

# Identification and Phylogenetic Relationship at *Cytochrome Oxidase Subunit I (COI)* Gene among Korean Terrestrial Planarian Taxa

Doo-Ho Moon<sup>1</sup>, Young Ah Lee<sup>1</sup> and Man Kyu Huh\*

*Department of Molecular Biology, Dongguk University 995 Eongwangno, Busanjin-gu, Busan 614-714, Korea*  
*<sup>1</sup>Pusan National University, Biological Education, Geumjeong-gu, Busan 609-735, Korea*

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Sequence data of *Cytochrome Oxidase Subunit I (COI)* gene of mitochondria were used to elucidate the taxonomy and phylogenetic relationships of the terrestrial planarian taxa in Korea. Published *COI* gene sequences from Family Bipaliidae in GenBank were also included in the phylogenetic analysis. The aligned data sets for Terricola ranged from 387 to 444 nucleotides (bp) as a result of differences in insert nucleotides. The phylogeny based on *COI* analysis was not congruenced with the morphological traits. *Bipalium nobile* included the remainder taxa (*Bipalium adventitium*, *Bipalium venosum*, *Bipalium kewense*, and *Bipalium multilineatum*). Internal nodes were strongly supported (>91%). The phylogenetic tree on *COI* analysis showed that most identified species were well separated from each other. The main phylogenetic analysis formed monophyletic groups. *COI* gene of mitochondria could have the resolving power for taxonomy information for the terrestrial planarian taxa in Korea.

**Key words** : *Cytochrome Oxidase Subunit I (COI)* gene, terrestrial planarians, phylogenetic tree

## Introduction

Within the free-living Platyhelminthes the triclads or planarians are perhaps the best known group, largely as a result of intensive research concerning cellular regeneration, taxis, pattern formation and, most recently, Hox gene expression [16]. Planarians are numerous, diverse, and globally distributed, occurring in marine, limnetic and terrestrial environments [4].

Systematics have recognized three major groups within the Tricladida for a long time: Paludicola (freshwater planarians), Terricola (land planarians), and Maricola (marine planarians). In a further entry on triclad taxonomy and phylogeny, Sluys [17] proposed the new infraorder Cavernicola (represented by three species), which grouped taxa formerly assigned to the Maricola but with apparent closer affinities to the Paludicola. The taxonomic rank of these groups has been shifted between that of suborder and infraorder, the latter being the accepted grouping today. The systematic and phylogenetic relationships of these infraorders have been discussed on the basis of morphological and ultrastructural characters by Ball [3] and Sluys [17].

Within triclads, Ball [3,4] considered Terricola (defined by its complex diploneural nervous system) as the sister group

of an undefined (no synapomorphies disclosed) clade made by Maricola and Paludicola (Haploneura). Whereas no synapomorphies were found for the Maricola, two presumed synapomorphies defined the Paludicola: their reduced pre-cerebral diverticula and the probursal condition of the intestine.

Mitochondrial DNA (mtDNA) is now commonly recognized as one of good phylogenetic tools for a wide animal taxa [16]. Many regions of mtDNA, such as coding for protein genes or participating in regulation as the control region, are used as makers for investigation of intraspecies and interspecies genetic diversity.

The mitochondrial *cytochrome oxidase subunit I (COI)* gene was selected for characterization of family Bipaliidae (Terricola) in Korea. Owing to its relative large size and the inclusion of both highly conserved and variable regions with a different range of mutational rates, it has previously been shown to be useful for a variety of molecular phylogenetic objectives [5,10].

The Terricola are taxonomically divided into three families (Geoplanidae Stimpson 1857, Bipaliidae Stimpson 1857, and Rhynchodemidae Graff 1896) which are divided into a number of subfamilies. Although some hypotheses on the ancestry of one or other subfamilies have been proposed [11,13,21], no cladistic study has been undertaken for this group of triclads. The general knowledge on terrestrial planarians in Korea is rather poor. In addition, the taxonomy

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### \*Corresponding author

Tel : +82-51-890-1521, Fax : +82-51-890-1529  
E-mail : mkhuh@deu.ac.kr

of the few known species is rather confused, in many cases with poor descriptions lacking the essential information to identify new individuals [10]. To address these uncertainties, we report here the *COI* gene sequences of four species of Terricola in Korea. Published *COI* gene sequences from three Bipaliidae were also included in the phylogenetic analysis. The aims of this paper are to analyse the phylogenetic relationships among the seven species in Terricola. New data are interpreted in the light of existing morphological evidence.

