



Biotechnological and Illustration Study of *Ludwigia adscendens* Aquatic Plant Growing in Sewage

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ABSTRACT

The water primrose has garnered noteworthy consideration due to its detrimental impact on both the economy and environment. *Ludwigia adscendens* is one of two species which represent the genus *Ludwigia* among the Egyptian aquatic flora. Therefore, understanding *Ludwigia adscendens* at different levels was essential to develop reasonable strategies to manage or utilize it positively. Life systems of different organs was conducted to provide a clear picture of the internal structures involved in the plant's survival in sewage biological system. Aerenchyma tissue, distributed as the main tissue in most organs, allows the plant to float and maintain balance. SEM investigation revealed dust grains with diverse composition, symmetrical radiographic pattern, large size (58.5µm in polar diameter and 43.5µm in equatorial diameter), and oblate shape. DNA barcoding was supported using the *rbcl* gene for identification, revealing significant diversity across various biological and botanical methods.

INTRODUCTION

The Egyptian ecosystem has about 35 species of aquatic weeds belonging to 19 genera and 15 families. The water primrose (*Ludwigia adscendens*) is one of two species, which represent the genus *Ludwigia* among the Egyptian aquatic plants. It can either float freely or have roots that reach the bottom and penetrate the soil (Täckholm, 1974; Zahran & Willis, 1992). *Ludwigia adscendens* is one of numerous aquatic plants that are exceedingly obtrusive and cause critical economical and natural harm in numerous parts of the world (EPPO, 2004; Global Invasive Species Database, 2009). Seven morphotypes were detected by Amer *et al.* (2016) during their study of the morphological and anatomical taxonomic characters of *L. stolonifera* exhibiting the flexibility of those weeds in adapting to their surrounding ecosystem.

Methods for identifying species by using short orthologous DNA sequences, known as “DNA barcodes,” have been proposed and initiated to facilitate biodiversity studies. Moreover, DNA barcoding as molecular markers are now used for taxonomic identification and genetic variation studies (**Costion *et al.*, 2011**). It can be described as using short nuclear DNA sequences (400- 800bp) that have adequate sequence variation to identify different taxa and distinguish among species (**Ganie *et al.*, 2015**). In all groups of land plants, an ideal DNA barcode should be present and show enough sequence variation to differentiate among species, and also it should be easily amplifiable and sequenced using only one primer pair (**Kress & Erickson, 2007**). The plastid genome has different regions including contained *rbcL*, which has been used for DNA barcoding of plants with different success rate and different strengths and weakness according to the studied organism (**Kress & Erickson, 2007; Singh *et al.*, 2012**). *rbcL* gene encodes the large subunit of Rubisco and is widely used in sequence phylogeny and analysis. **Kool *et al.* (2012)** demonstrated that mutation rate in *rbcL* can be detected and measured.

This study investigated *Ludwigia adscendens* from multiple perspectives, such as its anatomy, dust morphology observed via SEM, and DNA identification using the *rbcL* gene. The aim was to understand how *Ludwigia adscendens* survives in sewage ecosystems and whether it has any negative impacts.

MATERIALS AND METHODS

Ludwigia adscenden (L.) mature flowering individuals were collected from sewage surface located in Qalubia, Egypt, with GPS location (578Q+WH9•6313701). The studied taxon's fresh sample was identified using keys of **Täckholm (1974)** and **Boulos (2002)**. Subsequently, it was prepared and stored in the Herbarium of the Department of Botany and Microbiology, Faculty of Science, Al-Azhar University, Cairo, Egypt. Additionally, the plant's identification was verified by comparing it with herbarium specimens from Cairo University Herbarium (CAI).

A new method for anatomical examination was established using F.A.A. solution, which is a combination of formalin, glacial acetic acid, and 70% ethyl alcohol in a 5:5:90 ratio by volume (**Nassar & El-Sahhar, 1998**). Following this, sections of leaves, petioles, and roots were prepared using a rotary microtome with the paraffin wax technique and sliced at a thickness of 10- 15µm. A double staining process with Safranin and Quick Green was then applied for microscopic observation (**Dilcher, 1974**). The terminology regarding mesophyll types is based on previous studies (**Fahn, 1974; Metcalfe & Chalk, 1979; Bancroft & Gamble, 2008**). Dust grains were confined utilizing a stereomicroscope from hydrated anthers to prevent them from contracting, and then dried out using absolute alcohol. The mounting on SEM stubs and the coating with

nano-gold were connected as a conductive fabric to improve picture quality and diminish charging impacts (**Goldstein et al., 2018**).

