Morphological Description and Genetic Sequencing of Mitochondrial COI Gene in Iraqi New Record *Limax flavus* (L., 1758) (Mollusca: Gastropoda)

Nibras L. Aldoori, Ihsan A. Hussein and Maysaloon L. Aldoori

***Abstract*—**Morphological description and genetically diagnosing of *Limax flavus* (L., 1758) slug were done in this work. The morphological description results of the collected specimen confirmed that it is a new record founded in the west of Baghdad-Iraq. Detailed Morphological study showed that the specimen has yellow color with small dark sputum. Specimen long was ranging from 4.3 to 12.5 cm, also it has a slim mucous body, a pale keel and a thin, oval, darker mantel on the front dorsal side of the body with long ranging from 1.2 to 3.1 cm with blue pale tentacle. COI gene sequencing of this specimen was done to find the similarity with the same gene sequences, which published in National Center of Biotechnology Information (NCBI) database. Polymerase chain reaction (PCR) technique results revealed presence one band of mtDNA COI gene with 710 bp. Sequencing of the PCR product showed a high similarity (93%) with the accession number FJ606456.1 (specimen collected from Western Central Alps). The sequence result of COI gene in this work was deposited in the NCBI gene bank repository. In conclusion, the collected specimen species was the first record in Iraqi environment and the sequences results confirms that this specimen is *Limax flavus*.

***Keywords*—** Limax flavus, Gastropoda, Limacidae, Mollusca, Slug, COI gene, PCR.

I. Introduction

The Slugs (Mollusca: Gastropoda) are important in food chains, it consuming dead leaves, fungi, plants seed and some species prey on earth worm, then it may consume by birds and mammals (Hutchinson &Reis 2015). Slugs causing an economic damage to the plants, but in the other hand it medically important (Salam & El-wakeil 2012 ,Kamal & Almuktar 2010), but yet the *Limax* slug is one of the most significant group living Mollusca (Gamil 2013). *Limax flavus* was world–wide distribution (Branson 1980), and they are founded in British Island (Cameron *et al.* 1983), North of Mexico, and America (Pilsbry 1984), also was disturbed in Australia, China, Cook Islands, Canada, Europe, Japan, Ghana, Madagascar, Middle East, South Africa, New Zealand North, United States, Rarotonga, South America, the Island of St. Helena and Vanuata (Forsyth 2004; Roth, & Sadeghian 2006; Donnel *et al.* 2009).

Investigating the systematics and conservation biology of molluscs by using genetic tools is of increasing interest (Geist *et al.* 2008). The use of DNA as source was greatly hampers due to the unavailability of molluscan soft tissues or they have been preserved using formalin-containing solutions (Legrand *et al.* 2002). Slugs and snails which belong to Gastropods were secreting a trail of mucus from their pedal gland while moving across a surface (Denny 2012). Slugs have enormous quantities of mucopolysaccharides which make difficulty to DNA extraction. Some of recent extraction techniques designed to remove the mucus of snails by using some reagents that are hazardous to the environment (Winnepenninckx *et al.* 1993; Douris *et al.* 1998). However, the DNA isolation from molluscs proved to be a difficult task, because the polysaccharides present in the animals co-precipitates with the DNA and these polysaccharides inhibits the activity of many enzymes used in molecular biology, such as polymerases, ligases and restriction endonucleases (Sokolov 2000). Chelex-based extraction (Walsh *et al.* 1991) and CTAB procedure (Tel-Zur *et al.* 1999) are the most used protocols for genomic DNA extraction (Pereira 2011).

The identification of specimens was often impossible because of life stage diversity or damage to diagnostic traits (Hebert *et al.* 2003; Carr *et al.* 2010; Radulovici *et al.* 2010). Mitochondrial DNA markers have been widely used for identification of closely related species (Iidzuka & Aranishi 2008) and also for population genetic structure analyses (Kocher *et al.* 1989). Currently, the most widely used genes was cytochrome *c* oxidase subunit 1 (*COI*) for studying the phylogeny, systematics and the identification of species. There is only little information available on the amplification success of the *COI* gene fragment in gastropod tissues due to DNA extraction or preservation, storage media and storage duration (Huelsken *et al.* 2011). The *COI* gene region was effective tool for delineating marine species of molluscs and for revealing overlooked species and this was confirmed by Layton (2013). She investigates patterns of sequence variation at the *COI* gene in Canadian marine molluscs. The analysis of nucleotides sequences of nuclear and mitochondrial DNA (*COI* gene) provides us with a basis for taxonomic and phylogenetic consideration (Skujiene & Soroka 2003). The aim of this