## Materials and Methods

### Specimens

The Family Bipaliidae in Korea consists of four species; *Bipalium nobile*, *Bipalium adventitium*, *Bipalium multilineatum*,

and *Novibipalium venosum* (Table 1). Live specimens of terrestrial planarians were collected from beneath stones or fallen leaves lying in moist areas in five Korean localities (Figs. 1~4). 30 specimens were taken to the laboratory, reared in small captures at 8°C, photographed and preserved immediately in 96% EtOH. From each locality some individuals were fixed in Steinmann fluid and are kept in a reference collection at the Department of Molecular Biology (Dong-eui University, Busan). The animals were initially classified into general different morphotypes on the basis of external morphological data for each specimen based on Mateos et al [13].

We constructed a modified list of the important traits for Korean terrestrial planarians considering these criteria (Table 2). Voucher specimens are deposited in the Department of Science Education at Pusan National

Table 1. List of samples used in this study with sampling locality and genBank accession numbers

Family Bipaliidae	Region of origin	Source	Code
<i>Bipalium adventitium</i>	Leingston (USA)	GenBank	AF178306
<i>Bipalium adventitium</i>	Busan, Korea	This study	HM346597
<i>Bipalium nobile</i>	Busan, Korea	This study	HM346598
<i>Bipalium</i> sp.	Japan	GenBank	AF178307
<i>Bipalium multilineatum</i>	Yansan, Korea	This study	HM346600
<i>Novibipalium venosum</i>	Busan, Korea	This study	HM346599
<i>Novibipalium venosum</i>	Japan	GenBank	DQ666048

Table 2. Comparison of morphological traits between *B. adventitium*, *B. nobile*, *B. adventitium*, *N. venosum*, and *B. multilineatum* based on criteria of Mateos et al. [13]

	<i>B. adventitium</i>	<i>N. venosum</i>	<i>B. multilineatum</i>	<i>B. nobile</i>
Body length (mm)	70-200	30-50	70-200	100-800
Body width (mm)	2-4	2-3	1.5-2	3-4
Body shape	Little cylindrical	Little plump	Flattened	Flattened
Head length (mm)	2-3	2-3	2-3	3-4
Head width (mm)	4-5	3-5	3-4	5-7
Anterior side-end	Shark' pin	Hastate	Shark' pin	Palabola
Shape of head	Semilunar	Lunate head with a pair of well-developed and recurved auricles.	Semilunar with moderate auricles	Semilunar
Shape of eye	Aureolated	Aureolated	Aureolated	Aureolated
Color of head	Dark brown	Reddish brown	Brown with blakish margin	Brown
Body color	Light brown	Light yellowish Brown	Yellowish brown	Bright yellow
	Rim of head : dark brown		Rim of head : dark brown	
Dorsal stripe	1md		1md+2la	1md+2la+2mg
Ventral stripe	No		1md+2la	1md+2la
Nervous System	The central nervous system consists of two large ventral nerve cords connected by several commissures lying between the parenchymal muscle layers and the vasa deferentia.			
Alimentary system	The anterior ramus of the intestine runs forewords to within about 0.1 mm of the anterior tip of the animal, giving off several pairs of branched lateral diverticula.			

md: mid-dorsal, la: lateral, mg: marginal-occurring only on the prepharyngeal region of the body.

