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Biological characterization and pathogens detection of blood-feeding terrestrial leech, *Haemadipsa rjukjuana* at Gageodo in Korea

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Biological characterization and pathogens detection of blood-feeding terrestrial leech, *Haemadipsa rjukjuana* at Gageodo in Korea

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ABSTRACT

Leeches are segmented invertebrate and their habitats are marine, terrestrial and freshwater environments. Most of them can suck blood from vertebrate to invertebrate using their jaws. The Gnathobdellae, the jawed leeches armed with teeth, includes 2 family: Hirudidae and Haemadipisidae. Generally, Hirudidae refers to aquatic leech and Haemadipisidae refers to terrestrial leech. A blood-feeding terrestrial leech lives in high humid atmosphere like damp forest in South Asia, Southeast Asia and Australia. Since leeches have been used on human for medical purpose and the spectrum of arthropods vector has been expanded, research of leech has been conducted consistently whether if

the leech can transfer zoonotic pathogen. But, in Korea, there were no study of blood-feeding leech molecularly. Furthermore, no previous research of terrestrial leech was recorded.

This study is about blood-feeding terrestrial leech which lives on Mt. Docksil in Gageodo (The leech is called Docksilsan leech in this study) and it is composed of three parts. In the first experiment, observation was done on Docksilsan leech morphologically using electron microscopy (SEM). Six specimens were used to it. On the surface, their body length measured in 8 mm (mean), maximum body width 2.4 cm with two suckers in interior and posterior parts of body. Distinctive feature was that they have three jaws which located in Y-shaped lumen and 5 pairs of eye that last eye were divided by 2 annuli from 4^{th} eye. The posterior sucker consisted of 71-72 radial rays. Muscle layer consisted of circular muscle (external) and longitudinal muscle (internal). Intestine was composed of 6 parts covered with simple cubonial epithelium.

In the second experiment, in order to identify their species name, it was analyzed with 29 leeches by conventional PCR targeting 18S rRNA and cytochrome *c* subunit I (COI). Based on 18S rRNA, Docksilsan leech had 99.9% homologous to *Haemadipsa rjukjuana* isolate HARY (from Taiwan, HQ203097). Based on COI, the sequences were separated into two types, *H. rjukjuana* COI type A and COI type B. The type A and type B showed 94.6% and 94.3% similarity to the reference sequence of the *H. rjukjuana* L00115A (from Taiwan, HQ322443), respectively. These results suggest that the Docksilsan leech has in common the morphological feature of *H. rjukjuana* and also is closest to it genetically. In other words, Docksilsan leech, blood-feeding terrestrial leech which lives in Gageodo, is assumed that they are *H. rjukuana.*

Lastly, the last experiment was conducted to investigate the host preference and a possibility whether the Docksilsan leech could be a vector using 173 specimens by PCR. Result of amplification for 8 kinds of pathogens, *Anaplasma phagocytophilum*, *A. bovis*, *Ehrlichia chaffensis*, *E. canis*, *Borrelia burgdoferi*, *Bartonella* spp., *Rickettia* spp. and *wolbachia* spp., *Bartonella* spp. were detected with 8% infection rates (n=14/173). Two samples of them were closest to *B. grahamii* isolate KWDBG 41 (JN810847, South Korea) with 99.6% and 100.0% each. Other 5 samples were closest to *B. grahamii* V2 strain with 100.0% and other 6 samples had 100.0% homologous with *B. henselae* huston-1 and the rest of them were was presumed to be *Bartonella* sp. KM2563 (FJ667565, Taiwan) with 90.6% similarity.

In order to screen blood meal of Docksilsan leech, 173 specimens were used for PCR using cytochrome *b* gene. The result is that DNA of human (*Homo sapiens sapiens*, n=10), house mouse (*Mus musculus*, n=8), siberian weasel (*Mustela sibirica*, n=6), pale thrush (*Turdus paliidus*, n=3), grey-backed thrush (*Turdus hortulorum*, n=3), rufous-tailed robin (*Luscinia sibilans*, n=1), siberian rubythroat (*Luscinia calliope*, n=1)**,** oriental magpie robin (*Copsychus saularis*, n=1)**,** black-faced bunting (*Emberiza spodocephala*, n=1) and yellow-throated bunting (*Emberiza elegans*, n=1) were detected from extraction of leech. This result shows a host preference of Docksilsan leech as well as a fauna of Gageodo.

As the world climate has been changed, distribution of flora and fauna is altered and new vector or diseases is emerged. The research of blood meal screening of Docksilsan leech could monitor change of host distribution, especially migratory birds

which stay at Korea. Furthermore, the fact that *Bartonella* spp. were detected in Docksilsan leech implies a possibility of vector.

In conclusion, this research suggests that Docksilsan leech, blood-feeding terrestrial leech which live on Mt. Docksil in Gageodo, is closest to *Haemadipsa rjukjuana*. They were infected with *Bartonella grahamii* and *B. henselae* and sucked blood from human, mouse, weasel and 7 kinds of migratory birds. This research is remarkable for its originality to record and study of blood-sucking terrestrial leech, *H. rjukjuana*, is first conducted in Korea.

Key words: leech, blood-feeding terrestrial, *Haemadipsa rjukjuana*, pathogen, *Bartonella*, host

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ABBREVIATION

BPV	Bovine parvovirus
COI	Cytochrome subunit I
DNA	Deoxyribonucleic acid
DMSO	Dimethyl sulfoxide
EAV	Equine arteritis virus
EHV-1	Equine herpesvirus type 1
FCV	Feline calicivirus
*glt*A	Citrate synthase
HCl	Hydrogen chloride
ITS	Intergenic spacer
$MgCl_2$	Magnecium chloride
mtDNA	mitochondrial deoxyribonucleic acid
$(NH_4)_2SO_4$	Ammonium sulfate
PCR	Polymerase chain reaction
SEM	Scanning electron microscope
spp.	Species

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GENERAL INTRODUCTION

Leeches are segmented worm that belong to phylum Annelida. The majority of leeches live in freshwater while few species can live in marine or terrestrial environment. They are activated when the temperature is over 25℃ and the humidity is over 60% and the average life span is 2 to 3 years. They move like inchworm using two suckers at posterior and interior of the body (Kang 1995). Through the skin, they can sense environmental change and each of the segments works as a brain with neuronal ganglia. They can suck blood three to ten times larger than their own weight, which is generally 2 to 6 ml. Three or 4 months after last blood sucking, they look for fresh blood meal and can live up to 18 months without feeding (Nehili *et al.* 1994). When they suck blood, they use jaws which covered by teeth, leaving a Y-shaped incision. The leech secretes an anticoagulant which prevents blood coagulation in their digestive and store system. The anticoagulant includes hirudin, hyaluronidase, calin, destabilase, apyrase, eglin and etc. As a representative salivary component, hirudin acts as a thrombin inhibitor and they are used in cardiac surgery and plastic surgery (Abbas Zaidi *et al.* 2011, Electricwala *et al.* 1991). Hyaluronidase lowers viscosity of hyaluronan. Therefore, it increases tissue permeability and is used to speed drug absorption (Csoka *et al*. 1997). For the therapeutic importance of salivary components, leeches have been used by maxillofacial and microsurgeons and *Hirudo medicinalis* is the most frequently used species which is under family Hirudinidae (Abbas Zaidi *et al.* 2011).

They move actively by sucking blood from wild animal and human through spring to summer, during the winter they hibernate. They spawn eggs every autumn, even

though they are hermaphrodite, they cannot reproduce by themselves. Such as other earthworm, leeches also use a clitellum for eggs and make the cocoon. Leech looks like earthworm but they have blood-sucking habits. As their feature, they had been adjusted to their habits such as complex pharynx, jaw and distinctive digestive canal. These pharynxes, jaws, alimentary canal, genital structure, the number of eye spots and shape of segments are important criteria of leech identification. For example, five leech species, including *Haemadipsa zeylanica*, *Haemadipsa montana*, *Haemadipsa sylvestris*, *Haemadipsa ornata* and *Haemadipsa dussumieri*, were characterized by Moore (1924) based on various external characters such as color pattern, the presence or absence of furrow pits and a complete or partial annulus between the third and fourth eyespot pairs and a well developed prehensile lobe in the posterior sucker (Borda and Siddall. 2010). Lai *et al*. (2011) also distinguished *Haemadipsa rjukjuana* and *Haemadipsa japonica* by their morphological characters: *H. rjukjuana* has reddish, yellowish or grayish brown dorsum, no strip and 71-72 rays on venter of posterior sucker, while *H. japonica* has red brownish dorsum, mid-dorsal longitudinal dark stripe and about 74-76 rays on their sucker.

Evolutionarily, leeches can be divided into two groups, one is jawed leech (order Arhynchobdellida) and the other is jawless leech (order Rhynchobdellida). The jawed leeches consist of aquatic leech (family Hirudinidae) and terrestrial leech (family Haemadipsidae). Haemadipsidae includes the trignathous (three-jawed) leech which live in the Indian subcontinent (India, Nepal, Pakistan, Sri Lanka), south-east Asia (Myanmar, eastward to Taiwan and south extending to Sumatra) and up to north-east Asia (Japan) and the duuognathous (two-jawed) leech which live in Madagascar,

Australia, Indonesia, Papua New Guinea and South Pacific volcanic islands (Borda and Sidall. 2010). Habitat of *Haemadipsa* is damp forest, therefore, they are sensitive to humid atmosphere and seasonal rainfall (Richardson 1975). Borda and Sidall (2010) suggested that as the India continued this migration northward toward Asia, the precipitation is increasing and the rainforests is being expanded. As the ecological system changes, arthropods density and distribution also changes (Gratz 1999). Research of terrestrial leech has been conducted all over the world. And a new record of terrestrial leech is reported from a nearby country such as Taiwan and Japan. In Korea, 11 leeches which belong to the order Rhynchobdellida and 5 leeches which belong to order Arhynchibdellidae are officially recorded (Table 1). But, there had been no record of terrestrial leech, family Haemadipisdae. Furthermore, the molecular analyses had not been conducted.

Thus, this present study identified species name of terrestrial leech in Korea by morphological aspect, as well as molecular aspect. Furthermore, because leeches are haematophagous ectoparasites such as flea and tick, we investigate what animals was sucked blood by leeches and what pathogens naturally infected the terrestrial leech in Korea.

The studies were composed of three parts. First, in order to determine the species name of the terrestrial leech, morphological and histological analyses were conducted by electron microscope. Second, an in-depth species identification was conducted by molecular analysis. Third, to investigate which pathogens naturally infected the leech, experiments were done was conducted by using several specific primers which can detect zoonotic pathogens.

It would be expected that these results might be used for morphological and molecular feature of Korean blood-feeding terrestrial leech. Furthermore, the present research of pathogen and host animal may contribute to study that potential role of the haematophagous leech as a vector.

Table 1. The list of leeches which are recorded in Korea.

Class Hirudinea	
Order Rhynchobdellida	
Family Glossiphoniidae	*Alboglossiphonia heteroclita* (Linneaus)
	Alboglossiphonia lata (Oka)
	Batrachbdella paludosa (Carena)
	Glossiphonia complanata (Linneaus)
	Glossiphonia weberi Blanchard
	Helobdella stanalis (Linneaus)
	Hemiclepsis japonica Oka
	Hemiclepsis marginata (Muller)
	Toryx tagoi (Oka)
Family Pisciolidae	*Trachelobdella livanovi* Oka
	Trachelobdella sinensis Blanchard
Family Hirudinidae	*Hirudo mipponica* Whitman
	Whitmania acranulata Whitman
	Whitmania edentula Whitman
	Whitmania pigra Whitman
Family Erpobdellidae	*Erpobdella limeata* Muller
Family Haemadipsidae	*Haemadipsa rjukjuana* (Docksilsan leech)*

*a newly recorded leech which was founded in this study

CHAPTER I

Morphological study of blood-feeding terrestrial leech, *Haemadipsa rjukjuana* (Hirudinida: Arhynchobdellida: Haemadipisidae) at Gageodo in Korea

1. Introduction

The majority of blood-feeding terretrial leeches are about 50 species which belongs to the family Haemadipisidae described terrestrial leech species and about 10 species are in the family Xerodellidae (Sket and Trontelj 2008). The researchers suggested that about 30 genera belong to several families which is including Haemadipisidae are distinguished between blood-feeding land leeches, which only have two jaws. However, the family Haemadipisidae involves 17 genera which are divided into the duognathous (two jaws) and trignathous (three jaws) series, and include all the land leech species (Sawyer 1986). The taxonomic identification of blood-feeding land leeches was recently approached through the morphological studies (Trontelj *et al*. 1999). Based on these studies, the terrestrial leeches are separated into two families; Haemadipsidae and Xerobdellidae. The pharynx, jaw and mouthpart of blood-feeding leech have evolved, unlike other Oligochaeta which has a simple digestive canal, in response to adaptation to carnivorous and parasitic environment (Kang 1995). So their structure of digestive canal, reproductive organ and sucker as well as shape, number of eye and segment is important morphological feature which is used for classification of leech.

This study described the morphological characteristics of Docksilsan leech from Korea using gross, light and scanning electron microscope.

2. Materials and methods

2.1. Study sites

During July 25 to 28 of 2011, 40 leeches were collected on Mt. Docksil (a.s.l. 639 m, E 125°07′, N 34°04′) on Gageodo, Sinan-gun, Jeollanam-do of Korea (Fig. 1). The island is located at the southwestern part of Mokpo (136 km far from Mokpo) and its geographical coordinates is between N 34°06′ and 35°02′ and between E 125°05′ and 125°09′ (Fig. 1).

2.2. Sample collection and preservation

Collection was performed by walking along the forest path to attract leeches, and then the leeches on legs or other parts were removed with tweezers as soon as possible. The collected samples were preserved alive in falcon tube and kept in a cool humid state. Of the 40 leeches, 6 specimens were used to morphological and histological study.

2.3. Electron microscopic examination

The leeches were tentatively identified under the light microscope, before precise classification suing scanning electron microscope (SEM). For light microscopy, the specimens were placed in the lacto-phenol solution (20 ml glycerin, 10 ml lactic acid, 10 ml phenol, 10 ml D.W.) for 24 h. For the SEM, the parasites were washed five times with 0.2 M cacodylaye buffer (pH 7.3), fixed in 2.5 % glutaldehyde and post fixed in 1 % osmium tetroxide at 4℃. The samples were dehydrated in a graded ethyl alcohol series,

dried by CO_2 critical point, coated with gold and examined by SEM (S-4800, Hitachi) at 15kV.

2.4. Morphological classification

In order to morphological classification of Docksilsan leech, the number of jaw, ray and eye spots is used for identification depending on criteria of leech classification in published paper (Ngamprasertwong *et al*. 2007, Borda *et al*. 2008).

3. Results

3.1. External finding by gloss

This leech is recognized by the reddish, brownish yellow and gray body color with irregular black sports. The median stripes are not distinctly found. The nearly solid black venter with irregular margins is clearly distinguished this leech from other terrestrial leech species (Fig. 2 A, B and E). In fixative sample, body length measured in 8 mm (mean), maximum body width 2.4 cm, anterior sucker diameter 0.45 X 0.34 mm, posterior sucker diameter 1.68 X 1.58 mm. When the leeches were fixed in 100 % alcohol, they were stretched (Fig. 2 C and D). The leeches have two suckers at anterior and posterior parts of body (Fig. 2 F and G).

3.2. External finding by SEM

Under the SEM, the leech was covered by annuli (or segment) and proboscis pore and eye spots were on the anterior sucker (Fig. 3). The eye consists of 5 pairs, arranged respectively at II (2^{nd} annulus), III (3^{rd} annulus), IV (4^{th} annulus), V (5^{th} annulus) and VI (8^{th} annulus) in parabolic arc (Fig. 4). Anterior sucker circularly cumuliform with lateral buccal lobes and frill. Posterior sucker with 71-72 friction rays are circular, diameter leach to maximum body width, with definite radial prominence (Fig. 5 A). A pair of auricle is located at the front of the posterior sucker, trilobate with middle lobe smallest (Fig. 5 B and Fig. 6); Body is slenderly cylinderical and elongated with dorsum moderately depressed from the end of body to the head and venter more less flat in relaxed specimens.