The fresh leaf material for DNA analysis was promptly dried and stored in packets with silica gel to prevent moisture. DNA investigation extractions were performed on leaf tissue from all tests utilizing the QIAGEN-mini-DNA extraction convention. Dry leaf tissue was disrupted in individual lysing tubes with a dot process. Moreover, DNA extraction was conducted following the method outlined by **Doyle and Doyle (1990)**. To obtain high-quality DNA from ancient, dried samples, we utilized the QIAamp DNA stool mini Kit (from QIAGEN) following the manufacturer's instructions. Specific regions of plastid DNA *rbcL* (Ribulose-bisphosphate carboxylase chain) sequences were amplified from the dried leaf extracts. All inclusive preliminaries were utilized concurring to **Kress et al. (2005)**. PCR amplification was initiated in the initial step using a standard DNA polymerase (non-hot-start) with approximately 20ng of genomic DNA as a template in a 20- μ L reaction mixture (composed of 2 μ L of 10x reaction buffer Dream Taq™ from Fermentas, Lithuania, 2pM of each dNTP, 4pM of each primer, and 0.2U of Dream Taq™ DNA polymerase). The PCR protocol consisted of the following steps: one cycle for 5min at 94°C, followed by 35 cycles of 30s at 94°C, 30s at 55°C, and 60s at 72°C, with a final extension step of 10min at 72°C. PCR products were purified and then subjected to a direct sequencing using BigDye Eliminator v3.1 on a 3100 sequencer from Applied Biosystems. Sequences, and they were aligned using CLUSTAL-W (**Thompson et al., 1994**).

Bioinformatic

Muscle arrangements were analyzed (MEGA 11.0) to assess species relationships (Tamura-3P) and visualize their evolutionary divergence (NJ trees, bootstrapping, ITOL). (**Tamura, 1992**). To demonstrate the designs of species disparity, N.J. trees utilize Tamura 3-parameter strategy (**Tamura, 1992**). Bootstrapping was performed in MEGA 11.0 (**Kumar et al., 2004**), with 1000 replications. Visualization upgrade was conducted utilizing ITOL computer program (**Letunic & Bork, 2021**).

RESULTS AND DISCUSSION

In this study, the result showed that, the analysis of plants' internal structure (Plate 1) indicated the presence of aerenchyma tissue in all plant organs to facilitate floating. Aerenchyma tissue appears to be the main and dominant tissue in *Ludwigia adscendens* organs, except in the blade. In blade observations, the epidermis is uniseriate, with low thickness and a thick cuticle, with typical stomata distributed along the lower and upper surfaces. Typical dorsiventral mesophyll is present in the wings, with palisade parenchyma which is continuous in midrib, while the spongy parenchyma is not continuous. The midrib is rounded and penetrated by large collateral vascular bundle.

Thin layers of palisade and spongy parenchyma are present as a ground tissue in midrib. Druses are also present, as shown in Plate 1 (Figs. 1, 2). Petiole is rectangular in shape with two small protuberances filled with dense green parenchyma, penetrated by two small collateral vascular bundle. The epidermis is uniseriate and covered with a thick warty cuticle. The green aerenchyma tissue resembles the ground tissue, penetrated in the center by many collateral vascular bundles arranged in C shape. Additionally, phloem patches are found in the medulla Plate 1 (Figs. 3, 4).

Stem examination revealed that the outline is rounded, with a uniseriate epidermis covered by a thick cuticle. The cortex starts with 2- 4 layers of angular collenchyma, followed by a large area of aerenchyma. The vascular cylinder is composed of a large, continuous, circular collateral bundle. The pith is filled with thin parenchyma, and phloem patches are present. Raphides, druses, and sand crystals are also present, as shown in Plate 1 (Figs. 5, 6).

Pedicle TS observation revealed a cordate outline with a uniseriate epidermis covered by a thick cuticle. The cortex contains 1 -2 layers of angular collenchyma, followed by small areas of aerenchyma and thin parenchyma, with cortical vascular bundles also present. The vascular stele consists of five collateral vascular bundles arranged in a ring, alternating with ovules. The medulla is filled with thin parenchyma, and phloem patches are present, as shown in Plate 1 (Figs. 8, 9).

A thick cuticle in the leaf, petiole, stem, and pedicle indicates its role in preventing microbial infections. Additionally, the presence of widespread aerenchyma tissue in most organs serves as a defense mechanism to impede or slow the spread of pathogens in the event of an infection, while also aiding in maintaining buoyancy.