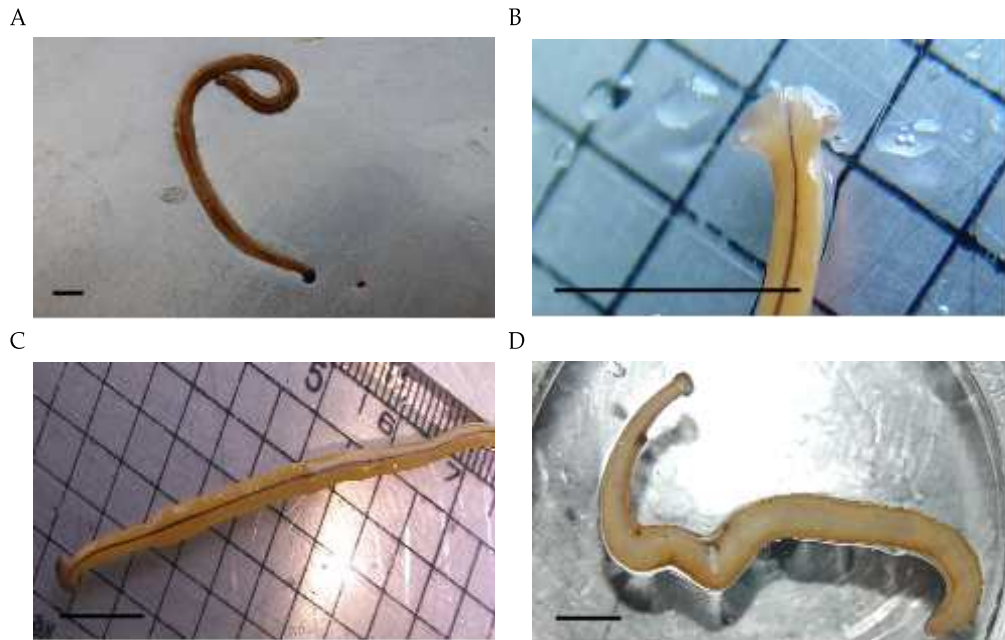


Fig. 1. *Bipalium adventitium* A, total view; B, head; C, dorsal view; D, ventral view. Scale bars=10 mm

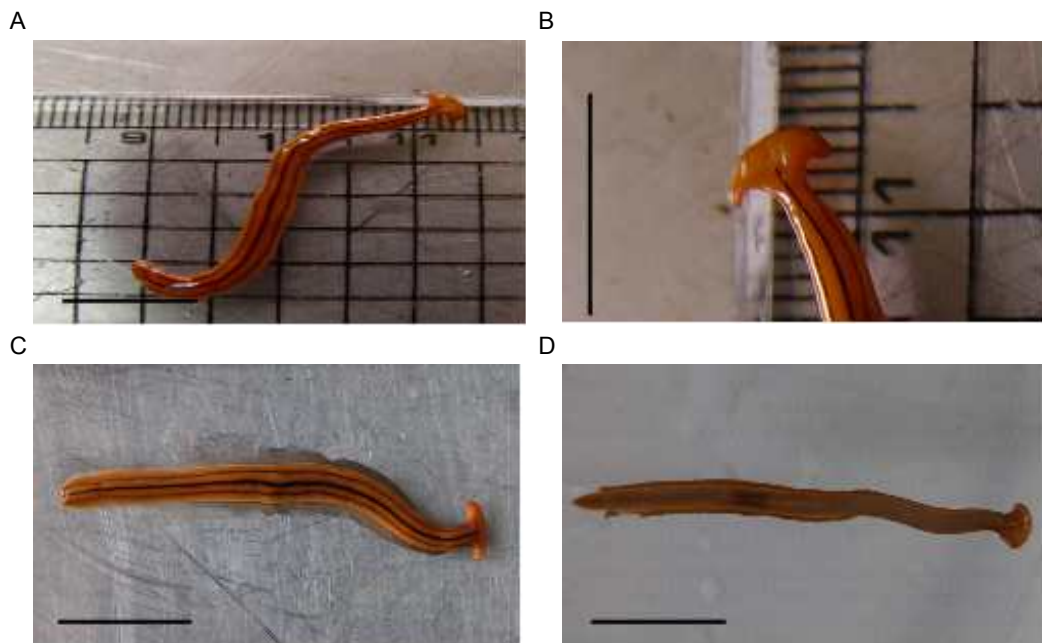


Fig. 2. *Novibipalium venosum* A, total view; B, head; C, dorsal view; D, ventral view. Scale bars=10 mm

University, Busan.

*Dugesia japonica* was used as a outgroup.

DNA extraction, gene amplification and sequencing

The High molecular weight DNA of the samples was extracted from live or ethanol fixed specimens using the DNA Zol Kit (Life Technologies Inc., Grand Island, New York,

USA) according to the manufacturer's protocol. New sequences obtained in this study are shown in an appendix.

Specific primers (Table 3) were used to amplify the entire length of the sequences for the *COI* gene by polymerase chain reaction (PCR) using standard techniques at a 2.5 mmol/l  $MgCl_2$  concentration.

