



# Identification of a New Species of the Medicinal Leeches in India using DNA Barcoding Technique - a Breakthrough

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## **Authors' contributions**

*This work was carried out in collaboration among all authors. Author GP wrote the first draft of the manuscript, designed the paper and explored the available literature and research work already done in the field. Author MD guided about the whole study, edited the draft and contributed by giving valuable inputs to make the paper impactful. Author RP managed the literature searches, helped in designing the study and gave valuable inputs in relation to ayurveda literature. Author MG performed all the molecular (DNA extraction, PCR, sequencing) as well as bioinformatics and phylogenetic analyses. She also submitted the leech sequence to NCBI and got the accession number. All authors read and approved the final manuscript.*

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## **ABSTRACT**

Leech therapy has always been an important treatment modality in Ayurveda. Medicinal leeches are known for their extensive therapeutic uses. Identification of leech species is an important research area to understand the mechanism of therapeutic gains and exploring more probable benefits. Researchers have done a swell job in identifying various species of leeches in different

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parts of Asia including India. For therapeutic purpose however, the clinicians in India have not bothered much about the exact identification of leech species being used at different centres. There is dearth of research papers in this aspect. Thus a study was planned to identify the species of leeches being used at our centre.

**Materials and methods:** DNA Barcoding technique was used where mitochondrial CO1 gene was amplified using LCO-HCO primers.

**Results:** DNA sequencing of Leech sample closely resembled to *Hirudinaria Bpling* species, which is a breakthrough in existing knowledge of medicinal leeches in India, as this species was recently reported to be found in Thailand in 2012 for the first time. No previous documentation of its existence is available in our country.

**Conclusion:** Further studies on large scale are required to explore their existence, morphological features and salivary contents in regard to their medicinal value. This will help in exploring any additional benefits to the existing knowledge of therapeutic uses of medicinal leeches. This study will pave the path to new avenues in therapeutic utility of leeches, as the newer species might have additional bioactive chemicals making them useful in wider variety of disorders.

**Keywords:** Leech therapy; *hirudinaria bpling*; ayurveda; *jalauka*; DNA barcoding; MLT.

## 1. INTRODUCTION

Leech therapy has always been one of the important tools in Indian system of medicine since its inception. All major texts contain elaborate description about their morphology, therapeutic benefits, undesired effects and the guidelines to use them. A good number of research papers are published globally on clinical utility of leeches in a wide range of diseases. Leeches are called by different names in different languages, for instance *Jalaukā* in Ayurveda and Mahabharat, *Jalaukā* in Sanskrit, *Jonk* in Hindi, *Juku* in Nepali and *Jaru* in Sindhi [1]. In Sanskrit literature, leeches are referred by different names like *Raktapa* (blood drinker), *Jalaukā* (water dwelling) and *Jala-sarpini* (water glider). Ancient Ayurvedic scholars like *Acharya Sushruta* and *Vagbhata* have devoted complete chapters on *jalaukāvchāran* (medicinal leech therapy) in their texts. Leeches have been quoted as the best way for blood sucking in sensitive people like children, aged, females, pregnant ladies and elite class like emperor etc [2,3]. *Shrunḡa* (cow horn), *Jalaukā* and *Alābu* (bottle gourd) have been told as best ways to let out the blood in *Vāta*, *Pitta* and *Kaphadosha* (the body humours) predominant individuals respectively. Although these three can be interchangeably used if needed in different *doshaprakriti* (body forms) people. Owing to its cool, sweet properties, the leech is best in pacifying *Pitta dosha* [4]. Leeches were used as Medicinal leech therapy (MLT) in Biomedicine for therapeutic blood sucking in many countries and regions globally, as evident from the vast literature available. They are usually found in abundance in warm and