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present work is to study the morphological description and the genetic identification at molecular level by using polymerase chain reaction (PCR) and mtDNA *COI* gene sequencing as DNA barcoding region in the new record *Limax flavus* (L., 1758).

II. Material and Methods

A. Specimen.

Mature mucus-rich *Limax flavus* (L., 1758) (Mollusca: Gastropoda) was collected from west of Baghdad Al-Karkh in the middle of Iraq from the period between August and September, 2016. Fresh specimen was used for DNA extraction and preserved in 70% ethanol for further studies.

B. Morphological Study.

The collected *Limax flavus* specimen was morphological described according to Thomas *et al*. (2010). The method was done by putting the specimen in boiling water for 1 min and then transferred to 75% ethanol. The classification of specimen was performed according to Cameron (1983), Wiktor *et al*. (2000) and Telebac *et al*. (2013).

C. DNA extraction protocol.

DNA from *Limax flavus* specimen head was extracted according to (Doyle & Doyle 1987; Gittenberger *et al.* 2006; Huelsken 2011) and used for the genetic analysis. The head of the specimen was separated and cut off with scalpel, grind and dissolved by incubation at 60ºC for 2 hours in a mixture of 300 µl proteinase K (20 mg/ml) and 500 µl CTAB (Cetyltrimethyl ammonium bromide) buffer (2% CTAB, 1.4 M NaCl, 20 mM EDTA, 100 mM Tris-HCl; for 10 ml of this buffer 0.4 g polyvinylpyrrolidone and 50 µl of β-mercaptoethanol were added before starting extraction). Five-hundred microliter of 24:1 (chloroform: iso amyl alcohol) was added and mixed well by shaking tubes and then centrifuged at 13000 g for 10 min . The aqueous phase was pipetted and placed into new eppendorf tube. Five-hundred microliter of cold isopropanol was added and left for standing in freezer for 15 min. The extraction then centrifuged at 13000 g for 3 min. Then the liquid was pipetted off and 700 µl of cold 70% ethanol was added. The extraction centrifuged at 13000 g for 1 min and the liquid was poured off. The tube was air dried for 20 min, and the pellet was re-suspended by adding 100 µl of 1X TE buffer (10 mM Tris-HCl, 1 mM EDTA). Four-microliter of RNase A was added to the extraction and incubated at 37 ºC for 1 hour. The DNA quality and quantity were tested by using Nanodrop (Act Gene, USA) and stored at -20 ºC for further use.

D. Polymerase chain reaction (PCR).

The mtDNA cytochrome *c* oxidase subunit I (*COI*) gene was used as DNA barcode and amplified using the PCR approach. Primers for *COI* gene was used depending on Folmer (1994), and synthesized in Alpha DNA Company (Canada). Primer sequences of the *COI* gene with approximately 710 bp products size were show in Table (1).

AccuPower® PCR PreMix (Bionner, Korea) was used for amplifiying *COI* gene by PCR. Two-microliter of each primer with 1 µM concentration and 4µl of DNA template with 100 ng/µl concentration were added to PCR PreMix tubes. The final volume for PCR reaction was made up to 20 µl with nuclease-free water. The reaction mixers were placed in thermal cycler (Esco, Singapore). The optimized PCR program was consisted of 1 cycle of template denature at 95°C for 3 min, initial denaturation at 95°C for 30 sec, annealing at 45°C for 1 min, extension at 72°C for 45 sec and final extension at 72ºC for 7 min. The last three steps for 35 cycles. The PCR products were resolved on 1.5% agarose gels prepared in 1x TBE buffer (Bioner, Korea). The 100 bp DNA ladder (Bioner, Korea) was loaded on the agarose gel. One-microliter of Bromophenol blue dye was also loaded with the reaction mixer sample. The gel electrophoresis was performed by using 75V for 2 hrs. Ethidium bromide dye (Promega, USA) was used for staining the agarose gel for 30 min. The agarose gel was documented with gel documentation system (Biocom, USA).

