with 100 µl of PBS. This experiment was conducted for a period of 60 days.

Collection of fish antiserum and determination of antibody titer

Blood were collected and sera were pooled from a random sample of five immunized fish per group by caudal vein puncture to record the initial antibody titre on the first day of immunization. Similarly the blood was collected after two months of post immunization and detected for agglutinating antibodies. The antibody titration was performed in microtitre plates as described above for rabbit. Antibody titre was recorded as $\log_2$ of the reciprocal of the highest dilutions showing agglutination.

Dot blot assay

Serum sample collected from treated and controlled fish were subjected for Dot blot assay which seems to be a convenient immunological tool for the detection of antigen specificity. Dot blot assay test was performed as per the method given by Swain et al. (2001) with slight

modification. Nitrocellulose paper strips (NCPs) were coated separately with 2 µl of serum sample and were dried at room temperature. Then the strips were blocked in PBS containing 5% skimmed milk powder at 37^{o}C for 30 min followed by washing thrice with PBS-T containing PBS (pH 7.2), 5% skimmed milk powder and 0.05% Tween 20. The strip was incubated with 1:100 dilution of primary antibody. Then it was washed several time in PBS-T and incubated further with secondary antibody i.e. anti rabbit horse radish peroxidase conjugate (Vector Lab, USA) for 30 min. Again the NCPs were incubated with ABC reagent (Anti-Bovine-Complex) for 30 min. Lastly the NCPs was transferred to new plate and then incubated with AEC reagents (3-Amino-9 ethyl- carbazole) for 10 min in dark. The NCPs strips were washed properly with distilled water, air dried at room temperature and blots were detected.

Challenge study

To find out the efficacy of the outer membrane protein against this pathogen the Group I, II and III fish were injected intraperitoneally with 0.1 mL of cell suspension of *A. hydrophila* CAHH14 strain at dose of 10^5 cfu/fish and the mortality rate was recorded up to 10 days. Relative percent survival (RPS) was calculated according to Amend (1981).

RPS= 1 – (% vaccinate mortality / % control mortality) x 100.

RESULTS

SDS-PAGE profile of OMP

The OMP profiles of CAHH14 strain of *A. hydrophila* have 14 bands with molecular weight ranging from 114.2 kDa to 12.8 kDa. The molecular weight of OMP bands found with high intensity included 114.2, 86.7, 67.8, 57, 42.5, 33.3, 30.5 and 12.8 kDa respectively. The other bands of molecular weight 72.4, 28, 25.6, 23.2, 19.3 and 15.7 kDa are having lower intensity (Fig. 1).

in vivo virulence study

LD_{50} value, after 5 days of observation period for viable cells of *A. hydrophila* injected to rohu was found to be 1.7 x 10^4 cfu/fish where as 100% mortality was recorded at 10^7 cfu/fish within 24 h, 85 % mortality was recorded at 10^5 CFU/fish within 5 days.

Determination of antibody titer and western blot analysis of RPAbs

The OMP was found to be highly immunogenic. Weak agglutination was observed in the sera of control and immunized rabbit at the first day of immunization. However, antibody titer was found to be highest after the 2nd booster dose in rabbit. Results of antibody titration in

rabbit against OMP antigen was shown in Fig. 2. In western blot analysis RPAbs detected 3 protein bands of molecular weight 114.2, 67.8, 33.3 kDa (Fig. 3).

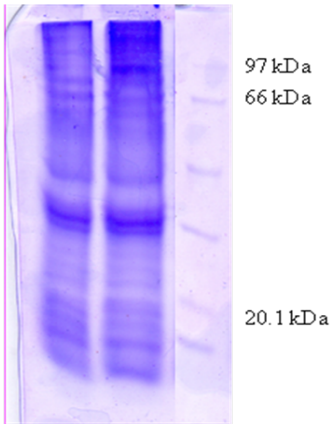

Fig. 1 SDS- PAGE profiles of the outer membrane protein of *A. hydrophila* CAHH14 Strain, Lane 1 and Lane 2- OMP ,Lane 3-ProteinMarker (97 -14 kDa).

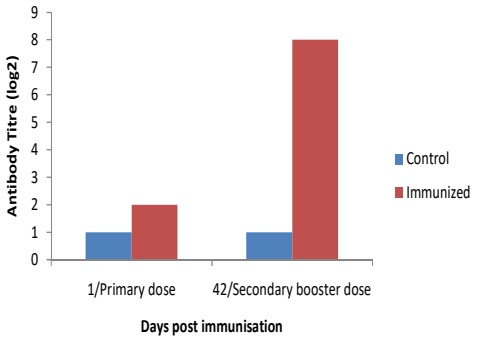

Fig. 2 Titre of agglutinating antibodies in rabbit immunized with outer membrane protein *of A. hydrophila* CAHH 14 strain.

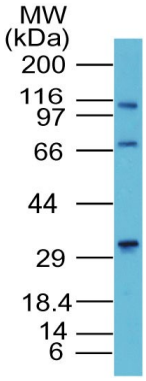

Fig. 3 Western blotting profile of OMP of *A. hydrophila* CAHH14 str

Determination of antibody titer and dot blot assay of fish antiserum

Antibody titer was found to be higher in immunized group of fish as compared to the controlled fish. However, maximum antibody titer was obtained in case of fish injected with antigen along with FCA as compared to fish injected without adjuvant after 60 days of post-immunization (Table 1). The titers remain constant between 0 and 2 for the sera obtained from control fish and 1st day sera of experimental fish.