temperate areas in the world. The word *Jalaukā* was found in holy book of Mahabharata and later in *Sushrutasamhitā*, *Ashtāngsangraha* and *Ashtāngahridaya* (various Ayurveda texts). Even the reference of leeches is found in proverbs mentioned in Bible. It is mentioned as a warning in Talmund that drinking water from river pools should be avoided for there is most dangerous leech known as '*Limmatisslinotica*', about 10 mm in length. This leech could result in haemorrhage from mouth, throat, nose if found access to these inner parts of body. It could not bite the inner surface of the human body although [5]. The major comprehensive work on taxonomy of Indian leeches was first presented by Harding and Moore (1927). The other works like Baugh (1960) and Sanjeev raj & Gladstone (1981) also contributed important work in this field [1]. Harding and Moore dealt with 51 species and subspecies of leeches found in Indian subcontinent. They recorded 38 species and 6 sub-species of leeches from India [6]. Later on other workers contributed immensely to leech fauna of Indian region viz. Bhatia (1930, 1934 and 1939), Challadurai (1934), Baugh (1960 a& b), Sanjeev raj (1951, 1954, 1959 & 1974). The work of Soos (1965-1970) on the leech fauna led to some changes in nomenclature of species and subspecies [5]. Mahesh Chandra presented a check-list of 52 species and 8 subspecies known from Indian origin [6].

Blood-letting or phlebotomy is achieved painlessly by the application of leeches. Majority of leeches used for medicinal purpose lead parasitic life, show many parasitic adaptations in their morphology, habits and habitat and suck blood of vertebrates [7]. There are more than 700 species identified at global level up to 2018

[8]. Out of them *Hirudo Medicinalis*, *Hirudo Nipponia*, *Hirudotroctina*, *Hirudo Quinquestriata*, *Hirudinaria Javanica*, *H. Manillensis*, *Poecilobdella Granulosa*, *Macrobodella Decora*, *Hirudo Verbena* and *Haementeria Officinalis* are most commonly used in clinical practice [9].

Medicinal leech therapy (MLT) is an important treatment modality and has been used in different regions of the world since long ago. The first documented application was observed in ancient Egypt [10]. The term 'Leech' originated from the word "Laece" which means the physician. The leech secretions have shown to have more than 100 types of proteins [11]. but majorly active role is performed by only a few of them like hirudin, calin, inhibitors of kallikrein, histamine-like substances, hyaluronidase, and collagenase [12]. The leeches produce therapeutic effects by different mechanisms like extracellular matrix degradation, inhibition of platelet functions, increasing blood flow and also producing analgesic, anti-inflammatory, anticoagulant and antimicrobial effects. These are achieved as a result of various proteins and bioactive molecules available in leech secretions, following a bite [13]. The secretions help to eliminate microcirculatory disorders, re-establish oxygenation and eliminate hypoxia by restoring the permeability of vessels supplying the tissues, decrease blood pressure, improve activity of immune system and also improve the overall bioenergetics status of the individual. [14]. Studies have shown more than 20 bioactive molecules in leech secretions and their modes of action have been identified too [15-17]. The medicinal leech therapy has been used in multiple clinical conditions. It has a convincing effect and potential in healing varied types of chronic wounds e.g. venous ulcers, diabetic foot ulcers, post-traumatic wounds, buerger's disease and cutaneous leishmaniasis [18]. Various studies have been published on use of MLT in reconstructive and plastic surgeries, which shows 65-80% success rate in post-operative venous congestion induced complications [19-22]. Clinical utility of leech therapy has also been explained in many other diseases like osteoarthritis [23], varicose veins [24], eczema [25], thrombosed pile masses [26], recurrent nasal vestibular frunculosis [27], scalp psoriasis [28], nevus of ota [29], peri-apical abscess [30] etc.