3.3. Internal finding by light microscopy

Three jaws are located in the Y-shaped lumen, crescent shaped, with many dentigrous element, in deep buccal cavity beyond velum. The basement of eye spot is infiltrated with melatonin pigment (Fig. 7 and Fig. 11). The specimen which described in Figure 7 was divided into 13 vertical sections. Each of the sections was numbered (Fig. 7).

External muscle layer was composed of circular muscle and internal muscle layer insisted of longitudinal muscle. This muscle layer is traditional muscle composition of Annelida. There were 3 jaws which were arranged with dentigrous elements and intestine is divided into 6 muscular ridges in which three continuous unicellular salivary glands (Fig 8). A pair of ovary located laterally at the 1/3 portion from head part and the nerve cord ventrally, lateral sinus and several pair of testis laterally (Fig. 9). Clop with 2 bland sacs is reached the posterior end of the leech (Fig. 10).

4. Discussion

The order Hirudinea includes 4 suborders: Acanthobdellae, Rhynchobdellae, Gnathobdellae (=Arhynchobdellae), Pharyngobdellae. The Acanthobdellae comprises the single genus Acanthobdella which is a small fish parasite (Sket and Trontelj 2008). The Rhynchobdellae are jawless, marine and freshwater forms, none is terrestrial. The Pharyngobdellae are freshwater or amphibious leeches which have lost the power of penetrating the tissues of a host and sucking blood. The Gnathobdellae, the jawed leeches armed with teeth, there is little to be said about the anatomy of members of the family Hirudidae, since *Hirudo*, a typical and widespread form, has been fully described. This order includes 2 family: Hirudidae and Haemadipisidae. They are blood-sucking forms which attack man and animals, lurking on vegetation in damp places. Within this order, the family Hirudidae is characterized by aquatic leeches and the family Haemadipsidae refers to terrestrial leeches. There are about 60 described terrestrial leeches species, 50 of which belong to the Family Haemadipsidae, while the rest are in the Family Xerobdellidae. Oka (1910) first named *Haemadipsa japonica* var. *rjukjuana,* a new land leech from Taiwan, based on a brief inspection of the external color pattern. *H. rjukjuana* is only recorded in East and South Asia, including the Indo-Chinese Peninsula, Malay Peninsula, Indonesia, Ryukyu Islands of Japan and Taiwan. *Haemadipsa sylvestris*, the Indian leech and *Haemadipsa zeylanica* (Yamabiru), the Japanese mountain or land leech are a member of the Haemadipsidae.

The outstanding characteristic of the Haemadipisidae is that they have become better adapted to terrestrial life than any other kind of leech. Because of their adjustment, they have distinctive morphological feature. The pharynxes, jaws, alimentary canal,

genital structure, the number of eye spots and shape of segments are representative criteria of leech identification. For example, five leech species, including *Haemadipsa zeylanica, Haemadipsa montana, Haemadipsa sylvestris, Haemadipsa ornata and Haemadipsa dussumieri*, were charaterized by Moore (1924) based on various external characters such as color pattern, presence or absence of furrow pits and a complete or partial annulus between the third and fourth eyespot pairs and a well developed prehensile lobe in the posterior sucker (Borda and Siddall 2010). Lai *et al*. (2011) also distinguished *Haemadipsa rjukjuana* and *Haemadipsa japonica* by their morphological characters: *H. rjukjuana* has reddish, yellowish or grayish brown dorsum, no stripe and 71-72 rays on venter of posterior sucker, while *H. japonica* has red brownish dorsum, mid-dorsal longitudinal dark stripe and about 74-76 rays on their sucker.

The Docksilsan leech in this study has features of *H. rjukjuana* species described by Lai *et al*. (2011) color pattern; dorsum reddish, yellowish or grayish with scattered elongated, more or less conntected lateral-posteriorly, irregular black spot, no stripe, 2) number of rays on venter of posterior sucker; 71-72, 3) epididymis; separated. Furthermore, some additional detection supports that this leech is *H. rjukjuana*. e. g. respiratory auricle are located at the front of the posterior sucker, trilobate with middle lobe smallest and the head part with sub-triangular pattern has a dorsal anterior sucker and the cuticle is covered with 97 even annuli in total. Eyes five paors, punctiform, arranged at 2^{nd}, 3^{rd}, 4^{th}, 5^{th} and 8^{th} annulus in parabolic arc and the 2 annuli are inserted between the fourth and fifth eye spot (Fig. 4 C). The Docksilsan leech has trignathous jaws with many dentigrous element which is an identification key of the family Haemadipsidae.

To sum it up, the morphological feature of Docksilsan leech such as internal organ, eye spot, jaw and respiratory auricle shows that the leech belongs to family Haemadipsa, especially *H. rjukjuana*. But morphological structure has so variation through each of the specimens that a specific species level identification of leech is limited. Therefore, molecular identification was conducted which described in next chapter to confirm the result of that the Docksilsan leech is closest to *H. rjukjuana*.

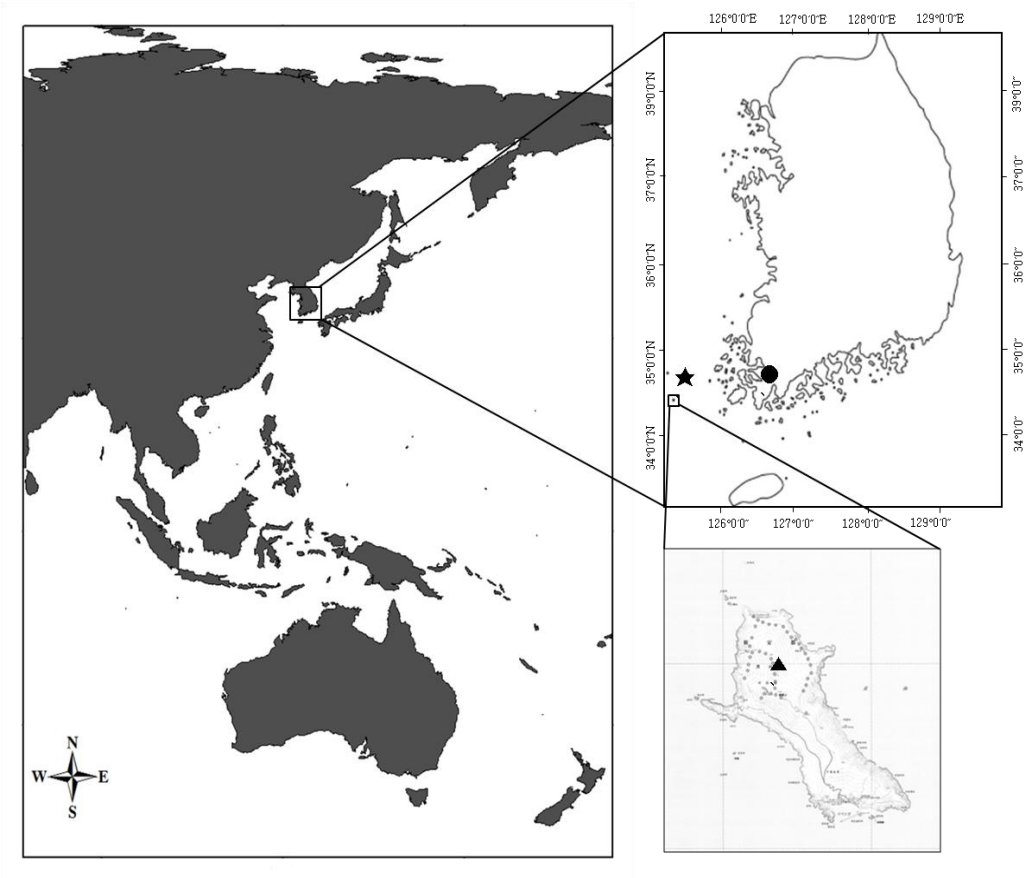

Fig. 1. Mt. Docksil (▲, altitude 639m) in Gageodo (Island, 125° 7″E, 34° 4″N), Korea. Gageodo is at Sinan-gun, Jeollanam-do and Korea`s south-western most island. Mt. Docksil is the highest peak in the Sinan-gun. **A,** the geographical map of Asia; **B,** the location of Mokpo (●), Heuksando (★) and Gageodo in Korea. Gageodo is 136 km distant from Mokpo and 70 km from Heuksando; **C,** a map showing the topography of the Gageodo and Mt. Docksil (▲).

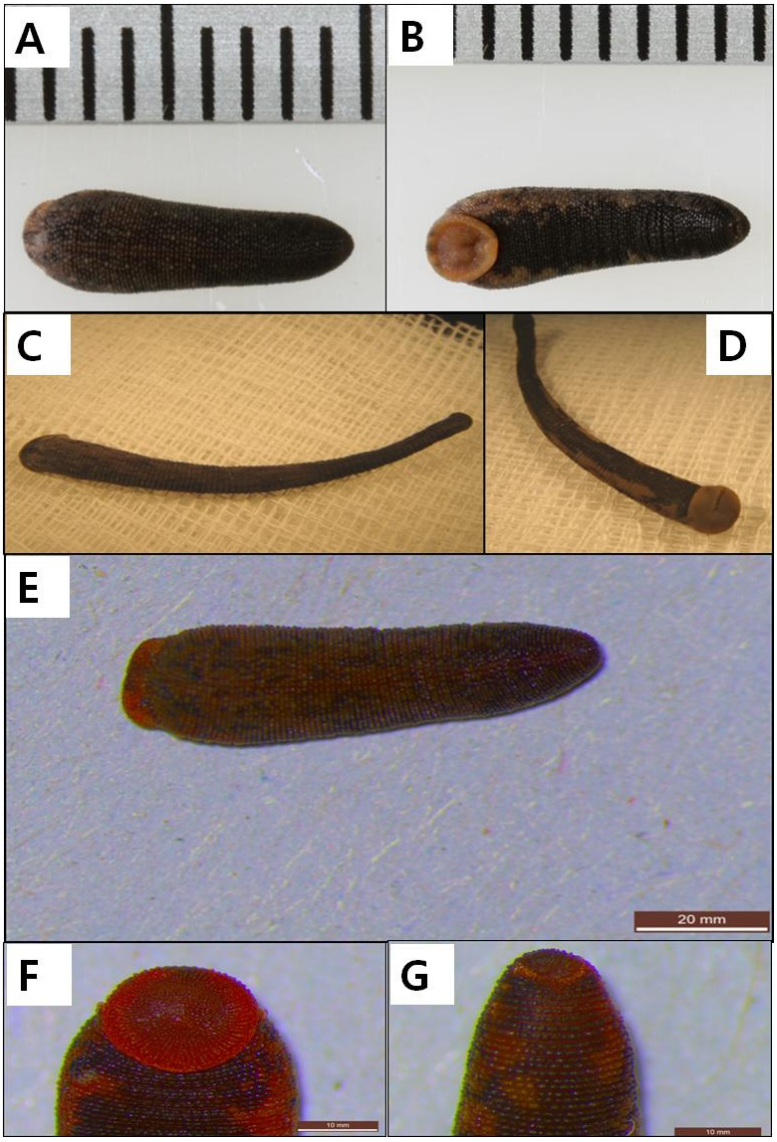

Fig. 2. The external feature of Docksilsan leech (*Haemadipsa rjukjuana*) under the light microscope. A, dorsal view; **B,** ventral view; **C and D,** specimens which fixed in 100% alcohol; **E,** dorsal view under the light microscope; **F and G,** posterior and interior suckers under the light microscope.

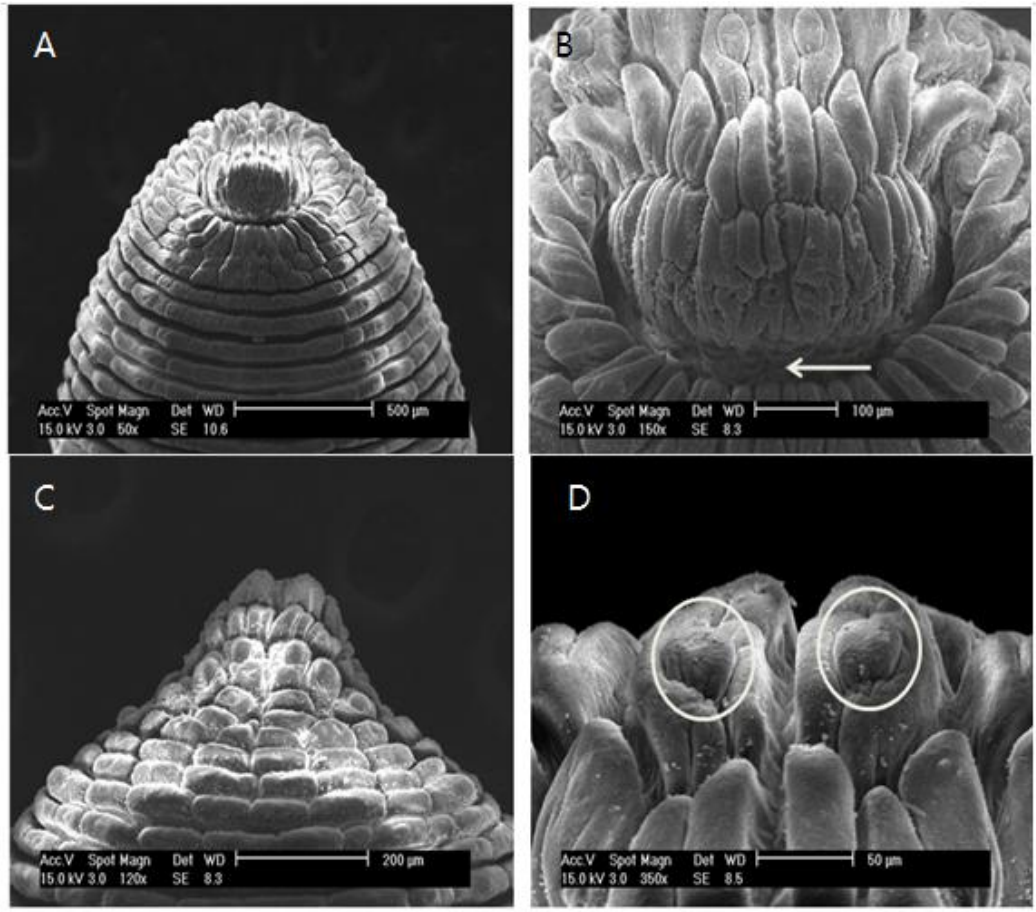

Fig. 3. The external feature of Docksilsan leech (*Haemadipsa rjukjuana*) under the electron microscope (SEM). A, The front part of dorsal view; **B,** Proboscis pore on interior sucker (arrow); **C,** The front part of ventral view; **D,** The first eye pair at interior part (circles).