PCR materials (50 ul volume) included 50 ng of genomic

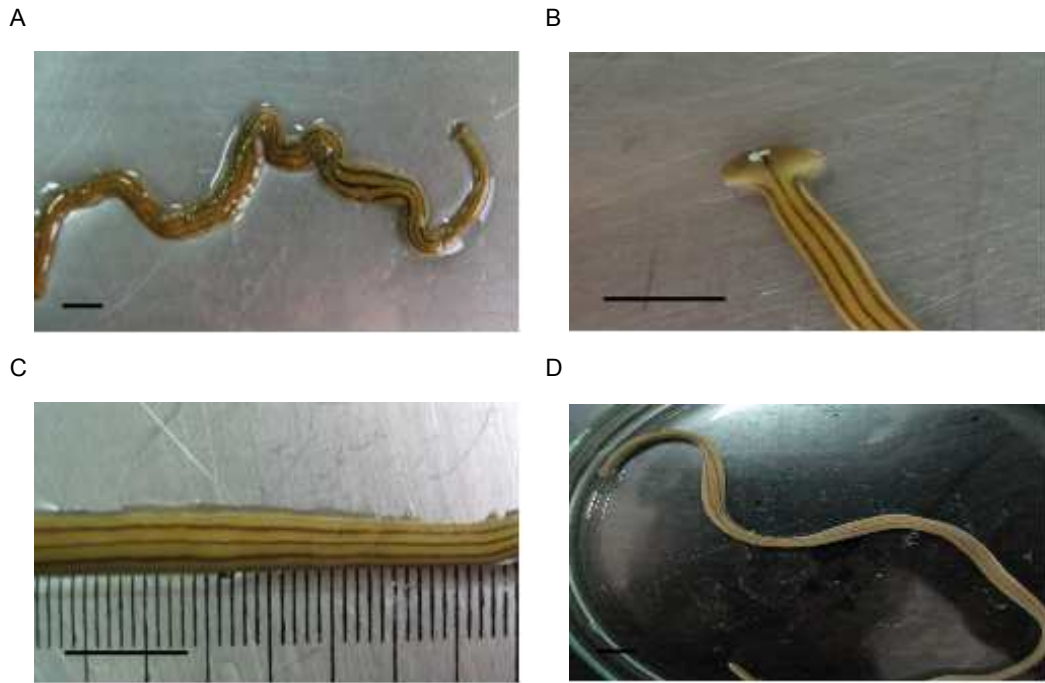


Fig. 3. *Bipalium multilineatum* A, total view; B, head; C, dorsal view; D, ventral view. Scale bars=10 mm

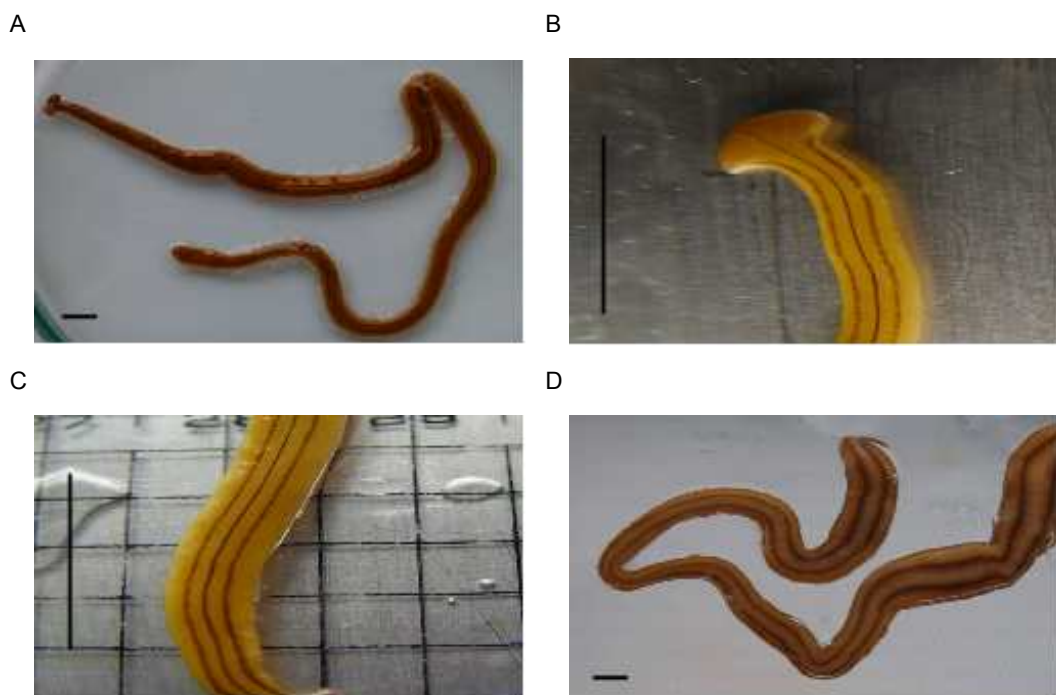


Fig. 4. *Bipalium nobile* A, total view; B, head; C, dorsal view; D, ventral view. scale bars=10 mm