All the immunized fish sera tested were positive for dot-ELISA, which was revealed by brown coloration of the nitrocellulose paper strips due to strong reaction of antigen with homologous antiserum whereas the control fish serum was found to be negative with no color development.

Table 1 Agglutinating antibody titre in the serum of fish immunized with OMP of *A. hydrophila* under different condition

Type of Vaccination	Agglutinating antibody titre (log_2)*
OMP with adjuvant	7.4
OMP without adjuvant	6.2
PBS control	1.1

* Geometric mean reciprocal log2 value of the highest dilution of serum which showed positive agglutination.

Challenge Study

Upon challenge of fish with CAHH14 strain of *A. hydrophila*, a good degree of protection was recorded in immunized fish. The percentage survival and mortality of the fish immunized with OMP, OMP along with adjuvant as well as in control fish was represented in Table 2.

Table 2 Percentage mortality and relative percent survival of challenged *L. rohita*

% mortality in control group	Immunized group	% mortality	RPS
96	OMP + FCA	12	87.5
	OMP immunized group	20	79.2

DISCUSSION

Success of aquaculture industry is generally related to environmental factor, biological factors and health status of the farmed aquatic organisms. To a great extent it depends on the way of infrastructure for diagnosis and treatment of fish diseases. Since the antibiotic treatment results in the development of the antibiotic resistance strains, so it seems to become ineffective (Radu et al., 2003). An alternative approach is prevention of diseases through vaccination. *A. hydrophila* has been considered as a significant heterogeneous aquatic pathogen affecting the cultured and wild fishes worldwide. Hence, it is high time to develop suitable vaccine against this potential pathogen in order to provide protective immunity to fish population in India for augmenting the production.

The most important attributes of a candidate molecule for vaccine development are they should be highly conserved among various members of the same species, should be expressed on the surface of pathogens so that antigen presenting cells can easily recognize them and should be immunogenic. In this regard, the OMP of Gram negative bacteria act as a suitable bacterial component for the development of vaccine (Hirst and Ellis, 1994). The presence

study was an approach towards the development of subunit OMP vaccine against *A. hydrophila* and its efficacy trial on the rohu to detect its immune potential.

The SDS-PAGE analysis of CAHH14 strain revealed 14 numbers of bands. In the present study the major bands found were somewhat similar to the finding of Esteve et al. (1994) who reported two major bands of OMP at 43 and 50 kDa regions. Maji et al. (2006) reported a 57 kDa and 23 kDa polypeptides in the OMP of *A. hydrophila* strain studied which had been also found in our strain. According to Subashkumar et al. (2007) the OMP molecular banding pattern of *A. hydrophila* mostly existed between 33 to 56 kDa in fish and diarrheal strains. Although we have found bands in this range but several bands were also found beyond this range.

The specificity of the polyclonal antibodies raised in rabbit against OMP of CAHH14 detected 3 prominent bands of molecular weight 114.2, 67.8, 33.3 kDa but not in control rabbit serum. These finding indicate that the above bands were immunodominant in nature and may be responsible for inducing immunity in fish (*L. rohita*) challenged with *A. hydrophila.* Rahman and Kawai (2000) reported the antigenecity nature of OMP of *A. hydrophila* which is able to induce protective immunity in goldfish. Majhi et al. (2006) studied on 57 kDa immunogenic polypeptide of the crude OMP antigen of *A. hydrophila* isolated from gold fish that provide significant protection to fish. They also reported a 23 kDa polypeptide should be considered important while preparing immune prophylactic tools against *A. hydrophila* infection in gold fish. Although we have not specifically studied on single polypeptide band but interestingly we have found protein bands of molecular weight 23.2 kDa as well as 57 kDa in the present strain. Similarly, Fang et al. (2004) reported that a 43 kDa major adhesion could confer protection to Blue gourami fish challenged with *A. hydrophila*.

The test fish, *Labeo rohita* treated with OMP antigen showed significantly more survival rate than the controlled fish challenged with *A. hydrophila*. However, the RPS value of 88% was found in fish injected with OMP along with adjuvant and 79% without adjuvant. This result coincided with the finding of Chandran et al. (2002) who reported a percentage survival of 80-90% in fish injected with OMP. The antibody titer in fish to *A. hydrophila* OMP antigen containing adjuvant was higher than that of fish immunized using antigen without adjuvant (2010). This indicates the positive effect of such immune stimulators for the expression of high antibody titer. The above finding were similar to the findings of Kalbassi et al. (2000) who had studied the humoral immune response of *Acipenser persicus* to four different *A. hydrophila* antigen. Ingram et al. (1985) reported successive booster dose of bacterial antigen

results in quicker antibody response. But in our study, we have found a single dose of OMP antigen with adjuvant given to the fish was able to produce high antibody titer that strongly correlated with protection.

Dot blot assay is regarded as a rapid and confirmatory test for the identification of bacterial pathogen (Swain et al., 2001, Sachan and Agarwal, 2002). In our test dot blot assay was carried out for the identification of specific bacterial antigen and all the serum collected from immunized fish showed positive results.

From the above study it was confirm that OMP of CAHH14 strain of *A. hydrophila* have protective immunogenicity in *L. rohita* and it may be useful to develop vaccine by selecting such OMP antigen. Further the OMP is to be fractionated and study should be conducted to assess the potentiality of each fraction to be used as immunoprophylatic agent. It was also concluded form the present study that a single dose of OMP antigen along with FCA is sufficient to elicit higher antibody titre and capable of producing high degree of protection in *L. rohita*. However, the duration of the protective immunity has not been studied and is an important factor to be reckoned.

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