E. DNA sequencing.

PCR products for amplified *COI* gene were subject to DNA sequencing. PCR products were purified by using AccuPrep®PCR Purification kit (Bioneer Corp., Korea) before sequencing. DNA sequencing was done by using genetic analyzer 3500 instrument (Applied Biosystems, USA). The used protocol was BDxStdSeq50_POP7_1, BigDye terminator V3.1, which provided by Applied Biosystems.

III. Results

A. Morphological description.

The *Limax flavus* specimen was collected from green gardens, which it's the natural place (Kearney 2010) The results of description of this specimen confirmed that it is a new record species found in west of Baghdad-Iraq. Morphological description of this specimen showed that it has yellow color with small dark sputum. The specimen long was ranging from 4.3 to 12.5 cm (Figure 1). The specimen also has a slim mucous body, a pale keel and a thin, oval, darker mantle on the front dorsal side of the body with long ranging from 1.2 to 3.1 cm with blue pale tentacle (Figure 2).

B. MtDNA COI gene amplification and sequencing.

PCR technique was performed by using two universal primers (LCO1490 and HC02198) to verify mtDNA *COI* gene. The primer pair successfully amplified a part of this gene from the collected specimen DNA. One band was observed with 710 bp (Figure 3).

Sequencing of *COI* gene of this specimen was done to find the similarity with the same gene sequences database which published in NCBI. The sequencing results revealed a high similarity (93%) with the *Limax flavus* accession number FJ606456.1; specimen collected from Western Central Alps (Figure 4). The calculated base composition average of these sequences was to be 166 (26.52%) adenine, 244 (38.98%) thiamine, 108 (17.25%) guanine and 108 (17.25%) cytosine. The sequence result of *COI* gene in this work was deposited in the NCBI gene bank repository and the accession number was MF034732 (www.ncbi.nlm.nih.gov/nuccore/MF034732).

IV. Discussion

All organisms are re-classified in depends on molecular levels. Barcode genes are useful for such studies. In

Iraq, there is no studies were done to re-classify the snails at molecular level. *Limax flavus* was founded in different parts in the world, but it's the first time was recorded in Iraq. This work was described *Limax flavus* genus as new recorded in Iraq. Morphological and genetic studies were done to confirm the classification of this genus. Descriptions of species in the majority of slug studies are depended on one individual or a small series of specimens. This fact hinders the estimation of inter- and intraspecific variations presented in these characters (Nitz *et al.* 2009). The apparent lack of diagnostic characters of external morphology, such as a well-developed shell is one of the major problems in slug research. The shape, body size, vestigial shell and coloration are all very variable and potentially misleading (Klee *et al.* 2007). Nearly all species of *Limax* genus are poorly known but there are some poorly studies on molluscans in Iraq (Shehab & et al 2015,Ameen 2018), and much historical identification is doubtful. Variable coloration was found in *Limax* species, ranging from creamy white through brownish to black. The long of living animal was up to 19.6 cm; sole length up to 19 cm (16.7 cm in ethanol), mantle length up to 5.8 cm (5.4 cm in ethanol); width up to 2.2 cm (up to 1.7 cm in ethanol), keel length in ethanol up to 4.6 cm. Discrimination of *Limax* species cannot be based on one or two morphological character sets alone, therefore the various characters value for discrimination and identification must be considered (Nitz *et al.* 2009). New possibilities of dealing with taxonomic problems were offered by using the application of molecular biology methods (Davis 1994). Using of molecular techniques to analyze relationships between populations and species has become widespread, and nucleotide sequence variations in the mtDNA offer a powerful tool in molecular phylogenetic of marine organisms (Carvalho & Pitcher 1995). MtDNA markers have been widely used for population genetic structure analyses (Kocher *et al.* 1989). The use of molecular biology methods in the studies of population genetics and evolution has increased dramatically during the last decade (Tripath *et al.* 2013). There is little information available on the amplification success of the *COI* gene fragment in gastropod tissues (Skujiene & Soroka 2003). The molecular tree study based on *COI* gene sequence data strongly supports the results based on morphology and behavior (Nitz *et al.*, 2009). Currently, one of the most widely used genes for phylogeny, systematics and the identification of species is *COI* (Huelsken *et al.* 2011). Nucleotide sequences of nuclear and MtDNA analyses were provides us a basis for taxonomic and phylogenetic considerations (Skujiene & Soroka 2003). The DNA sequences of cytochrome c oxidase subunit I (COI) and barcode gene, may serve as additional valuable character set for phylogenetic analyses and subsequent identification (Nitz *et al.* 2009). This study showed some differences in nucleotide sequences in comparison with the same nucleotide sequences for *Limax flavus* which published in NCBI data. Two primary evolutionary mechanisms that cause population differentiation are genetic draft and natural selection (Hufford & Mazer 2003). Development of ecological adaptation or ecotype will resulted by natural selection (Awodiran & Ogunjobi 2016). Also, these two forces may interact with other factors, such as breeding system, life-history traits, dispersal and other evolutionary and ecological processes to determine the genetic structuring patterns that are observed in the field (Gow *et al.* 2004). This study revealed a good similarity with *Limax flavus* specimen published in NCBI data and this help us for confirming that the collected genus is *Limax flavus*.