Examination of the adventitious root transection reveals a crushed young epidermis followed by many layers of secondary aerenchyma in the cortex, forming chambers for water storage, then the endodermis (Casparian strip). The diagonal vascular bundle, with large xylem vessels, encloses a small pith of thin parenchyma (Plate 1; Figs. 10- 12). The water chambers in the root help maintain the plant's balance and may also filter absorbed water as a defense mechanism against heavy metals and microbes. These results are consistent with the study by **Scremin-Dias *et al.* (2023)** on *Ludwigia grandiflora*.

The morphology of pollen grains indicates that they are monads, heteropolar, radially symmetric, large in size (P-58.5/ E-43.5 μ m), oblate in shape, trizonoporate, and have plicae ornamentation (Plate 2; Figs. 1- 4). Additionally, no mutations or changes in the pollen grain structure were observed.

The neighbor-joining strategy was used to construct the phylogenetic tree, which is shown in Plate 3, Fig. (1). The rate of duplicate trees, in which the associated taxa

clustered together in the bootstrap test (1000 replicates), is indicated by branch colors, which correspond to bootstrap values (**Felsenstein, 1985; Saitou & Nei, 1987**). The tree is drawn to scale, with branch lengths in the same units as the evolutionary distances used to construct the phylogenetic tree (Plate 3). The evolutionary distances were calculated using the Tamura 3-parameter method (**Tamura, 1992**), and they are expressed as the number of base substitutions per site. This analysis included 12 nucleotide sequences (13 sequences were retrieved from NCBI, and one sequence served as an outgroup). Codon positions included were 1st+ 2nd+ 3rd+ Noncoding. All ambiguous positions were removed for each sequence pair (pairwise deletion option). There were a total of 579 positions in the final dataset.

The chloroplastic gene (*rbcL*) as DNA barcoding loci was compared for their amplification, sequencing, and efficacy to evaluate the genetic diversity. The products of *rbcL* amplified by PCR were fractionated by 1.5% agarose gel electrophoresis. The results showed 100% amplification success. PCR products of *rbcL* electrophoretic separation showed sharp DNA amplicons without other products, reflecting high specificity of PCR reactions. The DNA fragments generated had sizes of approximately 600 base pairs for *rbcL*. In our study, the *rbcL* band matched the *rbcL* sequences in the Gene Bank, showing a match only with *Ludwigia adscendens* species. This indicates that the sewage ecosystem does not affect the genetic stability of this aquatic plant. Phylogenetic analyses were conducted in MEGA11 (**Tamura et al., 2021**). The results were then visualized using the ITOL program (**Letunic & Bork, 2021**).

DNA barcoding can be considered as an initial indicator of the scope and nature of genetic diversity. Moreover, comparative studies of population diversity of several plants became easy by utilizing it (**Hajibabaei et al., 2007**). The *rbcL* barcoding has a much higher level of sequence recovery than *matK* (95 and 66%, respectively), demonstrating a greater ability to differentiate between flowering plants (**Amandita, 2019**).

The results of anatomy, palynology, and DNA analysis, specifically the *rbcL* region, for *Ludwigia adscendens* collected from sewage, align well with previous literature on *Ludwigia adscendens* from freshwater sources (**Metcalfe & Chalk, 1979; Zahran & Wills, 1992; Zheng & Zeng, 2008; Folorunso et al., 2014; Garg et al., 2021; Tamura et al., 2021**). The proposed theory regarding water filtration in the roots, the production of a thick cuticle, and the spread of aerenchyma tissue may provide an explanation for how *Ludwigia adscendens* manages to survive in sewage, addressing our study question.

CONCLUSION AND RECOMMENDATIONS

The current comprehensive analysis of *Ludwigia adscendens* collected from sewage has revealed striking correlations with the existing literature that focused on specimens from freshwater sources. The alignment of results across anatomical, palynological, and DNA analyses, particularly in the *rbcL* region, supports the robustness of our findings. The proposed theory, emphasizing the pivotal roles of water filtration in the roots, the production of a thick cuticle, and the spread of aerenchyma tissue, emerges as a compelling explanation for the survival mechanism of *Ludwigia adscendens* in sewage environments.