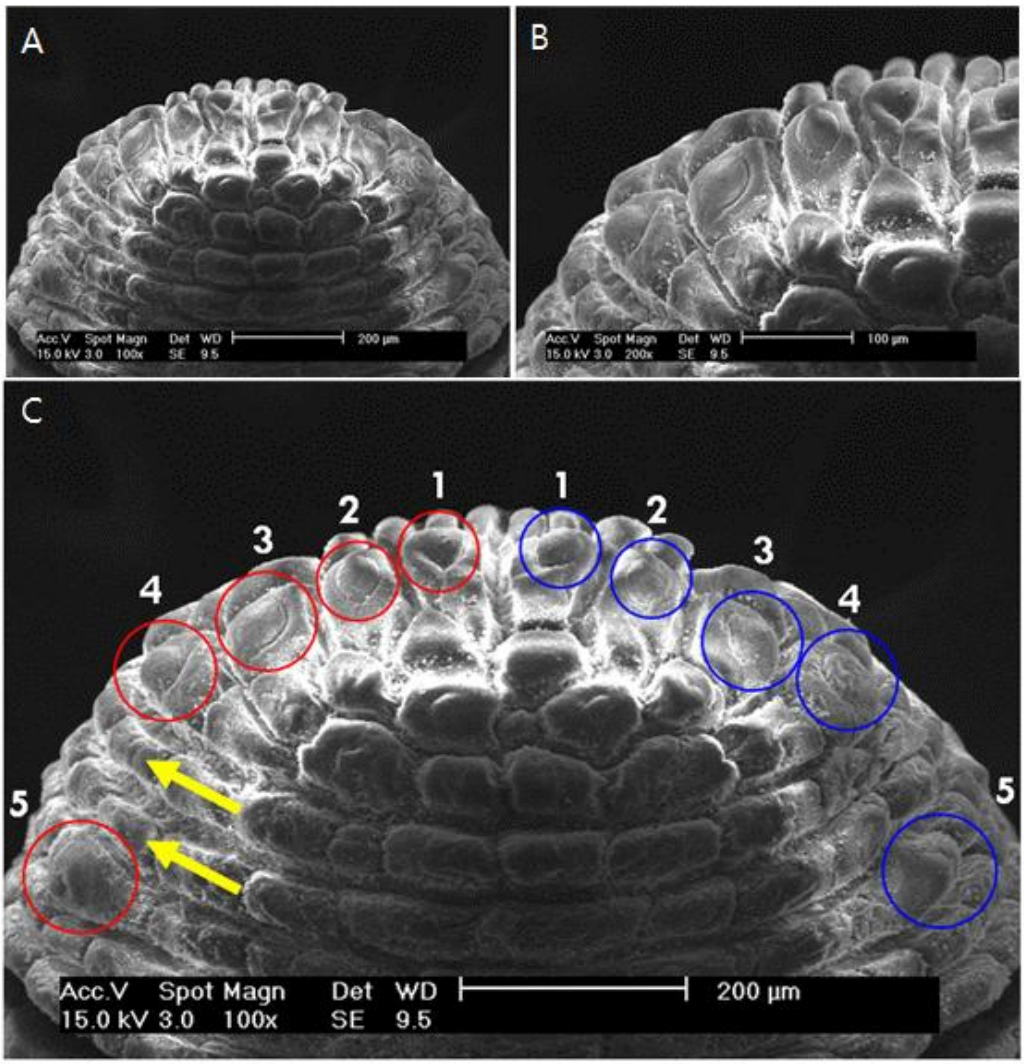

Fig. 4. The feature of eye spots of Docksilsan leech (*Haemadipsa rjukjuana*) under the electron microscope (SEM). A, The head part of ventral view; **B,** The enlarged photo of (A); **C,** Five pairs of eye spots (circle). The 4th and 5th eyes were divided by 3 annuli (arrows).

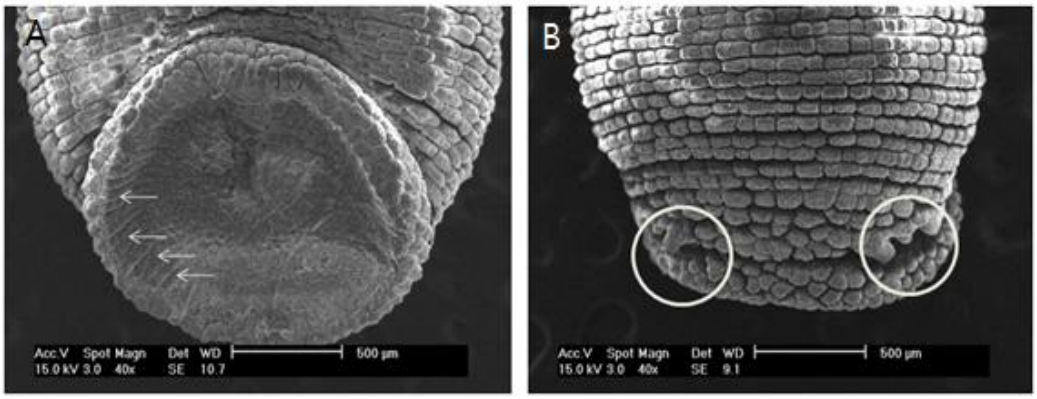

Fig. 5. The posterior part of Docksilsan leech (*Haemadipsa rjukjuana*) under the electron microscope (SEM). A, posterior sucker. They consist of 72-72 radial rays; **B,** respiratory auricle on ventral part.

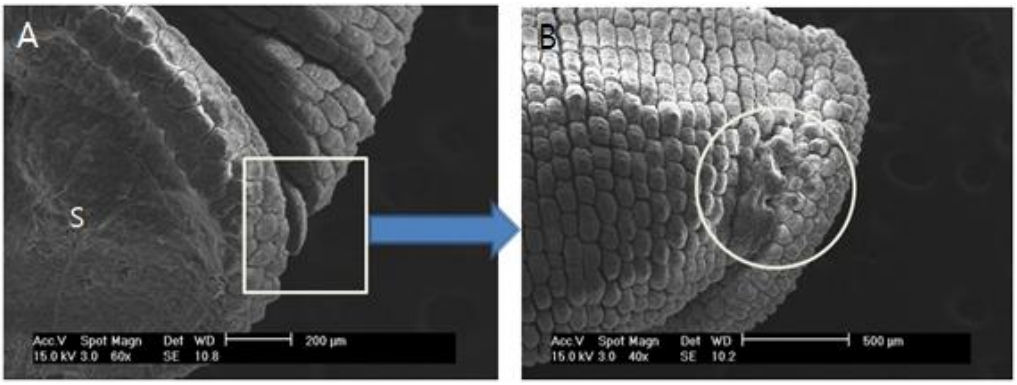

Fig. 6. Respiratory auricle of Docksilsan leech (*Haemadipsa rjukjuana*) under the electron microscope (SEM). A, respiratory auricle which is at near posterior sucker (square); **B,** the respiratory auricle in flank.

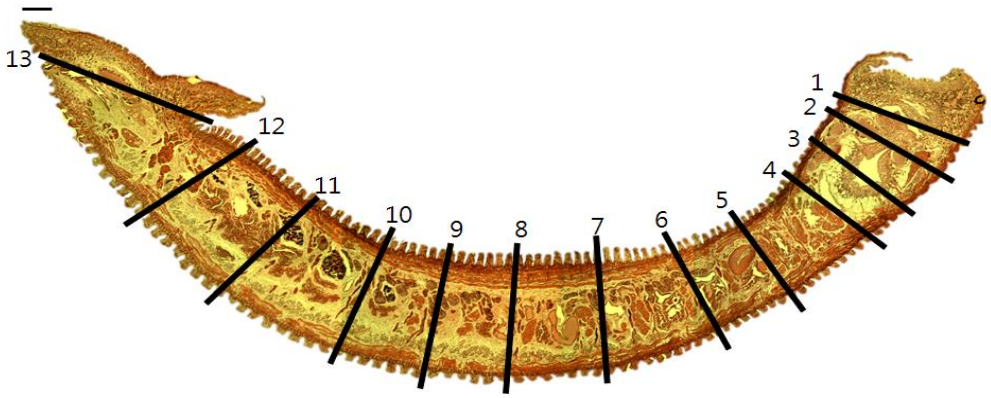

Fig. 7. Longitudinal section of Docksilsan leech (*Haemadipsa rjukjuana*). In order to examine the vertical section, they divided into 13 parts (No. 1 ~ No. 13). H-E stained. Bar=200μm.

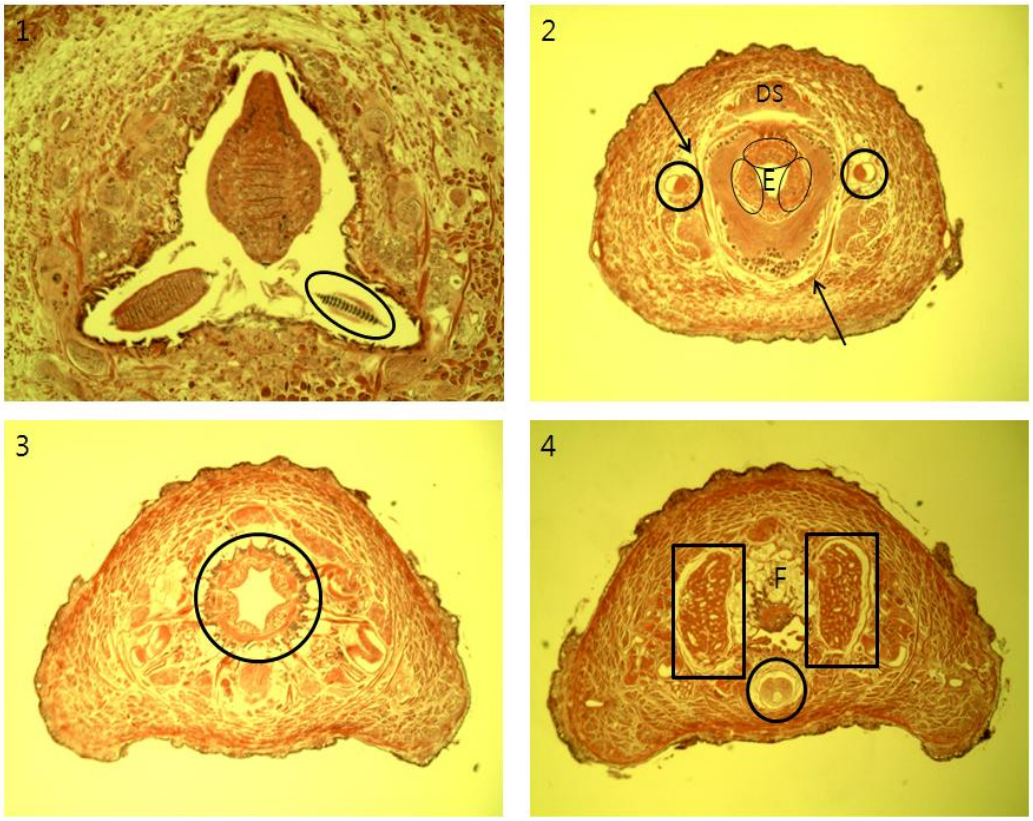

Fig. 8. Vertical sectioned view of Docksilsan leech (*Haemadipsa rjukjuana*) (No. 1~ No. 4). 1, Three jaws in the Y- shaped lumen; **2,** Esophagous with divided 3 muscles (circles). E=esophagial lumen. bold circles=lateral sinus, DS=dorso-ventral muscle; **3,** intestine. muscles divided into 6 parts (circle); **4,** nerve cord (circle) and ovary (square). F=fat tissue. H-E stained. Bar=200μm.

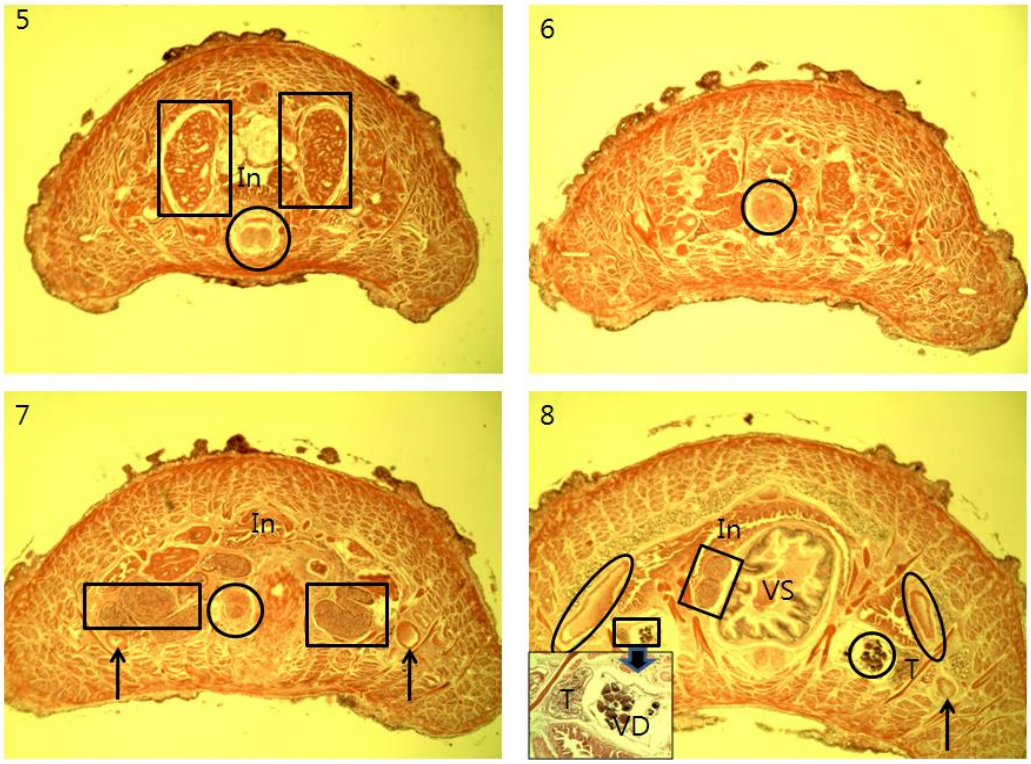

Fig. 9. Vertical sectioned view of Docksilsan leech (*Haemadipsa rjukjuana*) (No. 5~ No. 8). 5, Ventral nerve cord (circle) and Ovary (square). In=Intestine. **6,** Nerve cord (circle) **7,** Nerve cord (circle), Epididymis (square) is highly asymmetrical. Lateral sinus (arrow) **8,** VS=Vaginal sac, Lateral sinus (arrow), Crop diverticulum (long circle). T=Testis, VD=Vas deferens (circle). Salpinx (square). H-E stained. Bar=200μm.

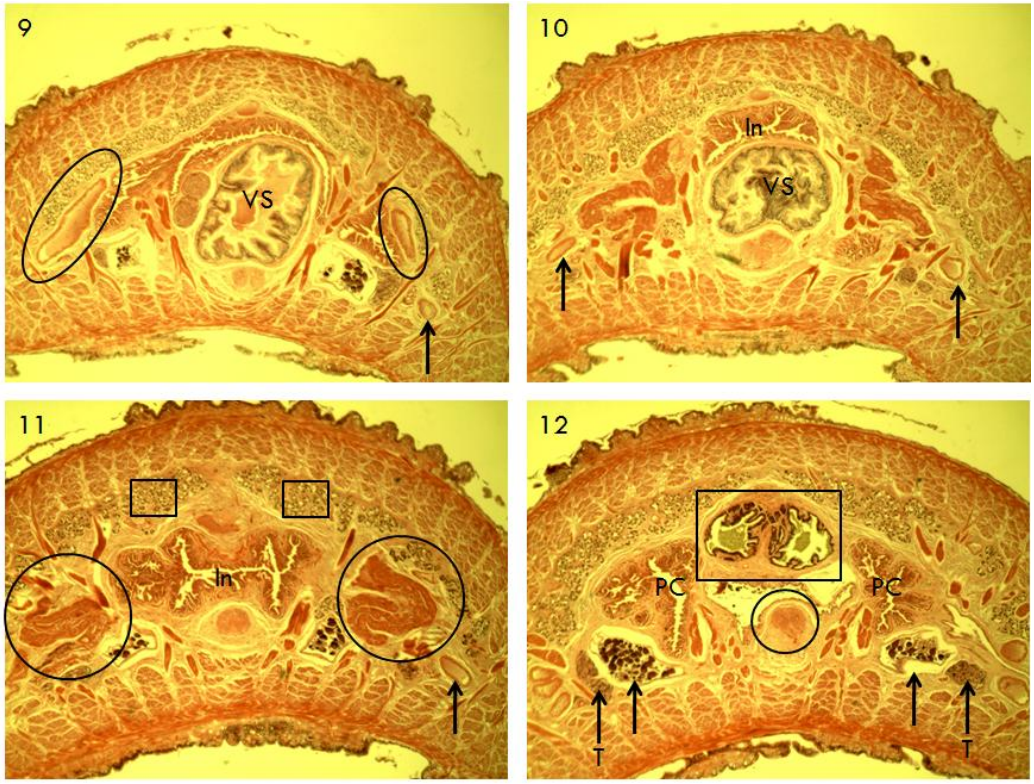

Fig. 10. Vertical sectioned view of Docksilsan leech (*Haemadipsa rjukjuana*) (No. 9~ No. 12). 9, Crop diverticulum (circle), Lateral sinus (arrow) **10,** VS=Vaginal sac, Lateral sinus (arrow), Fat tissue (square). **11,** Epididymis (circle), Lateral sinus (arrow). **12,** Intestine (square), Nerve cord (circle), PC=Post caeca, Vas deferens (arrow), T=Testis. H-E stained. Bar=200μm.