V. Conclusions

In conclusion, the collected specimen species was the first record in Iraqi environment and the sequences results confirms that this specimen is *Limax flavus*. *COI* gene sequence in this work was deposited in the NCBI gene bank repository (www.ncbi.nlm.nih.gov/nuccore/MF034732).

References

[1] Al-Ameen F.A.M. (2018). An Ecological survey of Benthic invertebrates in Three Sites in Shatt-Al- Kufa at AL- Najaf Province,Iraq . Journal of University of Babylon for Pure and Applied Sciences, Vol.(26),No.(8):230-239. https://doi.org/10.29196/jubpas.v26i8.1691

[2] AWODIRAN M. O. & Z. O. Ogunjobi Z. O. 2016. — Genetic diversity in two populations of *Limicolaria aurora* (Jay, 1839) from two ecological zones in Nigeria. *African Journal of Biotechnology* 15: 1306-1314. https://doi.org/10.5897/AJB2015.15154

[3] BRANSON, B. A. 1980. — The Recent gastropoda of Oklahoma Pat VIII Slug Families and Philomycidae," *Proceedings of the Oklahoma Academy* of *Science*, vol. 60, pp. 29-35, 1980.

[4] CAMERON R. A. D. 1983. — A field key to the slugs of British Isles (Mollusca–Gastropoda– Pulmonata). *Field Studies* 5: 807–824.

[5] CAMERON R. A. D., Eversham B., Jackson N. 1983. — A field key to the Slugs of the British Island. *Field Studies* 5: 807-827.

[6] CARR C, Hardy S. M., Brown T. A. Macdonald T. M. & Hebert P. D. N. 2010. — A tri-oceanic perspective: DNA barcoding reveals geographic structure and cryptic diversity in Canadian polychaetes," *PLoS ONE* 6: e22232. https://doi.org/10.1371/journal.pone.0022232

[7] CARVLHO G. R. & Pitcher T. J. 1995. — Molecular Genetics in Fisheries. Chapman & Hall, London. http://dx.doi.org/10.1007/978-94-011-1218-5.

[8] DAVIS G. M. 1994. — Molecular genetics and taxonomic discrimination. *The Nautilus* 2: 2-23.

[9] DENNY M. W. 1983. — Molecular biomechanics of mollucan mucous secretions *In* Wilbur K., Simkiss K., Hochka P.W., editors. Mollusca, Vol. 1, New York: Academic Press, 341–465p. https://doi.org/10.1016/B978-0-12-751401-7.50017-X

[10] DONNEL R. J. M., Paine T. D. & Gormally M. J. 2009. — Slugs A guide to the invasive and native fauna of California," University of California. Division of Agriculture and Natural Resources. Publication, 8336 p.