## 2. TYPES OF LEECHES

In Ayurveda, leeches are broadly classified into 2 broad categories- Poisonous and Non-poisonous

[31,32]. *Acharya Sushruta* has elaborated this classification further and mentioned six types each in both poisonous and non-poisonous categories. The category has been decided by their habitat and morphological features. Leeches dwelling in places having plenty of clean water and fragrance, water sources having lotus, lily, other fragrant flowers and algae are non-poisonous [31,33]. The poisonous ones live in dirty water contaminated by putrefied bodies and also by excretion of snakes, frogs, fish and other aquatic animals. Generally, the leeches which are expanded in the middle, which are ugly, flat or dull in movement and who do not stick well, suck little blood are unfit to use. Those having colour- red, white or too black, very thin or thick, very much mobile and slimy, stout and hairy in the middle or having streaks of varied colours like a rainbow, are of poisonous variety. Their bites cause oedema, excessive itching, Pyrexia, burning sensation, vomiting, feeling like intoxication and sinking. Therefore these must not be used for the therapeutic purposes. The non-poisonous leeches are found in areas like *Yavan* (Arab countries, Central Europe), *Pandyā* (South India), *Sahyā* (Mountain ranges near Narmada) and *Pautana* (Mathura). They are especially bulky, stout, fast suckers and gluttonous [34]. *Vāgbhata* has mentioned that they are round and blackish green like algae and have blue streaks [32]. The important features of both poisonous and non-poisonous leeches are summarised in the following tables [35].

We can therefore assume that 12 types of leeches were known at the time of *Sushruta*. Interestingly, the *Hirudininae* family of leeches also contains 12 known species within six genera named as *Dinobdella*, *Hirudinaria*, *Hirudo*, *Limnatis*, *Myxobdella* and *Whitmania* [36], which are found in temperate and tropical Asia, Africa and Caribbean islands. *Hirudininae* is one of the two sub-families of *Hirudinidae* family, the second one is *Haemidipsinae*. *Hirudininae* are also known as Buffalo leeches [36,37]. Extensive work has been done by various scientists in relation to taxonomy of leeches in India. Mahesh Chandra compiled a record of 52 species and 8 subspecies belonging to 25 genera and 5 families available in India, in a paper titled- A checklist of leeches which is found in records of Zoological Survey of India, 1983 [6]. Mandal and Mishra in 2017 have mentioned that 70 leech species have been recorded from India under 25 genera and 6 families [38].

**Table 1. Poisonous leeches [35]**

S.no.	SavishaJalaukā (poisonous leeches)	Features
1.	Krishnā	It resembles black in colour like that of anjana. It has pruthu (big) head.
2.	Karburā	It resembles varthamatsya (snake like fish). Its kukshi has elevations and depressions.
3.	Alagardā	It has mahāpārshva, its body is romasha and has Krishna mukha.
4.	Indrayudhā	It looks like that of rainbow. There are many striations or lines on its body.
5.	Samudrikā	Its colour resembles slightly asithapitikā. It has dotted skin and resembles many flowers.
6.	Gochandanā	Its end part resembles like that of govrushana. Its body has marked bifurcating lines and has anumukhi.

**Table 2. Non-poisonous Leeches [35]**

S. no.	NirvishaJalaukā (non-poisonous leeches)	Features
1.	Kapilā	It has manahshilā coloured striations at its body sides. Its dorsal surfaces are snigdha and color resembles like MudgaDhānya.
2.	Pingalā	Its body shape is vruttā, its colour matches to rakta and it has ashugati.
3.	Shankumukhi	Resembles yakrudvarna, it is sheegra pāyini, has dheergakāya and has shankhamukha.
4.	Mushikā	Its colour & shape resembles like that of mushikā and emits a foetid smell from the body.
5.	Pundrikamukhi	Its mukha resembles like that of pundarika and its colour matches with the color of mudgadhānya (greenish black).
6.	Savarikā	It has padmapatravarna and measures astadashāngula in length.

Overall 700 plus species are found globally, distributed in 91 genera and 7 families. *Americobdelidae* is endemic to America only. The families found in India are- *Piscicolidae*, *Glossiphonidae*, *Erpobdellidae*, *Hirudidae*, *Haemadipsidae* and *Ozobranchida* [39].

Families – 1. Erpobdellidae  
2. Hirudidae  
3. Haemadipsidae

### 3. MATERIALS AND METHODS

The studied sample was randomly selected from the large number of leeches present at the Leech farming centre at CBPACS, Najafgarh, NewDelhi.