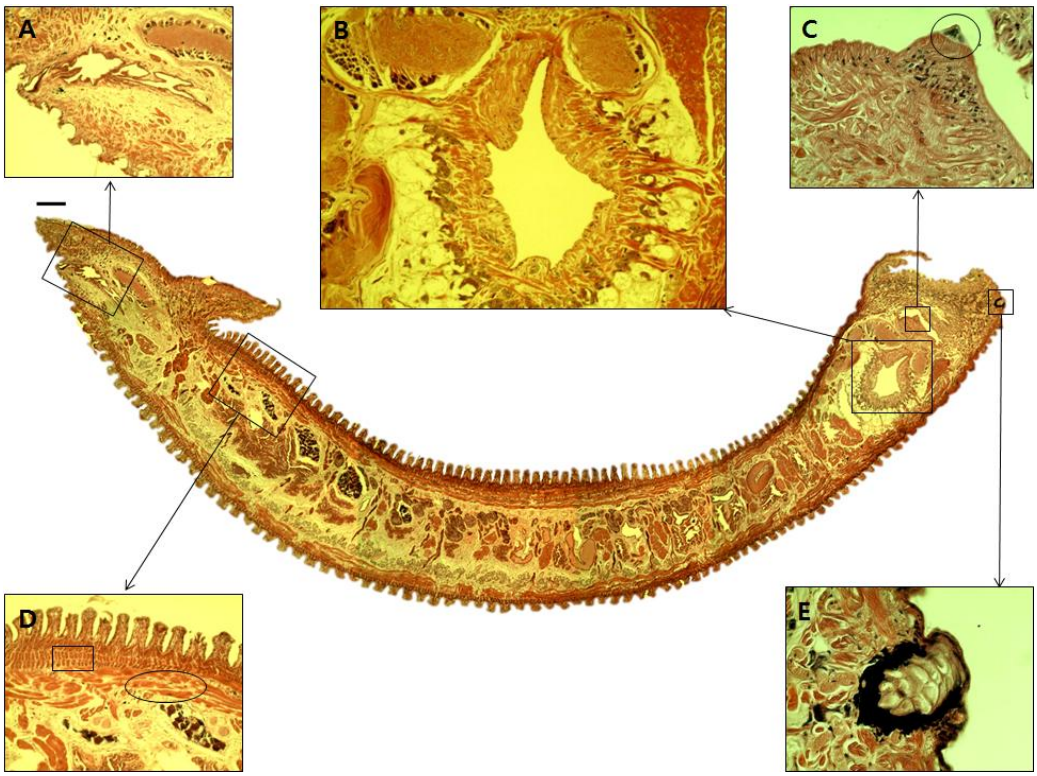

Fig. 11. Longitudinal section of leech with H-E stain. 1, Respiratory auricles. **2,** Esophagus. Simple cuboidal epithelium. **3,** Jaw. Note the carcified dentigerous ridge (circle). **4,** Cuticle and muscle. Note the outer circular (square), inner longitudinal (circle) and dorso-ventral muscle layer (arrow). **5,** Eye spot. Note the melatonine stained with black. Bar=200μm.

CHAPTER II

Molecular identification of blood-feeding terrestrial leech, *Haemadipsa rjukjuana* (Hirudinida: Arhynchobdellida: Haemadipisidae) at Gageodo in Korea

1. Introduction

Leeches are segmented worms belonging to the phylum Annelida. Unlike other oligochaeta such as earthworms, they have two suckers. The majority of leeches inhabits in freshwater environments, but may also reside in terrestrial environments. They are generally classified into two groups: Rhyncobdellae (jawless leeches) and Arhynchobdellae (jawed leeches). Jawed leeches are known for blood-feeding habits and can be found in terrestrial and aquatic environments. Blanchard (1896) established Haemadipsidae to distinguish blood-feeding terrestrial leeches from their aquatic sanguivorous and carnivorous counterparts in Hirudininae (Borda *et al*. 2008). In addition, blood-feeding terrestrial leeches are also separated into two families, Haemadipisidae and Xerobdellidae (Sket and Trontelj 2008) based on their phylogenetic analyses (Trontelj *et al*. 1999; Borda and Siddall 2004; Kutschera *et al*. 2007; Borda *et al*. 2008).

Terrestrial leeches belonging to the family Haemadipisidae are terrestrial haematophagous leeches. They exhibit high biodiversity especially in tropical regions with high humidity, such as the damp jungles and forests of South Asia, Southeast Asia and Australia. *Haemadipsa*, trignathous haemadipsid, is mostly abundant in South East Asia (Ngamprasertwong *et al*. 2007). Despite its high abundance throughout the range

countries, diversity and ecology of *Haemadipsa* leeches have been poorly understood. Recent studies (Ngamprasertwong *et al*. 2007; Lai *et al*. 2011) have been conducted to identify *Haemadipsa* leeches at species level. Lai *et al*. (2011) identified a new species using DNA barcoding which has been widely used (Lai *et al*. 2009; Siddall *et al*. 2007; Bely and Weisblat 2006). DNA barcoding system for animal species identification applies cytochrome *c* oxidase subunit I (COI) sequences as a genetic marker (Hebert *et al*. 2003). Additionally, other studies analyzed distantly related lineages of blood-feeding terrestrial leeches using nuclear 18S rNRA and 28S rRNA marker (Borda *et al*. 2008).

The presence of *Torix tagoi* Oka, *Hirudo nipponia* Murada and *Whitmania pigra* Murada were first reported in Korea during the first half of the 20th century (Kang 1995). In addition, *Glossiphonia weberi* and *Hemiclepsis japonica* were recorded by Paik and Park (1992). However, they described only freshwater leeches and did not apply molecular analysis. It has been known that some terrestrial leeches are living in islands of Korea , particularly in Gageodo, but yet to be documented scientifically. The goal of this study is to identify the taxonomical status of the terrestiral leeches newly found in the Gageodo of Korea based on molecular analysis. This is the first report on the presence of the terrestrial leech species in Korea.

2. Materials and methods

2.1. Study sites

During July 25 to 28 of 2011, 40 leeches were collected on Mt. Docksil (a.s.l. 639 m, E 125°07′, N 34°04′) on Gageodo, Sinan-gun, Jeollanam-do of Korea (Fig. 1). The island is located at the southwestern part of Mokpo (136 km far from Mokpo) and its geographical coordinates is between N 34°06′ and 35°02′ and between E 125°05′ and 125°09′ (Fig. 1).

2.2. Sample collection and preservation

Collection was performed by walking along the forest path to attract leeches, and then the leeches on legs or other parts were removed with tweezers as soon as possible. The specimens were preserved in 70% alcohol solution for DNA extraction. DNA was extracted from 29 of 40 leeches were used for PCR.

2.3. DNA extraction and PCR amplification

The DNeasy Blood & Tissue Kit (Quiagen) was used for lysis of tissue and DNA extraction. The extracted DNA was stored at -20℃. PCR amplifications of 18S rRNA and COI gene fragments were carried out using the primers listed in Table 2. The PCR amplification of 18S rRNA gene was performed by nested PCR. The initial PCR amplification was conducted using primers “A” and “B”, yielding a 1.8 kb fragment. The subsequent PCR amplification was performed using two sets of nested primers “A” and “Y” or “C” and “B” to yield two short overlapping DNA fragments with

approximately 1.2 kb in length each (Apakupakul *et al*. 1999). All PCRs were conducted using the *HiPi* PCR PreMix (Eplis-Biotech Inc.) in 20 ㎕ total volume. Amplification reactions consisted of 1unit/4 ㎕ *HiPi* Thermostable DNA polymerase in 250 mM Tris-HCl (pH 9.0), 80 mM $(NH_4)_2SO_4$, 10% DMSO, 8.75 mM $MgCl_2$, 0.05% bromophenol blue, 12% glycerol, stabilizer, 0.5 mM each primers and 10-15 ng of DNA. All PCR reactions were performed in a PTC-200 thermal cycler (MJ Research, Inc., Ramsey, Minnesota). Amplified PCR products were separated by electrophoresis on 1.5% agarose gel and visualized with Ethidium Bromide under a SL-20 Image visualizer™ (Seolin Sceintific Co., Ltd, Seoul, Korea)

2.4. DNA sequencing

The method of Sanger was used to PCR products sequencing by BigDye Terminator v3.1Cycle Sequencing Kit and ABI 2720XL DNA analyzer (Applied Biosystems, Foster City, CA, USA). For each reaction, PCR product DNA were mixed the reagent of terminator kit and cycle sequencing were performed for 1 cycle at 95℃ for 1min 30sec, and then 25 cycles at 95℃ for 30sec, 50℃ for 5sec, 60℃ for 4min. Clean up cycling reactions were conducted as follows. First, added 180 ul magnetic bead to each sequencing reaction and incubated at room temperature for 5 minutes. Placed the plate onto the magnetic plate to capture the particles and removed and discarded the liquid. Second, washed the plate using 100 ul 90% ethanol and incubated at room temperature for 5 minutes. Repeated this step for a total of two washes and placed them to the room temperature for 10 minutes for air dry. Third, added elution

solution and transferred purified sequencing reactions to a clean 96 well plate. Finally, ran on sequencer ABI 3730XL (Capillary array length: 50cm)

2.5. Phylogenetic analyses

The GenBank accession numbers of 18S rRNA gene and COI gene sequences related to Haemadipsoid leeches were listed in Table 3. For 18S rRNA sequences, the two separated fragments (1.2 kb for each) were edited and reconciled using BioEdit version 7.0.9.0 (Hall 1999). All sequences were manually edited at least three times. Alignment of sequences was carried out using MultiAlign (Corpet 1988). Consequently, both of 1.7 kb of 18S rRNA and 649 bp of COI sequences were used for species identification and phylogenetic analysis. Analyses of the sequences were comparatively completed using the Blast Search option for 18S rRNA and COI sequences of leeches in GenBank database. The phylogenetic relationships between haplotypes of the leeches were reconstructed using the neighbor-joining (NJ) method (Saitou and Nei 1987) under the Kimura 2-parameter (K-2-P) model (Kimura, 1980). As references, corresponding sequences were selected from GenBank database (Table 3). Also, corresponding sequences (n = 8) were used to root the phylogenetic tree as outgroup (Table 3). Confidence in estimated relationship was determined by using the bootstrap approach (Felsenstein 1985) obtained through 1,000 replicates with the same model as mentioned above. Both bootstrap analysis and phylogeny reconstruction were conducted using MEGA version 4.0 (Tamura *et al*. 2007).

3. Result

To determine the species identity and evaluate the genetic relationship of the terrestrial leeches in the Gageodo, both 1.8 kb of 18S rRNA and 710 bp of COI gene sequences were amplified from leech samples (Fig. 12). Of the 29 samples, 26 and 21 sequences were obtained for 18S rRNA and COI gene, respectively. The remaining samples were failed to yield clear sequences or proved to be other species, rather than *Haemadipsa* spp. (data not shown).

For determining the species identity of the Docksilsan leech, all sequences of the leeches were checked using the Blast search from GenBank database. The results showed that 18S rRNA nucleotide sequences have 99.9% identity to the *H. rjukjuana* isolate HARY (from Taiwan, HQ203097). They differed in only two base pairs of 1,706 bp. COI nucleotide also showed a similar pattern. The sequences were separated into two types, *H. rjukjuana* COI type A and COI type B. Among 21 COI sequences that we acquired, type A sequences were 13 and type B sequences were 8. The type A and type B showed 94.6% (614/649 bp) and 94.3% (612/649 bp) similarity to the reference sequence of the *H. rjukjuana* L00115A (from Taiwan, HQ322443), respectively.

For evaluating the genetic relationship of the Docksilsan leech, both 18S rRNA and COI gene nucleotide sequences were independently analyzed with other reference sequences available in GenBank database. The NJ trees of terrestrial leech 18S rRNA and COI supported that *H. rjukjuana* is monophyletic in a single clade with high bootstrap values. For 18S rRNA gene (Fig. 13), the genetic distance (*P*s) between *H. rjukjuana* Gageo and the *H. rjukjuana* HARY was 0.1%. In contrast, the *P*s between the *H. rjukjuana* HARY and the *H. sylvestris* and between *H. rjukjuana* Gageo and the *H.*

sylvestris were 0.4 and 0.5%, respectively. For COI gene (Fig. 14), the *P*s betweeen the *H. rjukjuana* L00115A and the *H. limuma* HACH is 14.6%. However, the *P*s between the *H. rjukjuana* L00115A and Docksilsan leech 1 or 27 were 5.6 and 6.0%, respectively. The pylogenetic tree also revealed COI sequences of the Docksilsan leeches had the closest relationship with *H. rjukjuana* in Taiwan.

4. Discussion

In general, leeches are divided into two groups, Rhynchobdellida Blanchard (jaw-less) and Arhynchobdellida Blanchard (jawed). Arhynchobdellid leeches have a muscular jaw for feeding and are classified into the Erpobdelliformes Caballero and the diverse Hirudiniformes Caballero (Borda 2007). Hirudiniformes are subdivided into five families: Americobdellidae Canallero, Cylicobdellidae Ringuelet, Haemadipsidae Blanchard, Haemopidae Richardson, and Hirudinidae Whitman (Sawyer 1986; Borda 2007). The remaining blood-feeding Hirudiniformes can be classified depending on their habitat preference as the semi-aquatic Hirudinidae and the terrestrial (or land) Haemadipsidae (Borda 2007). Also, Saywer (1986) further consolidated Haemadipsinae with Haemadipisidae to create a single family, which is divided into the dougnathous (two jawed) and trignathous (three jawed) series, and included all terrestrial leech species. The trignathous leeches of the genus *Haemadipsa* Tennent (1859) are widespread throughout the Indian subcontinent into south-east Asia and up to north-east Aisa (Borda and Siddall 2010).

Troxi tagoi Oka (1925), *Hirudo nipponia* Murada (1936), *Whitmania pigra* Murada (1936), *Glossiphonia werevi* and *Hemiclepsis japonica* have been documented in Korea (Paik and Park 1992). However these previous studies focused on morphological features of freshwater leeches but not terrestrial leeches (Park and Kim 1989). It has been previously known that terrestrial leeches inhabit in the Gageodo in Korea but none of researchers have done their taxonomy. This study documented the capture of terrestrial leeches on the Mt. Docksil of the Gageodo and their identification at species level. The Gageodo is a west-southernmost island of Korea and located over the

migratory routes of birds moving from Southeast Asia to Siberia. The total area and coastline length of the Gageodo is 9.18 km^2 and 22.0 km respectively. The island which is composed of rocks and evergreen forests has a maritime climate. Although, there is no weather station on Gageodo, according to the Heuksando weather station which is the most nearest location from Gageodo with 70 km away, the mean temperature is 3.3 to 6.0℃ during winter and 22 to 27℃ during summer. The annual average temperature is 13.6℃ and the annual total precipitation is 1,125.9 millimeters. From May to August, there are lots of foggy weathers and the fog tends to spread locally. Especially, Mt. Docksil (a.s.l. 639 m) in the Gageodo is the highest mountain in Sinan-gun and its internal forest generally shows a very humid condition throughout the year. Generally, terrestrial leeches are known to inhabit in tropical regions with high humidity, and in this regard the Mt. Docksil serves as an ideal habitat for leeches. The terrestrial leeches on the Mt. Docksil possess an interior and a posterior sucker and move like extremely elastic inchworm. They suck the blood of human legs for about 30 minutes, after their blood-feeding, a bleeding scar is formed. They were regarded as the family Haemadipsidae but there were no preliminary data comparable to other terrestrial leeches in Korea.