[11] DOURIS V., Giokas R., Lecanidou M., Mylonas M. & Rodakis G. C. 1998. — Phylogenetic analysis of mitochondrial DNA and morphological characters suggest a need for taxonomic re-evaluation within the Alopiinae (Gastropoda: Clausiliidae). *Journal of Molluscan Studies*, 64: 81-92. https://doi.org/10.1093/mollus/64.1.81

[12] DOYLE J. J. & Doyle J. L. 1987. — A rapid DNA isolation procedure for small quantities of fresh leaf tissue. *Phytochemical Bulletin* 19: 11-15.

[13] FOLMER O., Black M., Hoeh W., Lutz R. & Vrijenhoek R. 1994. — DNA primers for amplification of mitochondrial cytochrome *c* oxidase subunit I from diverse metazoan invertebrates. *Molecular Marine Biolology and Biotechnology* 3: 294-299.

[14] FORSYTH R. G. 2004. — Land snails of British Colombia. Royal British, Colombia museum, Canada, 188 p.

[15] GAMIL I. S. 2013. — Spermatogenesis and sperm ultrastructure in the land slug *Limax flavus* (Gastropoda: Pulmonata) from Egypt. *Advances in biological Research*, 76: 253–265.

[16] GEIST J., Wunderlich H. & Kuehn R. 2008. — Use of mollusc shells for dna-based molecular analyses. *Journal of Molluscan Studies* 74: 337–343. https://doi.org/10.1093/mollus/eyn025

[17] GITTENBERGER A. B., Kokshoorn, B. & Gittenberger, E. 2006. — A molecular phylogeny of Epitoniidae (Mollusca: Gastropoda), focusing on the species associated with corals. *In* Gittenberger, A. (ed.). The

evolutionary history of parasitic gastropods and their coral hosts, PhD Thesis, Leiden University, Chapter 8, 207-213.

[18] GOW J., Noble L. R., Rollinson D., Mimpfoundi R. & Jones C. S. 2004. — Breeding system and demography shape population genetic structure across ecological and climatic zones in the African freshwater snail, *Bullinus forskalii* (Gastropoda: Pulmonata), host for *Schistosomes*. *Molecular Ecolology* 13: 3561-3573. https://doi.org/10.1111/j.1365-294X.2004.02339.x

[19] HEBRET P. D. N., Cywinska A., Ball S. L. & deWaard J. R. 2003. — Biological identifications through DNA barcodes. Proceedings of the Royal Society of London Series B: *Biological Sciences*, vol. 270, pp. 313-321, 2003. https://doi.org/10.1098/rspb.2002.2218

[20] HUELSKEN T., Schreiber S. & Hollmann M. 2011. — COI amplification success from mucus-rich marine gastropods (Gastropoda: Naticidae) depends on DNA extraction method and preserving agent. *Die Mitteilungen der Deutschen Makrozoologischen Gesellschaft* 85: 17-26.

[21] HUFFORD K. M. & Mazer S. J. 2003. — Plant ecotypes: Genetic differentiation in the age of ecological restoration. *Trends in Ecology and Evolution* 18: 147-1415. https://doi.org/10.1016/S0169-5347(03)00002-8

[22] HUTCHINSON J. M. G. & Reis H. 2015. — Mating in *Ariunculus isselii* an aroid Slug without aspermatophore. *Journal of Molluscan Studies* 81: 247–258. https://doi.org/10.1093/mollus/eyu086

[23] IIDZUKA, Y. & Aranishi F. 2008. — DNA Fingerprinting for Ostreidae Oysters in Kyushu. *Laguna* 15: 69-76. (In Japanese).

[24] KAMAL D. & AlMUKTAR E. 2010 – An ecological study of the Brown Garden snail Cornu aspersum(Muller,1774) from selected habitats in Jadiriya in Baghdad vicinity . Journal of Baghdad sciens , vol.7(1):224-232. https://doi.org/10.21123/bsj.7.1.223-232

[25] KEARNEY J. 2010. — Kerry slug (*Geomalacus maculosus* Allman 1843) recorded at lettercraffor Co. Galway. *Irish Naturalists Journal* 31: 68 – 69.