**Identification of sample (methodology):** Leech samples were preserved in ethanol and kept in -20°C. For DNA extraction, approximately 0.5mm long fragment of leech sample was taken from its anterior end and washed three times with autoclaved water. We used phenol-chloroform-isoamylalcohol (PCI) method in which samples were crushed in 200µl lysis buffer containing 10mM each of Tris-HCL (pH 8.0), EDTA (pH 8.0) and NaCl. DNA was precipitated using

#### Leech tree [1,39]

#### Phylum – Annelida

#### Order- Hirudinea

#### Suborder- Rhyncobdellae

#### Arhynchobdellae

- I. Rhyncobdellae –
  - Families- 1. Piscicolidae
  2. Ozobranchidae
  3. Glossiphonidae
- II. Arhynchobdellae

Isopropanol and dissolved in TE buffer [40]. DNA was quantified using NanoDrop™ 2000 spectrophotometer. Resulting DNA concentration was approximately 230ng/μl which is very high for PCR. Therefore it is diluted to 20-30ng/μl in autoclaved water. To identify and study taxonomic relationship between samples, DNA Barcoding system was used (mitochondrial CO1 gene was amplified by using primers specific to this region. First, DNA was extracted from leech sample followed by PCR reaction using CO1 primers) - Hebert et al., 2003 [41]. It utilize nearly 600-700bp long mitochondrial COI gene. 2μl of extracted DNA was used in 20μl PCR reaction. PCRs were performed under the following conditions: initial denaturation at 95°C for 3 minutes, 39 cycles of denaturation (95°C, 45 seconds), annealing (F- LCO1490: GGTCAACAAATCATAAAGATATTGG, R-HC02198: TAAACTTCAGGGTGACCAAAAATCA; (Folmer et al., 1994) 55°C, and 45 seconds), extension (72°C, 1 minute) and a final extension at 72°C for 10 minutes. The PCR products were electrophoresed in 1% agarose gel. Samples for which PCR product was obtained, were cleaned using Exonuclease I and Shrimp alkaline phosphatase (New England Biolabs Inc.). Cleaned PCR products were sent for sequencing to Apical Scientific Sequencing. Resulting sequence was used for phylogenetic tree

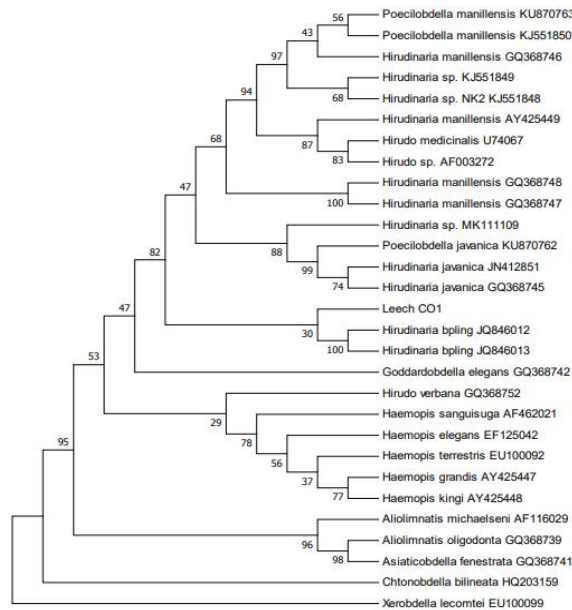
construction. Phylogenetic reconfirmation was done using the Maximum Likelihood method based on the Tamura 3-parameter model. Here query sequence was aligned with other closest leech sequences obtained from NCBI.

#### 4. RESULTS

On comparing the resultant CO1 sequence with known sequences on NCBI database [42], the closest hit to our query was *Hirudinaria bpling* with 87% identity.

The analysis involved 29 nucleotide sequences. The tree with the highest log likelihood (-4133.69) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. The values on each clade of phylogenetic tree represent authenticity of that clade. During phylogenetic tree construction the same tree was ran nearly 1000 times (bootstrap). These values represent percentage of times same clade was obtained. Higher percent value makes the clade more reliable.