Table 3. Synthetic primer sequencing (5'-3') used for the *COI* analysis in this study

Name	Sequence 5'-3'	Source
COI-F	AGCTGCAGTTTTGGTTTTTTGGA	Bessho et al. [5]
COI-R	ATGAGCAACAACATAATAAGTATCATG	Bessho et al. [5]

DNA, 100  $\mu$ M of each dNTP, 0.2  $\mu$ M of each primer, 1x enzyme buffer, and 2 unit of Taq polymerase. The amplification profile was 28 cycles of 94°C for 30 sec, 42°C for 60 sec, 72°C for 60 sec, preceded by an initial denaturation at 94°C for 90 sec and followed by a final extension at 72°C for 5 min.

PCR products were separated on 2.0% agarose gels and purified using the QIAquick Gel Extraction Kit (QIAGEN). The amplified fragments were cloned into a bluescript vector and sequenced using ABI Prism 377 Sequencer (Applied Biosystem, USA). At least seven individuals' clones of each species were analyzed.

#### Alignment and phylogenetic analysis

The chromatogram output for each sample was edited using the software Sequence Navigator 1.0.1 (Applied Biosystems Inc.), and the sequences were manually aligned.

A pairwise alignment was calculated using the ClustalX program. Phylogenetic relationship were estimated by MEGA version 4.1 [20] and PAUP version 4.0 beta treating all alignment gaps as missing. A maximum parsimony tree (MP) was inferred using heuristic search, branch-swapping options and tree bisection-reconnection. Confidence values for individual branches were determined by a bootstrap analysis with 100 repeated sampling of the data. In addition, a phylogenetic tree was constructed by the neighbor-joining (NJ) method [15] using the NEIGHBOR program in PHYLIP version 3.57 [8].

## Results

The complete sequences of *COI* regions were PCR amplified and sequenced for the four Korean distributed species of family Bipaliidae. The *COI* sequences of three species in same family included in this study were obtained from

GenBank (AF178306, AF178307, and DQ666048). The aligned data sets for *Terricola* ranges from 400 to 414 nucleotides (bp) as a results of differences in insert nucleotides (Table 4). However, aligned when three species of family Bipaliidae in genBank were included, nucleotide sequences of the length of *COI* vary from 401 to 421 bp.

Alignments of the Bipaliidae species were great similarity among the species' sequences and the unusual *COI* insert was not shown. Sequence variation within the *Terricola* is mostly due to nucleotide substitutions, although several small indels and one deletion can be found. Another source of sequence divergence is length variation due to stretches of short repeats that occur at the sequences of TTTTTT in all the *Terricola* (see sequences in GenBank).

Alignment of the DNA sequences did not require adding gaps.

Total alignment length is 401 positions, of which 20 are parsimony-informative characters, 63 variable but parsimony-uninformative, and 318 constant characters.

The sequences of *B. adventitium* in Korea are accorded with those (95%) of the Leingston region in USA.

G+C content for four Korean species of *Terricola* ranged between 31.4% and 35.0% (Table 5). The base furtherance did not showed the significant difference to the by a total taxon (Table 4). These values are similar to those (29.5%~34.2%) for the *Terricola* alignments of the *COI* region.

Genetic distance (*D*) based on the proportion of shared fragments was used to evaluate relatedness among species. The estimate of *D* ranged from 0.003 to 0.151 (Table 6).

The main phylogenetic analysis revealed two distinct clades (Fig. 5). The first clade includes three species (*B. adventitium*, *B. multilineatum*, and *B. nobile*). The second clade includes only *N. venosum* which was same clade with Japanese. Internal nodes are strongly supported (77%).