[26] KLEE B., Hyman I. T. & Haszprunar G. 2007. — Species boundaries in *Limax* (Gastropoda: Stylommatophora): extreme colour variations in and between species," Abstract. 9. Jahrestagung GfBS, Wien, Feb. 2007. *Organisms Diversity and Evolution* 7: 59–60.

[27] KOCHER T. D., Thomas W. K., Meyer A., Edwards S. V., Paabo S., Villablanca F. X. & Wilson A. C. 1989. — Dynamics of Mitochondrial DNA Evolution in Animals: Amplification and Sequencing with Conserved Primers. *Proceedings of the National Academy of Sciences USA* 86: 6196-6200. http://dx.doi.org/10.1073/pnas.86.16.6196.

[28] LAYTON K. 2013. — Examining patterns of genetic variation in Canadian marine molluscs through DNA barcodes. M.Sc. Thesis. University of Guelph, Canada.

[29] LEGRAND B., Mazancourt P. D., Durigon M., Khalifat V. & Crainic K. 2002. — DNA genotyping of unbuffered formalin fixed paraffin embedded tissues. *Forensic Science Internationa* 125: 205–211. https://doi.org/10.1016/S0379-0738(01)00641-7

[30] NITZ B., Heim R., Schneppat U. E., Hyman I. & Haszprunar G. 2009. — Towards a new standard in slug species descriptions: the case of *Limax* Sarnensis Heim & Nitz N. SP. (Pulmonata: Limacidae) from the western central Alps," *Journal of Molluscan Studies* 75: 279-294. https://doi.org/10.1093/mollus/eyp030

[31] PEREIRA J. C., Chaves R., Bastos, E., Alexandra Leitão A. & Guedes-Pinto H. 2011 — An Efficient Method for Genomic DNA Extraction from Different Molluscs Species. *International Journal of Molecular Science* 12: 8086-8095. doi: 10.33907/ijms12118086, 2011.

[32] POLSBRY H. A. 1984. — North America (North Mexico), *Academy of Natural sciences of Philadelphia Monograph* 2: 521-1113.

[33] RADULOVICI A. E., Archambault P. & Dufresne F. 2010. — DNA barcodes for marine biodiversity: moving fast forward?. *Diversity* 2: 450-472. https://doi.org/10.3390/d2040450

[34] ROTH B. & Sadeghian P. S. 2006. — Checklist of the Land Snails and Slugs of California," Contribution in science 3 . Santa Barbara Museum of Natural History, California, 82 p.

[35] SALAM A. & El-wakeil N. 2012. — Biological and ecological studies on land snail and their control. *In* Integrated best management and best control current and future Tactics, Eds. Larramendy , M. L. and S. Soloneski, 414-444 p. https://doi.org/10.5772/29701

[36] Shehab Z.H.,Al-Ameen N. E. & Jawad N. H. (2015) . Isolation and Identification of some Terrestrial Invertebrates Bacteria from Baghdad , Al-Jadiriya . Diyala Journal For Pure Sciens Vol.11 NO.:4 : 58-68.

[37] SKUJIENE G. & Soroka M. 2003. — A comparison of different DNA extraction methods for slugs (Mollusca: Pulmonata). *Ekologija* 1: 12-16.

[38] SOKOLOV E. 2000. — An improved method for DNA isolation from mucopolysaccharide-rich molluscan tissues. *Journal of Molluscan Studies* 66: 573-575. https://doi.org/10.1093/mollus/66.4.573

[39] TELEBAC B., Brajkovic M. & Curcic S. 2013. — Contribution to the knowledge of the slugs (Gastropoda–Stylommaphora– Limacidae Milacidae) fore Montenegro. *Bulletin of the natural History Museum* 6: 55–64. https://doi.org/10.5937/bnhmb1306055T

[40] TEL-ZUR N. A. S., Myslabodski D & Mizrahi Y. 1999. — Modified CTAB procedure for DNA isolation from epiphytic cacti of the genera *hylocereus* and *selenicereus* (cactaceae). *Plant Molecular Biolology Reporter* 17: 249–254. https://doi.org/10.1023/A:1007656315275

[41] THOMAS A. K., Donnel R. G. M., Pain T. D. & Harwood J. D. 2010. — A field guide to the slug. Kentucky University of Kentacky, Department of Intomology, USA, 34.