As the result of 18S rRNA gene analysis of the Docksilsan leeches (n=28/29 leeches), 26 sequences had 99.9% similarity to the *Haemadipsa rjukjuana* isolate HARY (from Taiwan, HQ203097) and the other two sequences were closer to the *Orobdella tsushimensis* (from Japan, AB663653, data not shown). As the result of COI gene analysis (n=24/29 leeches), 21 were closest to the *H. rjukjuana*. The 21 sequences were further divided into two types, Gageo 1 and Gageo 27, by only 1 bp. Both

haplotypes had a 94.6% or 94.3% similarity to the *H. rjukjuana* L00115A (from Taiwan, HQ322443). The remaining three sequences were similar to the *O. tsushimensis* (Janapn, AB679660, data not shown). For both genetic markers, the same result was obtained using COI gene.

Also, the phylogenetic trees also showed that the Docksilsan leeches were closest to *H. rjukjuana* (Fig. 13 and 14). It is not clear when the sanguivorous leeches start living on the Gageodo, but one possible hypothesis is that they were spread from other regions by human (e.g., fisherman or tourists) or animals (e.g., migratory birds). However, further surveys are required to prove these hypotheses. In addition, according to distribution map of Haemadipisidae (Borda and Siddall 2010), sub-distribution of the genus *Haemadipsa* which have three jawed are widespread throughout India subcontinent into Southeast Asia and up to Northeast Asia. But the boundary line of Northeast Asia is lined up to Japan. According to result of this study, the northern boundary line should be extended into Korea.

The present study supports the presence of blood feeding terrestrial leeches with a high genetic similarity to *H. rjukjuana* in Korea

Table 2. Nucleotide sequences of polymerase chain reaction primers and conditions for amplification of *Haemadipsa rjukjuana* 18S rRNA and COI gene

Target genes	Name of PCR primers	Primer sequences (5`-3`)	PCR product size	References
Nuclear 18S rRNA	A	AACCTGGTTGATCCTGCCAGT	1.2 kb	Apakupakul et al, (1999)
	Y	CAGACAAATCGCTCC		
	C	CGGTAATTCCAGCTC	1.2 kb	
	B	TGATCCTTCCGCAGGTTCACCT		
Mitochondrial COI	LCO 1490	GGTCAACAAATCATAAAGATATTGG	710 bp	Folmer et al. (1994)
	HCO 2198	TAAACTTCAGGGTGACCAAAAAATCA		

Table 3. Collection localities and GenBank accession numbers which were used for phylogenetic analyses of haemadipsoid leeches.

Taxon	Locality	GenBank accession No.	
		18S rNRA	COI
Haemadipsa rjukjuana Gageo (In this study)	Gageodo, South Korea	KC524508	-
Haemadipsa rjukuana Gageo 1(In this study)	Gageodo, South Korea	-	KC524509
Haemadipsa rjukjuana Gageo 27(In this study)	Gageodo, South Korea	-	KC524510
Haemadipsa rjukjuana L00112A	Taiwan	-	HQ322438
Haemadipsa rjukjuana L00115A	Taiwan	-	HQ322443
Haemadipsa rjukjuana L00098A	Ryukyu Islands, Japan	-	HQ322462
Haemadipsa hainana voucher L00153A	Hainan Island, China	-	HQ322473
Haemadipsa picta voucher L00151A	Taiwan	-	HQ322470
Haemadipsa rjukjuana isolate HARY	Taiwan	HQ203097	HQ203174
Haemadipsa limuna isolate HACH	China	HQ207191	HQ203169
Haemadipsa montana isolate HZKI	Nepal	HQ203105	HQ203182
Haemadipsa ornata isolate HPISUM	Sumatra	HQ203101	HQ203178
Haemadipsa trimaculosa isolate HAKY	Thailand	HQ203095	HQ203172
Haemadipsa interrupta	Thailand	EU100069	EU100091
Haemadipsa sumatrana	Borneo	AY425464	AY425446
Haemadipsa sylvestris	Vietnam	AF116005	AF003266
Chtonobdella bilineata	Australia	AF116006	AF003267
Chtonobdella whitmani	Australia	EU100065	EU100087
Idiobdella seychellensis	Seychelle Islands	EU100070	EU100094
Malagabdella fallax	Madagascar	EU100071	EU100096
Diestecostoma mexicana	Mexico	EU100068	EU100089
Xerobdella lecometi	Slovenia	AF099947	EU100099
Aliolimnatis michaelseni	Congo	AF116010	AF116029
Hirudo medicinalis	France	AY786464	EU100093

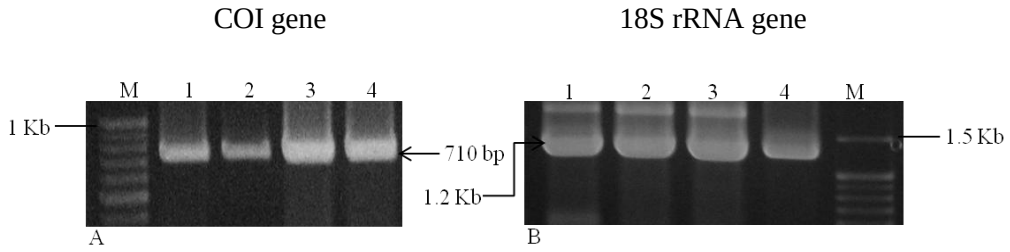

Fig. 12. **Results of PCR amplification of COI and 18S rRNA gene fragments from the Docksilsan leech ((*Haemadipsa rjukjuana*)). A,** COI gene PCR amplicons (710 bp); **B,** 18S rRNA gene amplicons (1.2 kb). Out of the total 1.8 kb 18S rRNA, PCR amplifications were conducted in two independent parts which are overlapped each other with approximately 1.2 kb in length; M, Elpis 100 bp marker; 1,2,3 and 4; the Docksilsan leech samples.

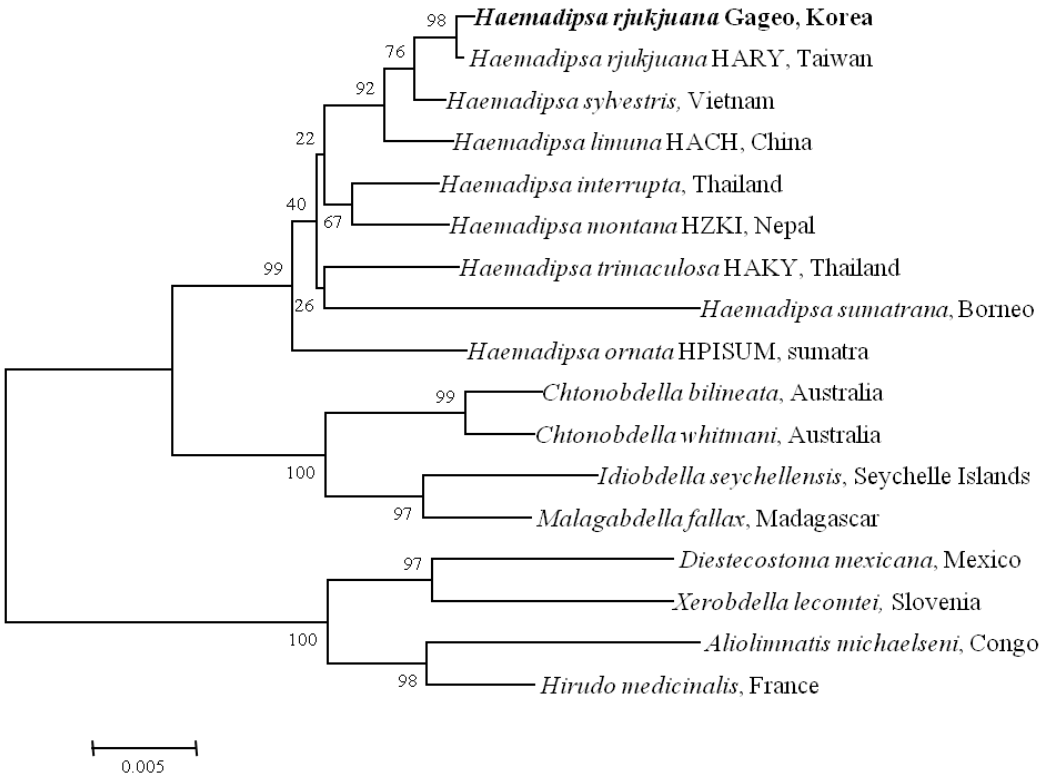

Fig. 13. **The phylogenetic tree illustrating the genetic relationship of Haemadipsoid leeches 18S rRNA gene sequences.** The tree used the neighbor-joining method implemented in Clustal X algorithm with Mega 4.0. 18S rRNA gene sequences of *Haemadipsa rjukjuana* obtained from the Mt. Docksil in the Gageodo, Sinan-gun, Jeollanam-do, Korea compared to previous reference sequences. Bold letters are sequences which were identified in this study.

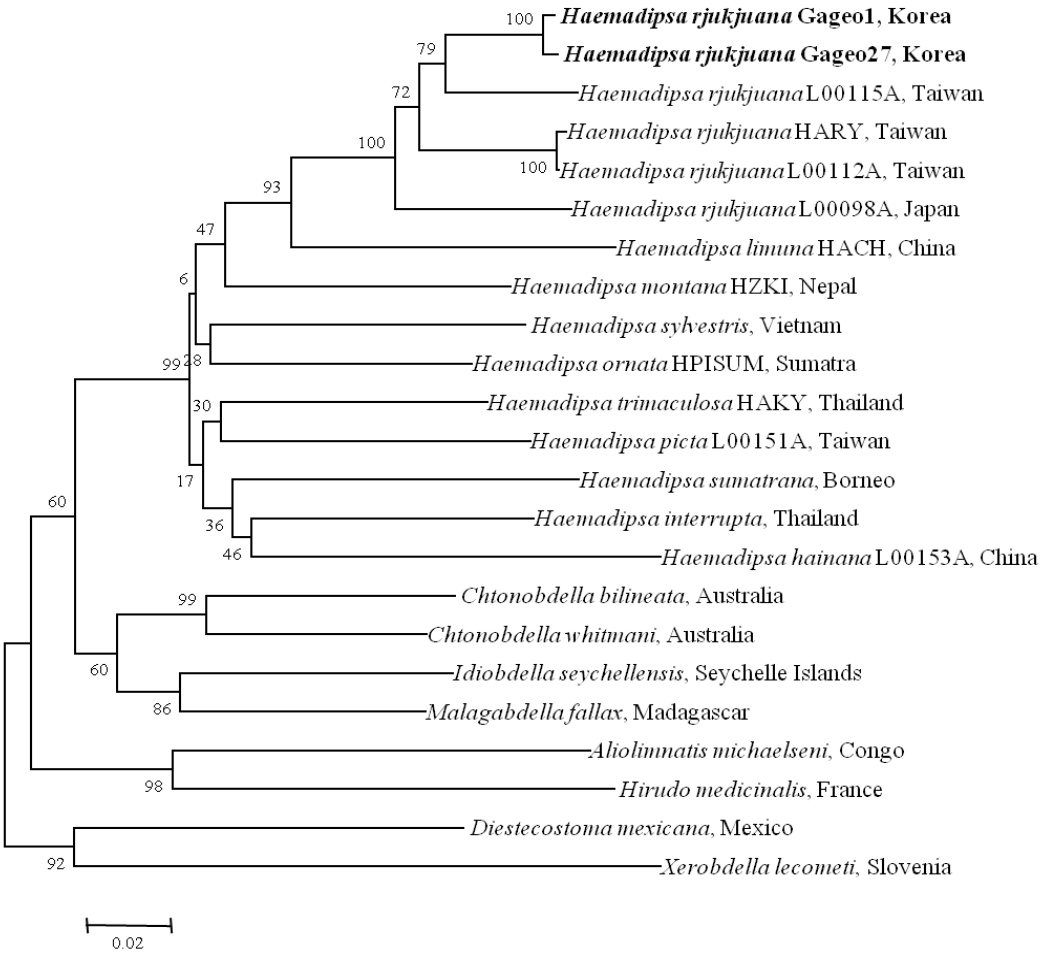

Fig. 14. Phylogenetic tree for COI gene showing the taxonomical status of Haemadipsoid leeches using the neighbor-joining method implemented. COI gene sequences of *Haemadipsa rjukjuana* obtained from Mt. Docksil in the Gageodo, Sinan-gun, Jeollanam-do, Korea. Bold letters are sequences which were identified in this study.

CHAPTER III

Molecular detection of *Bartonella* spp. and host animals from blood-feeding terrestrial leech, *Haemadipsa rjukjuana* at Gageodo in Korea

1. Introduction

Leeches are segmented invertebrates and have a blood-feeding habit. When they sucked the blood, they secrete important bioactive ingredients through their salivary gland, such as hirudin, hyaluronidase, calin, destabilase and apytase which have pharmacological activities (Abbas Zaidi *et al.* 2011). As their feature, the blood-feeding leeches are widely used in treatment of varicose venis, muscle cramps, thrombophlebitis, osteoarthritis and so on for reconstruction. Leeches suck the blood on surface of host as external parasites, then the sucked blood is mixed with a glandular secreted matrix (Abbas Zaidi *et al.* 2011). That blood mixture can remains several months in their intestine and is prevented from digestion, effectively (Nehili *et al.* 1994). As blood-feeding leeches were used to treatment for human, the risk of infection with pathogen through the medical leeches has been focused. The risk is a possibility of transmission of pathogen which can survive in the gut of the leech as long as the blood cells do (Nehili *et al.* 1994). When the leeches sucked the host having a certain pathogens, the pathogens can transmit to host through the salivary gland.

The possibility that leeches can vector of pathogen has studied in more recent past, especially due to leech therapy. A medical leech, *Hirudo*, may cause a severe disease by transmitting a Syphilis (*Treponema*), erysipelas (*Streptococcus* sp.), tetanus (*Clostridium tetani*), hog cholera (hog-cholera virus) and hospital wound infection

(*Aeromonas hydrophila*) (Adams 1988; Dickinson 1984; Kaestner *et al.*1982; Shope 1957). Besides medical leech, there are reports that *Ozobranchus* (turtle leech) may be a mechanical vector for the fibropapilloma-associated turtle herpesvirus (Greenblatt *et al.*2004) and *Rickettsia* was infected in *Torix tagoi*, *T. tukubana*, *Hemiclepsis marginata* and *H. japonica*. (Kikuchi and Fukatsu 2005). These report suggest that various bacteria or viruses can remain in the leech`s gut as long as they are alive. Although leeches are potentially hazardous to human health, reported research of diseases transmitted by leeches are very limited (Al-Khleif *et al.* 2011). Furthermore, the remained blood in leech can be detected by PCR amplification. This method is used to monitor terrestrial mammal biodiversity which were sucked blood by leech, which is a useful tool for investigation of animal diversity and understanding of leech feeding habits (Schnell *et al.* 2011).

In this study, a survey of pathogenic prevalence on sanguivorous terrestiral leech, *Haemadipsa rjukjuana* (Hirudiniformes: Haemadipsidae), including a blood meal of them were conducted by PCR assay. To analyze which pathogens were infected in terrestrial leech, *Anaplasma phagocytophilum*, *A. bovis*, *Ehrlichia chaffensis*, *E. canis*, *Borrelia burgdoferi*, *Bartonella* spp., *Rickettia* spp. and *wolbachia* spp. were amplified by each species specific primers. For blood meal screening, mitochondrial cytochrome *b* gene was used to amplify the host blood DNA from field-collected specimens of *Haemadipsa rkujkuana*.