Table 4. The chi-square test for homogeneity of base frequencies across taxa of Family Bipaliidae using *COI* sequences

Base		A	$\chi^2$	C	$\chi^2$	G	$\chi^2$	T	$\chi^2$
<i>B. adventitium</i>	Obs.	87	0.748	46	0.618	94	0.417	173	1.016
	Exp.	95.45		43.97		89.69		170.89	
<i>N. venosum</i>	Obs.	88	0.094	46	0.084	94	0.019	173	0.539
	Exp.	95.69		44.08		89.92		171.32	
<i>B. multilineatum</i>	Obs.	102	0.207	45	0.185	81	0.885	173	0.003
	Exp.	95.69		44.08		89.92		171.32	
<i>B. nobile</i>	Obs.	105.3	0.026	39.1	0.016	90.23	0.015	165.4	0.176
	Exp.	95.45		43.97		89.69		170.89	

Obs.: the value of observation, Exp.: the value of expectation

Table 5. The base frequencies across taxa of Family Bipaliidae using *COI* sequences

Base	A	C	G	T	Total sites
<i>B. adventitium</i>	0.2175	0.1150	0.2350	0.4325	400
<i>N. venosum</i>	0.2195	0.1147	0.2344	0.4314	401
<i>B. sp.</i>	0.2544	0.1122	0.2020	0.4314	401
<i>B. nobile</i>	0.2632	0.0977	0.2256	0.4135	400
Mean	0.2386	0.1099	0.2242	0.4272	400.5

Table 6. Genetic distances among four species of Family Bipaliidae based on *COI* analysis

Taxa	<i>B. adventitium</i>	<i>N. venosum</i>	<i>B. multilineatum</i>	<i>B. nobile</i>
<i>B. adventitium</i>	-			
<i>N. venosum</i>	0.0025	-		
<i>B. multilineatum</i>	0.1428	0.1421	-	
<i>B. nobile</i>	0.1454	0.1429	0.1505	-

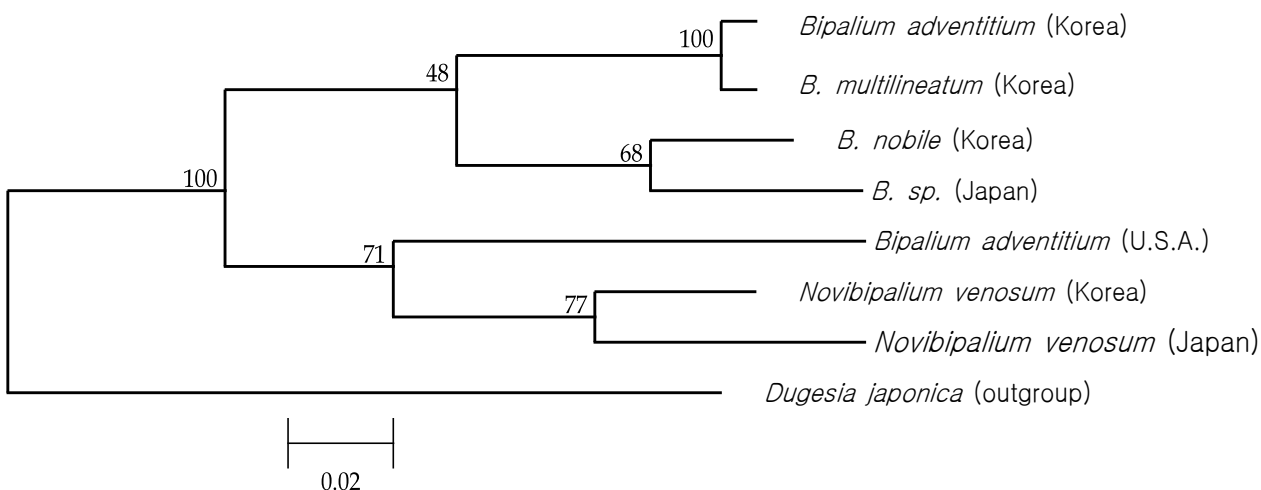


Fig. 5. A phylogenetic analysis for Family Bipaliidae based on *COI* analysis. Tree obtained by Maximum-likelihood (ML) with the values of bootstrap.

However American *B. adventitium* was not from same species in Korea.

### Discussion

Given the proliferation of genetic markers, comparisons between techniques are inevitable. There is a need technique which is best suited the issues being examined. In previous study, *COI* was used to determine the genetic relationships among species and the results compared to pedigree relationships available [1,5].