[42] TRIPATHI P. N. K., Kaur P & Kumari A. 2013. — Optimization of DNA isolation and PCR protocol for RAPD analysis from mucopolysaccharide rich foot muscles of *Lymnaea acuminate* (Gastropoda: Pulmonata) from Indian Himalayan regions. *International Journal of Zoological Research* 3: 35-42.

[43] WALSH P.S., Metzger D.A. & Higuchi R. 1991. — Chelex 100 as a medium for simple extraction of DNA for PCR-based typing from forensic material. *Biotechniques* 10: 506–513.

[44] WIKTOR A., De-Niu A. & Ming W. 2000. — Stylommatophoran slugs of China (Gastropoda– Pulmonata). *Prodromus folia Malacologica* 8: 3–35. https://doi.org/10.12657/folmal.008.002

[45] WINNEPENNINCKX B., Backeljau T. & De Wachter R. 1993. — Extraction of high molecular weight DNA from molluscs. Trends in Genetics, vol. 9, 409 p https://doi.org/10.1016/0168-9525(93)90102-N

TABLE I: PRIMERS FOR COI GENE AMPLIFICATION

Primer	Sequence (5´ → 3´)
LCO1490 (F)	5´-GGTCAACAAATCATAAAGATATTGG-3´
HC02198 (R)	5´-TAAACTTCAGGGTGACCAAAAAATCA-3´

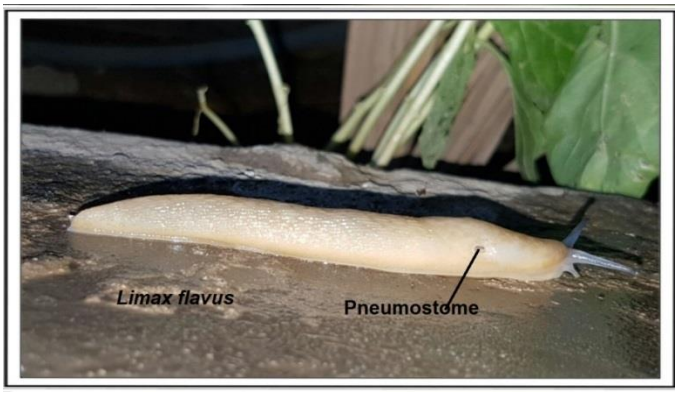

Fig. 1: External feature of the collected *Limax flavus* (L., 1758)

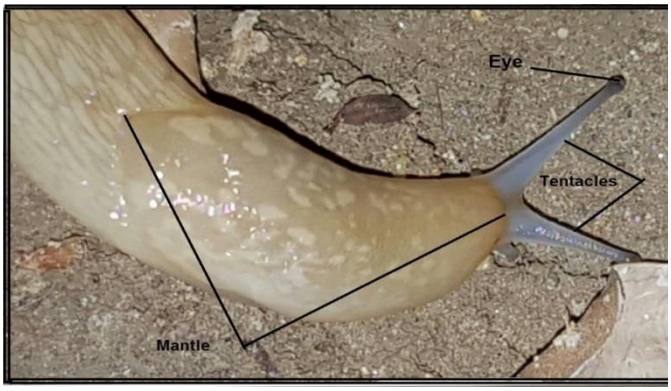

Fig.. 2: The tentacles and mantle of the collected Limax flavus (L., 1758)

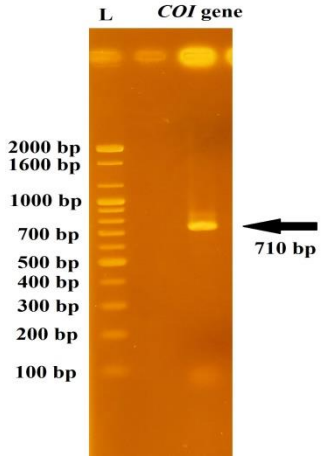

Fig. 3: Gel electrophoresis for mtDNA COI gene in the collected *Limax flavus* specimen (Gel electrophoresis was performed by using 1.5% agarose gel concentration at 75 Volts for 2 hours).