2. Materials and Methods

2.1. Sample collection and preservation

From 2011 July 25 to 28 and July 9 to 11, a total of 173 leeches were collected on the Mt. Docksil in Gageodo, Sinan-gun, Jeollanam-do (125° 7"E, 34° 4"N), Korea. The collection method was by walking through the forest to attract leeches and collected them directly by tweezers. The specimens were preserved in 70% or 100% alcohol solution for DNA extraction.

2.2. DNA extraction and PCR amplification

The DNeasy Blood & Tissue Kit (Quiagen) was used for lysis of tissue and DNA purification. The extracted DNA was stored at -20℃. For detection of zoonotic pathogens, *Bartonella* spp., *Anaplasma phagocytophilum, A. bois, Ehrlichia chaffensis, E. canis, Borrelia burgdorferi*, *Rickettia* spp. and *Wolbachia* were amplified. Target gene of each pathogens and PCR amplification is listed in Table 3. For analysis of host animals, human mitochondrial DNA cytochrome *b* gene was amplified by conventional PCR. PCR amplification of pathogens and host animals were accomplished using the primers listed in Table 3. Amplification reaction mixture used *HiPi* PCR PreMix (Eplis) with: 13 μl distilled water, 1 μl of each primer (10 pmole), 1 μl DNA template (total volume, 20 μl). All amplification reactions were performed by PTC-200 thermal cycler (MJ Research, Inc.). Amplified products were separated by electrophoresis on 1.5% agarose gel and visualized by ethidium bromide.

2.3. DNA sequencing

The method of Sanger was used to PCR products sequencing by BigDye Terminator v3.1Cycle Sequencing Kit and ABI 2720XL DNA analyzer (Applied Biosystems, Foster City, CA, USA). For each reaction, PCR product DNA were mixed the reagent of terminator kit and cycle sequencing were performed for 1 cycle at 95℃ for 1min 30sec, and then 25 cycles at 95℃ for 30sec, 50℃ for 5sec, 60℃ for 4min. Clean up cycling reactions were conducted as follows. First, added 180 ul magnetic bead to each sequencing reaction and incubated at room temperature for 5 minutes. Placed the plate onto the magnetic plate to capture the particles and removed and discarded the liquid. Second, washed the plate using 100 ul 90% ethanol and incubated at room temperature for 5 minutes. Repeated this step for a total of two washes and placed them to the room temperature for 10 minutes for air dry. Third, added elution solution and transferred purified sequencing reactions to a clean 96 well plate. Finally, ran on sequencer ABI 3730XL (Capillary array length: 50cm)

2.4. Phylogenetic analyses

The GenBank accession numbers of 16S-23S intergenic spacer (ITS) region sequences related to *Bartonella* sp. for sequence comparisons are listed in Table 4. The phylogenetic relationships between haplotypes of the leeches were reconstructed using the neighbor-joining (NJ) method (Saitou and Nei 1987) under the Kimura 2-parameter (K-2-P) model (Kimura 1980). Confidence in estimated relationship was determined using the bootstrap approach (Felsenstein 1985) obtained through 1,000 replicates with

the same model as mentioned above. Both bootstrap analysis and phylogeny reconstruction were conducted using MEGA version 4.0 (Tamura *et al*. 2007).

3. Results

To analyze which pathogens were naturally infected in Gageodo blood feeding terrestiral leeches, polymerase subunit beta (*rpo*B) gene and 16S-23S intergenic spacer (ITS) region of *Bartonella* spp., 16S rRNA gene of *Anaplasma phagocytophilum*,16S rRNA gene of *A. bois*, 16S rRNA gene of *Ehrlichia chaffensis*, 16S rRNA gene of *E. canis*,16S rRNA gene of *Borrelia burgdorferi*, citrate synthase (*glt*A) gene of *Rickettia* spp. and 16S rRNA gene of *Wolbachia* were amplified. As a result of amplification, only *Bartonella* spp. was detected from 14 samples of 173 leeches based on ITS partial sequences (Fig. 15 and Table 6). Sequence analysis was conducted for the all 14 samples and the result showed that 7 samples were closest to *Bartonella grahamii* with 99.6 to 100.0% identity, 1 sample had a 90.6% similarity with *Bartonella* sp. KM2563 (FJ667565, Taiwan) and the rest were presumed to be a *B. henselae* huston-1 strain with 100.0% similarity on the GenBank database. Of 7 *Bartonella grahamii* sequences, 2 samples were closest to *B. grahamii* isolate KWDBG 41 (JN810847, South Korea) with 99.6 and 100.0% identity respectively and the rest 5 samples had 100.0% homologous with *B. grahamii* V2 strain. To confirm the genetic relationship of *Bartonella* naturally infected Gageodo terrestrial leech, obtained ITS region sequences were compared with other sequences available in the GenBank database (Fig. 16). *B. grahamii* and *B. henselae* (LE 1-1, 14, 23, 36) clustered France, UK population which includes also other Korean strain and the *Bartonella* spp. (LE 1-4) clustered Taiwan, Australia and Japan population.

The result of sequencing of mt DNA cytochrome *b* gene fragment to identify host animals, total 35 sequences were acquired of 173 samples (Table 7). The rests of the

samples did not amplify or prove to be other species. According to sequencing analysis of 35 amplicons, human (*Homo sapiens sapiens*, n=10), house mouse (*Mus musculus*, n=8), siberian weasel (*Mustela sibirica*, n=6), pale thrush (*Turdus paliidus*, n=3), grey-backed thrush (*Turdus hortulorum*, n=3), rufous-tailed robin (*Luscinia sibilans*, n=1), siberian rubythroat (*Luscinia calliope*, n=1)**,** oriental magpie robin (*Copsychus saularis*, n=1)**,** black-faced bunting (*Emberiza spodocephala*, n=1) and yellow-throated bunting (*Emberiza elegans*, n=1) were detected. Of total 35 sequences, about 68.5 % are mammals including human and about 31.4 % were migratory birds which pass through the Gageodo in Korea.

4. Discussion

Most vector-borne disease including dengue, lyme disease, malaria, endemic typhus and bartonellosis are transmitted by sucking arthropods such as flea, mosquito and tick (Gratz 1999). Prevalence of zoonotic pathogens which infected arthropod vector and host animal has been conducted on numerous studies (Kim *et al.* 2003; Ko *et al*. 2011; Nieto *et al*. 2007). Leech is also haematophagous ectoparasite, which sucks blood of human and animals including fish, frogs, turtles or birds. Once they suck blood, the blood digestion is very slow, which can remain in the gut up to 27 weeks (Sawyer 1986, Al-Khleif *et al.* 2011). Actually, recent researchers consist that leech might be a promising candidate as vector (Schnell *et al.* 2012). Furthermore, research of infection about *Steptococcus* sp., *Clostridium tetani*, classical swine fever virus, *Aeromonas hydrophila*, bovine parvovirus (BPV), feline calicivirus (FCV), equine arteritis virus (EAV), equine herpesvirus type 1 (EHV-1) and *Rickettia* in leech were reported (Adams 1988; Dickinson and Lent 1984; Shope 1957; Al-Khleif *et al.* 2011; Kikuchi and Fukatsu 2005). In present study, *Bartonella* spp., including *B. grahamii* and *B. henselae*, were detected from leech`s DNA extraction and the total infection rate is 8 % (n=14/173) (Table 6). The *Bartonella* were amplified based on 16S-23S ITS region. The 16S-23S ITS region is useful housekeeping gene for identifying *Bartonella* spp. due to the hypervariable region and much lower sequence similarity than other target gene (Gil *et al.* 2010). When *Bartonella* is amplified, actually, the PCR products have different size to their species because of the variable region (Fig. 15).

Bartonella spp. is small, gram-negative bacteria which infect red blood cells and invade endothelial cell. *Bartonella* is infected host or reservoir via two routes. First, it is

transmitted by blood-sucking arthropods, such as cat flea, human flea, bat flea, body lice, sand fly, ked and other biting fly. Second is animal scratch and bites (Billeter *et al.* 2008; Eisen and Gage 2010). Several *Bartonella* were identified as zoonotic agent without doubt, but most *Bartonella* spp. has been detected or cultured from numerous other arthropods and various arthropods which might be a potential vector for *Bartonella* has been explored (Billeter *et al.* 2008). Tick, as a representative example, has been became a considerable potential vector for *Bartonella* species. A number of researches have been proven to be positive for *Bartonella* spp. from diverse tick population using PCR (Kim *et al.* 2005; Chang *et al.* 2002; Loftis *et al*. 2005; Rar *et al.* 2005). In other words, natural *Bartonella* infections begin with feeding of blood-sucking arthropods, and the spectrum of arthropod vectors has been expanded.

Bartonella spp. also have a natural cycle as other pathogens, which contains a vector and a host. The host can be the natural reservoir hosts, new competent reservoir hosts or incidental hosts (Jacomo *et al*. 2002). Host is important to *Bartonella* infection as well as to vector species as their immune response and host specificity. Apart from *Bartonella*, a research of blood meal (host) is very important to understanding their host preferences and vectorial capacity. Furthermore, it is used to investigate host distribution and control of disease diffusion. (Garlapati *et al.* 2012; Schnell *et al.* 2012).

In this present study, the DNA of human, mouse, weasel and seven kinds of birds were detected from terrestrial leech which live on mountain in Gageodo (Table 7). The detection on human seems to be derived due to method of sampling which attract leech by walking and remove them from leg of human. Gageodo is a isolated island which is 173 Km far from land, Mokpo (Fig. 1). So the fauna in there is very limited-

representatively, cows, goats and wild weasels. Although the result of mammal is univariately, the birds are remarkable for its diversity. The birds which were detected in this study are all migratory birds which come from north. Gageodo has been known a major stopover location for migratory birds which pass through West Sea. As the climate change worldwidely, the weather of Korea is also changing. As the change, a distribution of flora and fauna undergoes various influences, and a chance of inflow of new vectors and disease increase. Blood meal screening could follow the change of migratory birds which stop Gageodo as well as current state of distribution. Furthermore, *Bartonella* spp. were also detected from leech extraction. As other arthropods vector, if leech may has a role of vector, the zoonotic pathogen could be transmitted to other host. But, because these results were determined by PCR, there is a critical point whether this result can prove vector competence of leech. A proven vector competence and vector potential is different (Billeter *et al.* 2008). The vector competence is completed when the zoonotic agent is transferred from vector to host reliably, and vertical or horizontal transmission should be practicable (Billeter *et al.* 2008; Kikuchi and Fukatsu 2005). So it is seem to need further research for experimental transmission of zoonotic pathogens by leech.

Finally, this study suggests that a Docksilsan leech, *Haemadipsa rjukjuama*, could be a potential vector. And blood meal screening shows fauna of Gageodo, especially migratory birds, as well as sucking habits of the leech.

Table 4. Nucleotide sequences of polymerase chain reaction primers and conditions for amplification of pathogens and host animals

Target gene	PCR method	Pimer names	Nucleotide sequences (5`-3`)	Annealing temperature	References
Bartonella spp. *rpo* B	1st	1400F	CGCATTGGCTTACTTCGTATG	57	Ronesto *et al*. 2007
		2300R	GTAGACTGATTAGAACGCTG		
	2nd	1400F	CGCATTGGCTTACTTCGTATG	60	
		1400D	TTCCCGTACCAACAAATGG		
Bartonella spp. ITS	1st	QHVE1	TTCAGATGATGATCCCAAGC	58	Houpikian and Raoult 2001
		QHVE4	AACATGTCTGAATATATCTTC		
	2nd	QHVE12	CCGGAGGGCTTGTAGCTCAG	58	
		QHVE14	CACAATTTCAATAGAAC		
Anaplasma phagocytophilum 16S rRNA	1st	AEI-F	AAGCTTAACACATGCAAGTCGAA	56	Oh *et al.*2009
		AE1-R	AGTCACTGACCCAACCTTAAATG		
	2nd	EE3	GTCGAACGGATTATTTTTATAGCTTGC	56	
		EE4	CCCTTCCGTTAAGAAGGATCTAATCTCC		
Anaplasma bovis 16S rRNA	1st	AEI-F	AAGCTTAACACATGCAAGTCGAA	56	Oh *et al.*2009
		AE1-R	AGTCACTGACCCAACCTTAAATG		
	2nd	ABkf	TAGCTTGCTATGGGGACAA	59	Kang *et al.*2011
		ABlr	TCTCCCGCACTCCAGTCTG		Kawahara *et al.* 2006

Ehrlichia chaffensis 16S rRNA	1st	AEI-F	AAGCTTAACACATGCAAGTCGAA	56	Oh *et al.*2009
		AE1-R	AGTCACTGACCCAACCTTAAATG		
	2nd	HE3	TATAGGTACCGTACTTATCTTCCCTAT	56	Anderson *et al.* 1992
		HE1	CAATTGCTTATAACCCTTTTGGTTATAAAT		
Ehrlichia canis 16S rRNA	1st	AEI-F	AAGCTTAACACATGCAAGTCGAA	56	Oh *et al.*2009
		AE1-R	AGTCACTGACCCAACCTTAAATG		
	2nd	HE3	TATAGGTACCGTACTTATCTTCCCTAT	56	Murphy *et al.* 1998
		ECAN5	CAATTATTTATAGCCTCTGGCTATAGGC		
Borrelia bugdoferi 16S rRNA	1st	B1	CAGTGCGTCTTAAGCATGC	59	Park 2004
		B6	CAACCATGCAGCACTGTATAT		
	2nd	B3	GCAGCTAAGAATCTTCCGCAATGG	60	
		B8	CCTTAAATACCTTCTCCC		
Rickettia sp. *glt*A	1st	RpCS. 877p	GGGGACCTGCTCACGGCGG	54	Roux *et al.*1997
		RpCS. 1258n	ATTGCAAAAAGTACAGTGAA		
	2nd	RpCS. 896p	GGCTAATGAAGCAGTGATAA	58	
		RpCS. 1233n	GCGACGGTATACCCATAG		
Wolbachia 16S rRNA	1st	16S-F	TTGTAGCCTGCTATGGTATAACT	56	O'Neill *et al.* 1992
		16S-R	GAATAGGAGTTTTCATGT		
mt DNA cytochrome *b* gene	1st	L14841	AAAAAGCTTCCATCCAACATCTCAGCATGATG	50	Kocher *et al.* 1989
		H15149	AAACTGCAGCCCCTCAGAATGATATTTGTCCTCA		

Table 5. Reference sequences of *Bartonella* 16S-23S intergenic spacer (ITS) which were used in the phylogenetic analyses

Taxon	Locality	Accession No.
B. grahamii isolate KWDBG41	South Korea	JN810843
B. grahamii isolate KWDBG29	South Korea	JN810847
B. grahamii ITS1, strain V2	United Kingdom	AJ269785
B. elizabethae	France	L35103
B. japonica	Japan	AB498007
B. silvatica	Japan	AB498008
B. birtlesi	France	AY116640
B. capreoli	Japan	AB498009
B. chomelii	Japan	AB498010
B. doshiae	United Kingdom	AJ269786
B. henselae Houston-1	USA	L35101
B. koehlerae	France	AF312490
B. quintana	France	L35100
B. schoenbuchensis	France	AY116639
B. taylorii	United Kingdom	AJ269788
B. tribocorum	France	AF312505
B. vinsonii subsp. *arupensis*	France	AF312504
B. vinsonii subsp. *berkhoffii*	USA	AF167988
B. vinsonii subsp. *vinsonii*	France	L35102
B. bacilliformis	USA	CP000524
B. bovis	France	AY116638
B. queenslandensis	Australia	EU111765
B. mayotimonensis	France	FJ376735
B. australis strain	Australia	DQ538396
B. tamiae strain	Thailand	DQ395180
B. rochalimae	Peru	DQ683199
B. coopersplainsensis	Australia	EU111770
Candidatus *B. thailandensis*	-	FJ411485
Bartonella sp. KM2563	Taiwan	FJ667565
B. phoceensis	-	AY515123
B. alsatica	-	AF312506
B. rattimassiliensis	-	AY515121
B .rattaustraliani	Australia	EU111760

Table 6. Sequence similarity to their closest relatives of *Bartonella* spp. ITS gene from Docksilsan leeches (*Haemadipsa rjukjuana*) to the present study using GenBank database.