A discrete Gamma distribution was used to model evolutionary rate differences among sites. Evolutionary analyses were conducted in MEGA7 with 1000 bootstrap replicates.



**Fig. 1. Phylogenetic analysis of query sequence (denoted as Leech CO1) using MEGA 7. CO1 sequences from this study were aligned with known sequences from different taxa of Leech. Xerobdella lecomtei is taken as outgroup**

## 5. DISCUSSION

The leeches have been used for therapeutic purposes all over the world including India. Leeches have been discussed in detail in the important treatise of Ayurveda, firstly in Sushruta Samhitā and later in *AshtāngSangraha* and *Hridaya*. In *SushrutaSamhitā*, 12 species of leeches have been explained under two broad categories of poisonous and non-poisonous comprising of six species each. In present times, leeches are extensively used in India for medical purposes by Ayurveda clinicians and to a lesser extent by some western medicine doctors. The Zoologists have explored different zones of their existence in India and have so far recorded 70 species, 6 families and 25 genera. The Indian clinicians have not bothered much about the exact identification of leeches, although Indian cattle leeches namely *H. Granulosa*, *H. Menilensis* and *Verbena* are commonly used. Most of the doctors use the leeches based on their gross appearance and long history of their use in patients, assuming the available leeches to be of medicinal variety. The common cattle leeches are easily available in different zones of India. The medicinal leeches are found to be effective and their various mechanisms of action like vasodilation, bacteriostatic role, analgesic, anti-inflammatory, anti-oedematous and anticoagulation are attributed to the active chemicals reported in the saliva of leeches [43]. Accurate interpretation of bioactive compounds depends upon the determination of species precisely. A thorough investigation of various species of medicinal leeches may reveal more powerful peptides or bioactive compounds. It has been recorded that sequence of amino-acids in peptides like anti-statins, anticoagulants are substantially different between closely related species of medicinal leeches [44]. There are also chances of variation in chemicals or proteins found in the saliva of different species being used. The mode of action of different medicinal leeches depends upon the bio-active chemicals or proteins available in the salivary secretions. Therefore it becomes important to know about the exact species of leeches in use. Our study planned for this very purpose has found a new species, not yet reported in India i.e. *Hirudinaria Bpling*. *H. bpling* (Phillips, 2012) belongs to the genus *Hirudinaria* and is one of the three species found in the family *Hirudinidae*. The other two species are- *Hirudinaria Javanica* (Wahlberg, 1856) and *H. Manillensis* (Lesson, 1842). Morphological and molecular evidence reported in a study conducted at Thailand, which also

included the use of scanning electron microscopy (SEM), supported it to be a new species in the family of *Hirudinidae*. It has shown to possess some differentiating features which separate it from other two known species like unusually large vaginal caecum that opens directly into the female bursa, presence of a median longitudinal furrow in the upper lip (ventral surface), salivary papillae, well developed jaws and dorsal colour pattern [45]. The species of leeches within the genus *Hirudinaria* Whitman, 1886, are widely distributed over tropical south and south-east Asia. The karyotype examination conducted at Thailand, of the three known species of this genus, revealed different number of chromosomes among them. *H. bpling* had highest chromosome number ( $n=14$ ,  $2n= 28$ ) in comparison to *H. Javanica* ( $n=13$ ,  $2n=26$ ) and *H. Manillensis* ( $n=12$ ,  $2n=24$ ) [37]. These variations may also result in different therapeutic benefits of *H. Bpling* species, which must be evaluated further.

## 6. CONCLUSION

Our study has found *Hirudinaria Bpling*, a leech species which is novel edition to existing knowledge of available leeches in India. This study may open new avenues of clinical utility of leeches in this country in future. Now after these study findings, further study of morphological features, taxonomy and analysis of salivary contents is required to explore more about this species. Study of larger sample size is mandatory to find out the existence of different leech species and its percentage, available in Delhi and its surroundings.

## CONSENT

It's not applicable.

## ETHICAL APPROVAL

It's not applicable.

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## COMPETING INTERESTS

Authors have declared that no competing interests exist.

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