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Query 1    TGGAACAGGTTTATCTTTATTAATCCGATTAGAGCTTGGAACGGCTGGTGTTTTATTAAA 60
           ||||||||||||||||||||||||| ||||||||||||||||||||||||||||||||| |
Sbjct 39   TGGAACAGGTTTATCTTTATTAATTCGATTAGAGCTTGGAACGGCTGGTGTTTTATTAGA 98

Query 61   TGACCATTTTTTTAATGTAATTGTTCCCGCCCCCCCCTTTGTAAAAATTTTTTTTATAGT 120
           ||||||||||||||||||||||||| | || |   | ||||||| ||||||||||||||
Sbjct 99   TGACCATTTTTTTAATGTAATTGTTACTGCTCATGCATTTGTAATAATTTTTTTTATAGT 158

Query 121  AATACCAATTATAATTGGTGGGTTTGGAAATTAAAAAATTCCCTTCTTAATTGGAGCACC 180
           ||||||||||||||||||||||| |||||||| | || | |||| || |||||||| |||||
Sbjct 159  AATACCAATTATAATTGGTGGATTTGGAAACTGAATAGTTCCGTTGTTAATTGGTGCACC 218

Query 181  CGAAATAAGGTTCCCACGAATAAATAACATAAGATTTTGACTTCTCCCCCCTTCTTTTAT 240
             || ||||||||||||||||||||||| |||||||||||||||||| || ||||||||||
Sbjct 219  TGATATAAGGTTCCCACGAATAAATAATATAAGATTTTGACTTCTTCCGCCTTCTTTTAT 278

Query 241  TTTATTAATTTGTTCAAGAATAGGAGAAGGTGGTGCCGGGACCGGGTGAACTGTATACCC 300
           ||||||||||||||||||||||| |||||||||||||||||| ||||||||||||||||||
Sbjct 279  TTTATTAATTTGTTCAAGAATAGTAGAAGGTGGTGCCGGGACAGGGTGAACTGTATACCC 338

Query 301  CCCCTTAAGGGGGCCTTTAGGTCCTGGGGGTGCTCCATTTGATTTACCTATTTTTTCATT 360
             || |||||||||||||||||| |||||||| || |||||||| ||||||||||||||||
Sbjct 339  ACCATTAAGGGGGCCTTTAGGTCATGGGGGTGCTTCAGTTGATTTAGCTATTTTTTCATT 398

Query 361  ACATTTACCTGGAAAATCTTCTATTTTAGGTGCAATTAATTTTATTACAACTATTTTTAA 420
           ||||||| |||||| ||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 399  ACATTTAGCTGGAATATCTTCTATTTTAGGTGCAATTAATTTTATTACAACTATTTTTAA 458

Query 421  TAAACAATCTCCTGGAATAACAATAAAACGTGTAAGACTATTTGTGTGGTCTATTTTAGT 480
             || || ||||||||||||||||||| |||||||||||||||||| ||||||||||||||
Sbjct 459  TATACGATCTCCTGGAATAACAATAGAACGTGTAAGACTATTTGTATGGTCTATTTTAGT 518

Query 481  AACTGTTTTTCTATTACTTCTTTCTTTACCTGTTTTAGCTGGGGCTATTACAATCCTGGT 540
           ||||||||||||||||||| |||||||||||||||||||||||||||| || ||  |
Sbjct 519  AACTGTTTTTCTATTACTTCTATCTTTACCTGTTTTAGCTGGGGCTATTACTATGCTACT 578

Query 541  AACTGATCATAATTTTAATACAAGGTTTTTTGATCCCGCTGGTGGAGGGAATCCAATTTT 600
           |||||||| ||||||||||||||||||||||||||| |||||||||||| ||||||||||
Sbjct 579  AACTGATCGTAATTTTAATACAAGGTTTTTTGATCCAGCTGGTGGAGGGGATCCAATTTT 638

Query 601  ATATCAACATTTGTTTTGGTTTTTTG 626
             ||||||||||||||||||||||||
Sbjct 639  ATATCAACATTTGTTTTGGTTTTTTG 664
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Fig. 4: *Limax flavus* specimen sequences alignment of the mtDNA *COI* gene with the reference gene from NCBI database (accession number FJ606456.1)