The Terricola is defined by three synapomorphies: complex pharyngeal musculature, creeping sole, and diploneural nervous system. The last two have always been considered

very important synapomorphies for the Terricola [4,18,19] as they represent adaptations to the terrestrial lifestyle [2]. In four terricola taxa in Korea, the central nervous system consists of two large ventral nerve cords connected by several commissures lying between the parenchymal muscle layers and the vasa deferentia [14] (Table 2). The diploneural nervous system is formed by the longitudinal nerve cords, which take a much more internal position, and by a thickened subcutaneous nerve plexus [11]. There is ample morphological evidence that the ventral nerve cords of the Terricola are homologous to those of the Maricola and Paludicola, including dugesiids [12]. Moreover, it is highly likely that the subcutaneous plexus of land planarians represent a quantitative differentiation of similar plexuses de-

scribed in dugesiids (e.g. the subepidermal and submuscular plexuses) related to the presence of a creeping sole. The later structure is developed to varying extents in the different families and subfamilies. The fact that the creeping sole and the diploneural nervous system are clear adaptations to the terrestrial life-style jointly with the low bootstraps or the lack of support backing the monophyly of the Terricola in NJ, MP [9] and ML (this work) trees would be congruent with a parallel independent evolution of both structures from freshwater ancestors not bearing them. However, unless a denser sampling of species is carried out, namely of bipaliids and rhynchodemids, a unique event followed by diversification is also likely, and may be more parsimonious.

In Sluys' [17] analysis on the phylogenetic relationships of the triclades, he stated that research in this group 'is in a state of flux'. The advent of molecular studies, largely based on 18S rDNA sequences, has increased the rate of flux. This reflects the apparent instability of the morphological homology from recurrent convergences or homoplasy but, at the same time, also reflects the inconsistencies of most molecular data often based on incomplete sampling and, so far, on a single (namely 18S rDNA) or a few genes [6,7].

Our results show that the previous infra generic classification of genera *Bipalium* and *Novibipalium* is doubtful. The separation of the subgenera is not supported here because the two representatives of subg. *B. nobile* and *B. adventitium* are more distantly related to each other than *B. multilineatum* (Fig. 5). The morphological and anatomical characters of *B. nobile* are not shared with those of *B. adventitium* than those of *B. multilineatum*. The same trends at the results of *COI* (Table 4). Although *COI* gene of mitochondria could be had the resolving power for taxonomy information for the terrestrial planarians in Korea, further investigations, such as the search then the identification of a specific terrestrial planarian makers, would be needed to clearly specify the gene.

To further support the taxonomy conclusion for Korean species within the Terricola, and to resolve the contradictions posed, further studies will be needed, including a denser sampling of Terricola taxa for molecular data, complementary sequences from independent genes, and the broad and through morphological database already available for the Terricola, used together in a combined total evidence approach.

Terrestrial planarians in Korea have not still the scientific names. We recommend that *B. adventitium* is a Geomeunhanjulye-yukgiplanaria, *B. multilineatum* a

Sejuljobundabinyeo-yukgiplanaria, *B. nobile* is a Ginbinyeo-yukgiplanaria, and *N. venosum* is a Sejulbandalmeori-yukgiplanaria.

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초록 : 한국 내 육지플라나리아 간 치토크롬 산화효소의 동정과 계통유전학적 관계

문두호<sup>1</sup> · 이영아<sup>1</sup> · 허만규\*

(동의대학교 분자생물학과, <sup>1</sup>부산대학교 생물교육학과)

미토콘드리아 산화효소(COI) 유전자의 서열을 이용하여 한국 내 육지플라나리아의 분류와 계통관계를 규명하였다. 유전자은행에서 Bipaliidae과의 종에 관한 기 발표된 서열을 계통분석을 위해 포함시켰다. 육지 플라나리아의 서열 배당은 387 bp에서 444 bp로 나타났으며 이런 차이는 염기 삽입에 기인하였다. COI 분석에 근거한 계통학적 분지도는 형태적 형질에 의한 결과와 일치하지 않았다. *Bipalium nobile*가 나머지 분류군(*Bipalium adventitium*, *Bipalium venosum*, *Bipalium kewense*, *Bipalium multilineatum*)을 포함하는 관계로 나타났다. 내부 가지의 분지군은 강하게 지지되었다(>91%). The phylogenetic tree on COI 분석에 의한 계통도는 잘 분리되었다. 이들은 단계원을 형성하였다. 미토콘드리아 산화효소 유전자는 한국 내 육지 플라나리아 분류군을 동정하는데 유력한 도구가 될 수 있다.