Sample No.	The results of GenBank database search	Similarity (%)	The total number of identifying samples
1-1	*Bartonella grahamii* isolate KWDBG41, South Korea	99.6	1
14	*Bartonella grahamii* isolate KWDBG41, South Korea	100.0	1
23	*Bartonella grahamii* V2	100.0	5
36	*Bartonella henselae* huston-1	100.0	6
1-4	*Bartonella* sp. KM2563, Taiwan	90.6	1
	total		14/173

The result of sequencing of 14 amplified bands which showed on fig. 15, those samples were clustered 5 groups; three groups were *B. grahamii*, one is *Bartonella* sp. and the others is *B. henselae*. The table below shows the representative sample of each group

Table 7. The result of detection of host animals using mtDNA cytochrome *b* gene fragments (450 bp)

Scientific names	Category	The number of identifying samples
Homo sapiens sapiens	Mammal	10
Mus musculus	Mammal	8
Mustela sibirica	Mammal	6
Turdus paliidus	Aves	3
Turdus hortulorum	Aves	3
Luscinia sibilans	Aves	1
Luscinia calliope	Aves	1
Copsychus saularis	Aves	1
Emberiza spodocephala	Aves	1
Emberiza elegans	Aves	1
total		35

Of 173 samples, 35 samples were identified from Docksilsan leech using conventional PCR and sequencing.

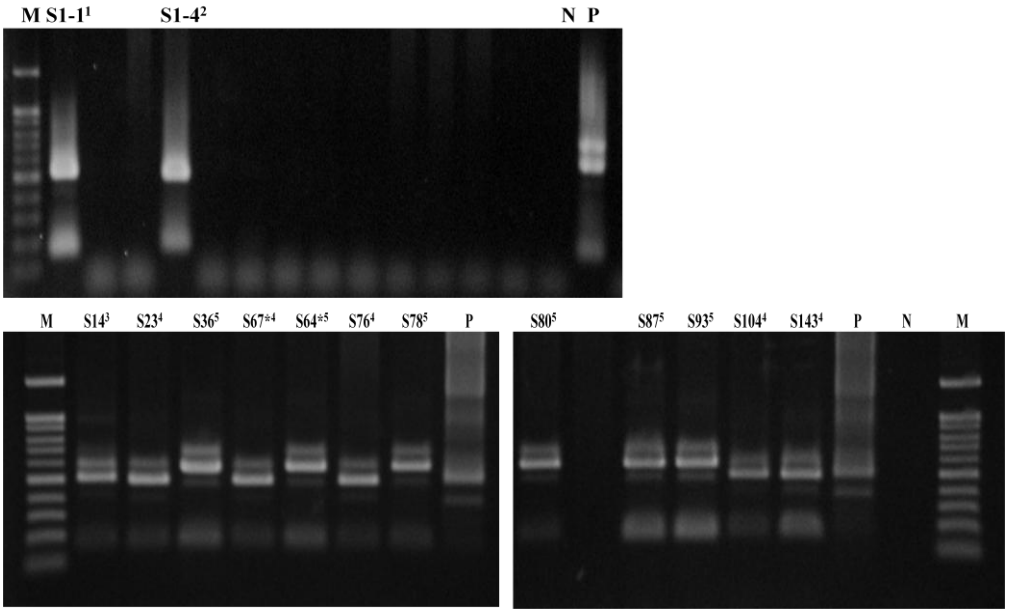

Fig. 15. The result of PCR for detection of *Bartonella* spp. 16S-23S intergenic spacer (ITS) from Docksilsan leeches (*Haemadipsa rjukjuana*). Two samples were amplified among the 173 samples. Of the173 samples, 14 samples were amplified. S1-1, S1-4, S14, S23, S36, S64, S67, S76, S78, S80, S87, S93, S104 and S143, Docksilsan leech sample No.; P, *Bartonella grahamii*; N, water control; M, 100 bp size marker.

*The loading order of S64 and S67 were inverted

[1] S1-1, group1

[2] S1-4, group2

[3] S14, group3

[4] S23, 67, 76, 104 and143, group4

[5] S36, 64, 78, 80, and 93, group5

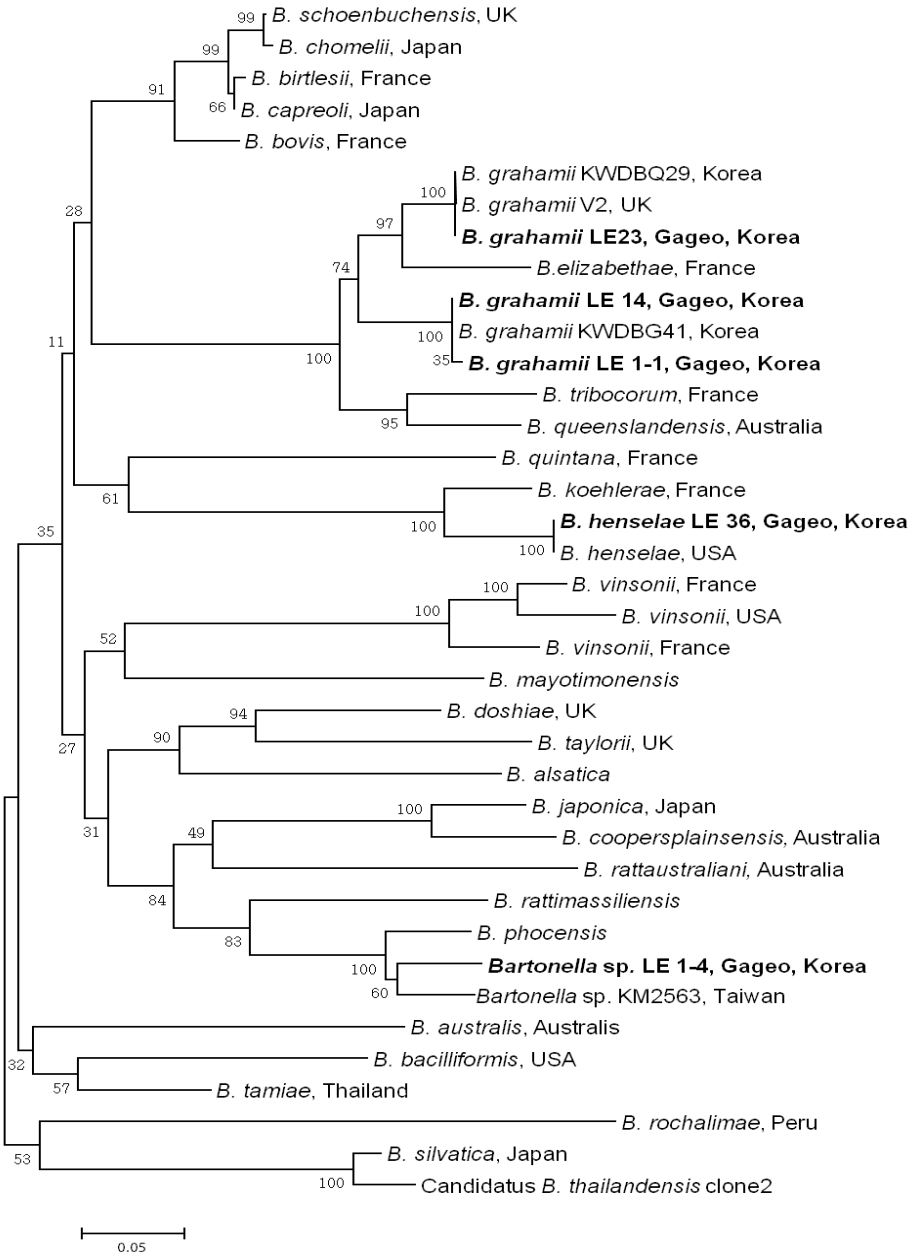

Fig. 16. Phylogenetic tree of Bartonella detected in Docksilsan leeches (*H. rjukjuana*) in Gageodo based on 16S-23S internal spacer (ITS) sequences. Trees using the neighbor-joining method implemented in Clustal X algorithm with Mega 4.0.

GENERAL CONCLUSION

This study was carried out to investigate the species name of blood-feeding terrestrial leech which live in Gageodo and to explore their potential of vector.

The Docksilsan leech has three jaws which located in Y-shaped lumen and 5 pairs of eye, and the last eye was divided by 2 annuli from 4th eye. Posterior sucker consist of 71-72 friction rays. Muscle layer were composed of circular muscle (internal) and longitudinal muscle (external). Intestine was divided into 6 muscular ridges which covered with simple cuboidal epithelium.

The result of identification of the leech molecularly, Docksilsan leech has a 99.9% similarity with *Haemadipsa rjukjuana* isolate HARY (from Taiwan, HQ203097) based on 18S rRNA. Based on cytochrome *c* subunit I (COI), the sequences were separated into two types, *H. rjukjuana* COI type A and COI type B. The type A and type B showed 94.6% and 94.3% similarity to the reference sequence of the *H. rjukjuana* L00115A (from Taiwan, HQ322443), respectively. As their morphological and molecular feature, this result suggests that the Docksilsan leech is highly analogous to *H. rjukjuana*.

Bartonella spp. were detected from DNA of Docksilsan leech as 8% infection rate (n=14/173). Two samples of them were closest to *B. grahamii* isolate KWDBG 41 (JN810847, South Korea) with 99.6% and 100.0% each and other 5 samples were closest to *B. grahamii* V2 strain with 100.0% and other 6 samples had 100.0% homologous with *B. henselae* huston-1 and the rest one was presumed to be *Bartonella* sp. KM2563 (FJ667565, Taiwan) with 90.6 % similarity. And DNA of human (*Homo*

sapiens sapiens, n=10), house mouse (*Mus musculus*, n=8), siberian weasel (*Mustela sibirica*, n=6), pale thrush (*Turdus paliidus*, n=3), grey-backed thrush (*Turdus hortulorum*, n=3), rufous-tailed robin (*Luscinia sibilans*, n=1), siberian rubythroat (*Luscinia calliope*, n=1)**,** oriental magpie robin (*Copsychus saularis*, n=1)**,** black-faced bunting (*Emberiza spodocephala*, n=1) and yellow-throated bunting (*Emberiza elegans*, n=1) were detected. The birds are all migratory birds which come from north and stop at Korea. This exploration of blood meal suggests that the fauna of Gageodo as well as host preference of Docksilsan leech. It is important that the leech which lives in Gageodo could has a role of vector like other blood-sucking arthropods vector. This result can be used to understand feeding habits of Docksilsan leech and to control transmission of pathogen.

This research is remarkable for its originality to record and study of blood-sucking terrestrial leech, *H. rjukjuana*, is first conducted in Korea.

REFERENCES

Abbas Zaidi, S. M., Jameel, S. S., Zaman, F., Shazia J., Sultana, A., Khan, S. A. (2011). A systematic overview of the medicinal importance of sanguivorous leeches. Alternative Medicine Review. **16**, 59.

Adams, S. L. (1988). The medicinal leech. A page from the annelids of internal medicine. Annals of Internal Medicine. **109**, 399-405.

Al-Khleif, A., Roth, M., Menge, C., Heuser, J., Baljer, G. and Herbst, W. (2011). Tenacity of mammalian viruses in the gut of leeches fed with porcine blood. Journal of Medical Microbiology. **60**, 787-792.

Apakupakul, K., Siddall, M. E., and Burreson, E. M. (1999). Higher level relationships of leeches (Annelida: Clitellata: Euhirudinea) based on morphology and gene sequences. Molecular Phylogenetics and Evolution. **12**, 350-359.

Bely, A. E., and Weisblat, D. A. (2006). Lessons from leeches: a call for DNA barcoding in the lab. Evolution & Development. **8**, 291-501.

Billeter, S. A., Levy, M. G., Chomel, B. B. and Breitschwerdt, E. B. (2008). Vector transmission of *Bartonella* species with emphasis on the potential for tick transmission. Medical and Veterinary Entomology. **22**, 1-15.

Blanchard, R. (1896). Hirudinees. Bollettino dei Musei Torino. **11**, 1-24.

Borda, E. (2007). 'Systematics and biodiversity of Arhynchobdellida (Oligochaeta: Hirudinida) with a focus on the evolutionary history of bloodfeeding terrestrial leeches.' (ProQuest Information and Learning Company: Michigan, USA. p12-47)

Borda, E., Oceguera-Figueroa, A., and Siddall, M. E. (2008). On the classification, evolution and biogeography of terrestrial haemadipsoid leeches (Hirudinida: Arhynchobdellida: Hirudiniformes). Molecular phylogenetics and Evolution. **46**, 142-154.

Borda, E. and Siddall, M. E. (2004). Arhynchobdellida (Annelida: Oligochaeta: Hirudinida): phylogenetic relationships and evolution. Molecular Phylogenetics and Evolution. **30**, 213-225.

Borda, E. and Siddall, M. E. (2010). Insights into the evolutionary history of Indo-Pacific bloodfeeding terrestrial leeches (Hirudinida : Arhynchobdellida : Haemadipsae). Invertebrate Systematics. **24**, 456-472.

Corpet, F. (1988). Multiple sequence alignment with hierarchical clustering. Nucleic Acids Research. **16**, 10881-10890.

Chang, C. C., Hayashidani, H., Pusterla, N., Kasten, R. W., Madigan, J. E. and Chomel, B. B. (2002). Investigation of *Bartonella* infection in icodid ticks form California. Comparative Immunology, Microbiology and Infectious Diseases, **25**, 229-236.

Chen, J. H. *Haemadipsa rjukjuana* Oka, 1910. BitoTaiwanica. Retrieved May. 2, 2013. http://clitellates.biota.biodiv.tw/pages/1207

Csoka, T. B., Frost, G. I. and Stern, R. (1997). Hyaluronidases in tissue invasion. Invasion & Metastasis. **17,** 297-311.

Dickinson, M. H. and Lent, C. M. (1984). Feeding behavior of the medical leech, *Hirudo medicinalis*, L. Journal of Comparative and Physiological Psychology. **154**, 449-455.

Eisen, R. J. and Gage, K. L. (2012). Transmission of flea-borne zoonotic agents. Annual Review of Entomology. **57**, 61-82

Electricwala, A., Sawyer, R. T., Jones, C. P. and Atkinson, T. (1991). Isolation of thrombin inhibitor from the leech Hirudinaria manillensis. Blood Coagulation & Fibrinolysis. **2,** 83-90.

Felsenstein, J. (1985). Confidence limits on phylogenies: an approach using the bootstrap. Evolution. **39**, 783-791.

Folmer, O., Back, M., Hoeh, W., Lutz, R., and Vrijenhoek, R. (1994). DNA primers for amplification of mitochondrial cytochrome *c* oxidase subunit I from diverse metazoan invertebrates. Molecular Marine Biology and Biotechnology. **3**, 294-299.

Gratz, N. G. (1999). Emerging and resurging vector-borne diseases. Annual Review of Entomology. **44**, 51-75.

Garlapati, R. B., Abbasi, I., Warburg, A., Poche, D. and Poche, R. (2012). Identification of bloodmeals in wild caught blood fed *Phlebotomus arhentipes* (Diptera: Psychodidae) using cytochrome *b* PCR and reverse line blotting in Bihar, India. Journal of Medical Entomology. **49**, 515-521.

Gil, H., Garcia-Esteban, C., Barabdika, J. F., Peig, J., Toledo, A., Escudero, R., Gado, I., Rodriguez-Vargas, M., Garcia-Amil, C., Lobo, B., Roales, P., Rodriguez-Moreno, I., Olmeda, A. S., Garcia-Perez, A., L., Anda, P. (2010). Variability of *Bartonella* genotypes among small mammals in Spain. Applied and Environmental Microbiology. **76**, 8062-8070.

Greenblatt, R. J., Work, T. M., Balazs, G. H., Sutton, C. A., Casey, R. N. and Casey, J. W. (2004). The *Ozobranchus* leech is a candidate mechanical vector for the

fibropapilloma-associated turtle herpesvirus found latently infecting skin tumors on Hawaiian green turtles (*Chelonia mydas*). Virology. **321**, 101-110.

Hall, T. A. (1999). BioEdit: a user-friendly biological sequence alignment deitor and analysis program for Windows 95/98/NT. Nucleic Acids Symposium Series. **41**, 95-98.

Hebert, P. D. N., Ratnasingham, S., and deWaard, J. R. (2003). Barcoding animal life: cytochrome *c* oxidase subunit 1 divergences among closely related species. Proceedings of the Royal Society B: Biological Sciences. **270(**Suppl. 1) S96-S99.

Jacomo, V., Kelly, P. J. and Raoult D. (2002). Natural history of *Bartonella* infections (an exception to Koch`s postulate). Clinical and Diagnostic Laboratory Immunology. **9**, 8-18.

Kang, K. W. (1995). 'Leech.' (Academybook: Seoul, Korea. p4-10)

Kim, C. M., Kim, M. S., Park, M. S., Park, J. H., and Chae, J. S. (2003). Identification of *Ehrlichia chaffensis*, *Anaplasma phagocytophilum*, and *A. bovis* in *Haemaphysalis longicornis* and *Ixodes persulcatus* ticks form Korea. Vector Borne and Zoonotic Diseases. **3**, 17-26.

Kim, C. M., Kim, J. Y., Yi, Y. H., Lee, M. J., Cho, M. R., Shah, D. H., Klein, T. A., Kim, H. C., Song, J. W., Chong, S. T., O'Guinn, M. L., Lee, J. S., Lee, I. Y., Park, J. H. and Chae, J. S. (2005). Detection of *Bartonella* species from ticks, mites, and small mammals in Korea. Journal of veterinary Science. **6**, 327-334.

Kimura, M. A. (1980). A simple method for estimating evolutionary rate of base substitutions through comparative studies of nucleotide sequences. Journal of Molecular Evolution. **16**, 111-120.

Kikuchi, Y. and Fukatsu, T. (2005). *Richettsia* infection in natural leech populations. Microbial Ecology. **49**, 265-271.

Ko, S., Kim, H. C., Yang, Y. C., Chong, S. T., Richards, A. L., Sames, W. J., Klein, T. A., Kang, J. G., and Chae, J. S. (2011). Detection of *Rickettsia felis* and *Rickettsia typhi* and seasonal prevalence of fleas collected from small mammals at Gyeonggi Province in the Korea. Vector-Borne and Zoonotic Diseases. **11**,1243-1251.

Kutschera, U., Pfeiffer, I., and Ebermann, E. (2007). The European land leech: biology and DNA-based taxonomy of a rare species that is threatened by climate warming. Naturwissenschaften. **94**, 967-974.

Lai, Y. T., Chang, C. H., and Chen, J. H. (2009). Two new species of Helobdella Blanchard, 1896 (Hirudinida: Rhynchocdellida: Glossiphoniidae) from Taiwan, with a checklist of Hirudinea fauna of the Island. Zootaxa. **2068**, 27-46.

Lai, Y. T., Nakani, T., and Chen, J. H. (2011). Three species of land leeches from Taiwan, *Haemadipsa rjukjuana* comb.n., a new record for *Haemadipsa pocta* Moore, and an updated description of *Tritetrabdella taiwana* (Oka). Zookeys. **139**, 1-22.

Moore, J. P. (1924). Notes on some Asiatic leeches (Hirudinea) principally from China, Kashmir, and British India. Proceeding of the Academy of Natural Sciences of Philadelphia. **76,** 343-338.

Ngamprasertwong, T., Thirakhupt, K., and Panha, S. (2007). Two new species of land leeches from Thailand (Hirudiniformes: Haemadipsidae). The National History Journal of Chulalongkorn University. **7**, 155-159.

Nehili, M., Iik, C., Mehlhorn, H., Ruhnau, K., Dick, W. and Njayou, M. (1994). Experiments on the possible role of leeches as vectors of animal and human pathogens: a light and electron microscopy study. Parasitology Research. **80**, 277-290.

Nieto, N. C., Dabritz, H., Foley, P., Drazenvich, N., Calder, L., Adjemian, J., Conrad, P. A., and Foley, J. E. (2007). Ectoparasite diversity and exposure to vector-borne disease agents in wild rodents in central coastal California. Journal of Medical Entomology. **44**, 328-335.

Loftis, A. D., Gill, J. S., Schriefer, M. E., Levin, M. L., Eremeeva, M. E., Gilchrist, M. J. R. and Dasch, G. A. (2005). Detection of *Rickettsia*, *Borrelia*, and *Bartonella* in *Carios Kelleyi* (Acari: Argasidae). Journal of Medical Entomology. **42**, 473-480.

Oka, A. (1910). Synopis der jepanischen Hirudineen, mit diagnosen der neuen species. Annotationes Zoologicae Japonensis. **7**, 165-183.

Park, K. H. and Kim, C. H. (1989). Taxonomic studies on freshwater leeches (=Hirudinea) of Korea. Chungnam Journal of Science. **16**, 149-158.

Paik, E. I.. and Park, S. R. (1992). A Taxonomic study on two new records of freshwater leeches, Glossiphoniidae (Hirudinea: Rhynchobdellae) from Korea. Research bulletin. **44**, 497-502.

Rar, V. A., Fomenko, N. V., Dobrotvorsky, A. K., Livanova, N. N., Rudakova, S. A., Fedorov, E. G., Astanin, V. B. and Morozova, O. V. (2005). Tick-borne pathogen detection, Western Sineria, Russia. Emerging Infectious Diseases. **11**, 1708-1715.

Richardson, L. R. (1975). A contribution to the general zoology of the land leeches (Hrudinoidea: Haemadipsoidea superfam. nov.). Acta Zoologica Academiae Scientiarum Hungaricae. **21,** 119-152.

Saywer, R.T. (1986). 'Leech Biology and Behavior: Feeding, Biology, Ecology and Systematics v. 2.' (Oxford University Press: Oxford, UK. p1-10)

Shope, R. E. (1957). The leech as a potential virus reservoir. The journal of Experimental Medicine. **105**, 373-382.

Saitou, N., and Nei, M. (1987). The neighbor-joining method: a new method for reconstructing phylogenetic trees. Molecular Phylogenetics and Evolution. **4**, 406-425.

Schnell, I. D., Thomsen, P. F., Wilkinson, N., Jensen, L. R. D., Willerslev, E., Bertelsen, M. F., and Gilbert, M. T. P. (2012). Screening mammal biodiversity using DNA from leeches. Current Biology. **22**, R262-R263.

Seleznev, K. G., Shchetinina, E. A., Trophimenko, N, P., Nikonov, G. I., and Baskova, I. P. (1992). Use of the medicinal leech in the treatment of ear diseases. Journal for oto-rhino-laryngology and its related specialties. **54**, 1-4.

Siddall, M. E., Trontelj, P., Utevsky, S. Y., Nkamany, M., and Macdonald, K. S. (2007). Diverse molecular data demonstrate that commercially available medicinal leeches are not *Hirudo* medicinalis. Proceedings of the Royal Society B: Biological Sciences. **274**, 1481-1487.

Sket, B., and Trontelj, P. (2008). Global diversity of leeches (Hirudinea) in freshwater. Hydrobiology. **595**, 129-137.

Tamura, K., Dundley, J., Nei, N., and Kumar, S. (2007). MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. Molecular Phylogenetics and Evolution. **24**, 1596-1599.

Trontelj, P., Sket, B., and Steinbruck, G. (1999). Molecular phylogeny of leeches: congruence of nuclear and mitochondrial rDNA data sets and the origin of bloodsucking. Journal of Zoological Systematics and Evolutionary Research. **37**, 141-147.

국 문 초 록

가거도 흡혈성 육상거머리의 생물학적 특성 및 매개병원체 검출

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거머리는 체절로 이루어진 거머리강 환형동물문에 속하는 동물을 총칭한다. 거머리는 바다, 육지 및 담수 등 여러가지 환경에서 생활하며 대부분의 거머리가 흡반에 달린 이빨(teeth)을 이용하여 동물의 피를 흡혈한다. 치열을 가진 악질과 거머리(Gnathobdellidae)는 수생거머리 Hirudidae (aquatic leech)와 육상거머리 Haemadipisidae (terrestrial leech)로 나뉜다. 흡혈성 육상거머리는 습지와 같이 습도가 높고 축축한 곳에서 흔히 발견되며, 세계적으로는 남아시아, 동남아시아 및 오스트레일리아에 널리 분포한다고 알려져 있다. 거머리의 흡혈성을 이용하여, 오래 전부터 거머리를 대체의학으로 사용함과 더불어 최근 들어 질병을 매개하는 새로운 흡혈성 외부기생충이 연구됨에 따라, 거머리 또한 사람에게 질병을 전파할 수 있는 매개체(vector)가 될 수 있을 것인가에 대한 조사가 이루어져 왔다. 하지만 국내에서는 거머리를 분자학적으로 연구한 사례가 없을뿐더러, 특히 육상거머리에 대한 기록은 전무하다.

본 연구에서는 가거도 독실산에 서식하는 흡혈성 산거머리(일명 독실산 거머리)에 대하여 형태학적 종 동정과 분자 생물학적 분류, 흡혈된 숙주동물 동정 및 병원체 감염 여부를 조사 하였으며 총 3 가지 파트로 구성되어 있다.

첫 번째, 전자현미경(SEM)을 이용하여 총 6 마리의 독실산 거머리의 형태학적 특성을 관찰하였다. 육안으로 관찰하였을 때, 독실산 거머리의 길이는 평균 8 mm 였고, 신장되었을 때 최대 길이는 평균 2.4 cm 였으며, 몸체 앞 뒤에 흡반이 달려 있었다. 전자현미경을 통해 관찰한 결과, 거머리 분류에 기준이 될 수 있는 특징적인 형태로는 Y 자 모양으로 된 공간에 이빨이 달린 세 개의 턱(jaw)이 있었고, 5 쌍으로 된 안점(eye) 중 4 번째 안점과 5 번째 안점이 2 개의 체절(annuli) 로 나뉘어져 있었다. 흡혈에 사용되는 후반부 흡반은 71-72 개의 방사형 늑(ray)로 이루어져 있었다.

내부 조직학적 구조는 종단면과 횡단면으로 절단하여 위치 별 장기를 확인하였다. 각피 밑에 위치하는 근육층은 바깥층이 환상근(circular muscle)으로, 내층은 종주근(longitudinal muscle) 로 구성되어, 환형동물의 전형적인 근육 분포를 보였다. 식도에 이어지는 장의 근육은 6 분원으로 구성되어 있었으며, 장벽은 단순입방상피(simple cuboidal epithelium)으로 이루어져 있었다.

두 번째, 형태학적 특징과 더불어 유전학적으로 거머리의 종 정보를 알아내기 위해 29 개체를 이용하여 분자학적 실험을 수행하였다. 거머리의 gDNA 를 추출 한 후 18S rRNA 유전자와 cytochrome oxidase subunit I (COI) 유전자를 증폭하여 염기서열을 분석한 결과, 독실산 거머리의 18S rRNA 염기서열은 *Haemadipsa rjukjuana* isolate HARY(대만, HQ203097)와 99.9% 일치하였다. COI 유전자를 증폭한 결과, 2 가지 유형으로 나뉘었으며, 각각의 염기서열은 *H. rjukjuana* isolate L00115A(대만, HQ322443)과 94.6%와 94.3%로 일치하였다. 독실산 거머리의 형태학적 소견과 유전학적 유사성을 종합하여 볼 때, 가거도 독실산에 서식하는 흡혈성 육상 거머리는 대만에서 서식하는 해마딥사 류큐아나(*H. rjukjuana*)와 가장 유사한 것으로 판단된다.

세 번째, 독실산거머리의 질병매개 가능성과 숙주동물에 관한 연구를 수행하였다. 독실산 거머리 173 개체를 이용하여 8 종류의 병원체(*Anaplasma*

phagocytophilum, *A. bovis*, *Ehrlichia chaffensis*, *E. canis*, *Borrelia burgdoferi*, *Bartonella* spp., *Rickettia* spp. and *wolbachia* spp.)를 조사한 결과, 14 마리에서 바토넬라(*Bartonella* spp.)가 검출되었다 (검출율=8%, 14/173). 검출된 염기서열을 분석한 결과, 개는 *Bartonella grahamii* KWDBG 41 (한국, JN10847)과 각각 99.6%와 100.0%로 일치하였고, 5 개는 *B. grahamii* V2 strai 과 100% 일치하였고, 개는 *B. henselae* huston-1 과 100% 일치하였으며, 나머지 하나는 *Bartonella* sp. KM2563 (대만, FJ667565 와 90.6% 일치하였다.

독실산 거머리가 흡혈한 숙주동물을 조사하기 위해서, 위와 동일한 173 개체를 이용하여 cytochrome *b* 유전자를 증폭한 결과, 사람(Human, *Homo sapiens sapiens*) 10 개체, 쥐(House mouse, *Mus musculus*) 8 개체, 족제비(Siberian weasel, *Mustela sibirica*) 6 개체, 흰배지빠귀(Pale thrush, *Turdus pallidus*) 3 개체, 되지빠귀(Grey-backed thrush, *Turdus hortulorum*) 3 개체, 울새(Rufous-tailed robin, *Luscinia sibilians*) 1 개체, 진홍가슴(Siberian rubythroat, *Luscinia calliope*) 1 개체, 오리엔탈-까치로빈(Oriental magpie robin, *Copsychus saularis*) 1 개체, 촉새(Black-faced bunting, *Emberiza spodocephala*) 1 개체, 노랑텃맷세(Yellow-throated bunting, *Emberiza elegans*) 1 개체의 유전자가 검출되었다. 이 결과는 독실산 거머리가 흡혈하는 숙주동물의 종을 알 수 있을 뿐만 아니라 가거도에 서식하는 야생동물상을 조사하는데 사용될 수 있다.

또한, 전 세계적으로 기후가 변화되면서, 지역별 야생동물의 분포 및 빈도가 변화되고 있고, 새로운 질병매개체(vector) 및 질병이 야기되고 있다. 거머리를 통한 숙주동물 분석은 가거도의 야생동물 분포의 변화, 특히 그 중에서도 가거도를 경유하는 철새를 모니터링 하는데 유용할 것이라 생각된다. 또한, 본 연구에서 바토넬라가 거머리에서 발견된 것은 거머리 또한 다른 질병매개체(진드기, 벼룩 등)와 같이 인수공통 전염원을 매개할

수 있는 가능성을 밝힌 바, 지속적인 거머리 연구를 통하여 다른 도서나 육지의 거머리 및 질병 전파를 관리 할 수 있어야 한다.

결론적으로 가거도 독실산에 서식하는 독실산 거머리가 해마딥사 류큐아나(*Haemadipsa rjukjuana*)와 형태학적, 유전학적으로 가장 유사하며, 사람을 비롯한 쥐, 족제비, 철새를 흡혈하였고, 검체 중 8%가 바토넬라에 감염되어 있었다. 본 연구는 국내에서는 최초로 흡혈성 육상 거머리에 대하여 연구한 결과라는 점에서 독창성을 지니며, 거머리를 통한 매개질병 전파의 위험성을 제시한다.

주요어: 거머리, 흡혈성, 육상 거머리, 해마딥사 류큐아나, 병원체, 바토넬라, 숙주

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