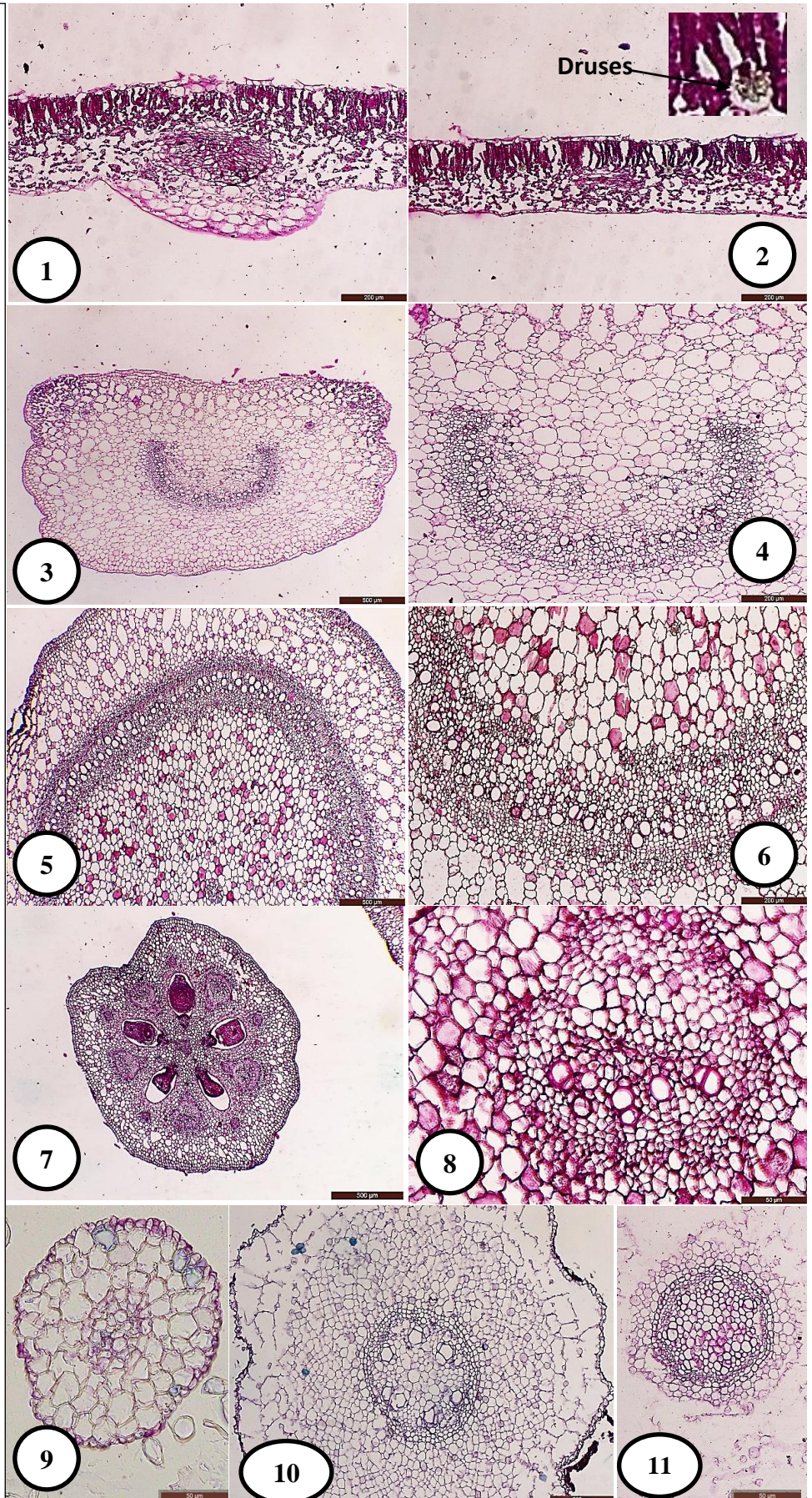
Based on the present findings, further research into the genetic adaptations and metabolic pathways of *Ludwigia adscendens* in sewage environments is warranted. Additionally, conducting an ecological impact assessment is essential to gauge potential consequences. Exploring practical applications for water treatment and conservation strategies should be prioritized. These steps would contribute to a more comprehensive understanding of *Ludwigia adscendens* and aid in developing sustainable solutions for sewage management.

Plate 1.

Figs. (1, 2) showing midrib TS with main vascular bundle and mesophyll. Figs. (3, 4) exploring leaf petiole shape and U-shaped vascular steel. Figs. (5, 6) revealing rounded stem TS and ring shaped vascular cylinder. Figs. (7, 8) showing pedicel transection, and the outline cordat with 5 ovules.

Figs. (9- 11) showing TS of adventitious root structure in different stages.

Used magnification powers: 4, 10 and 40x.



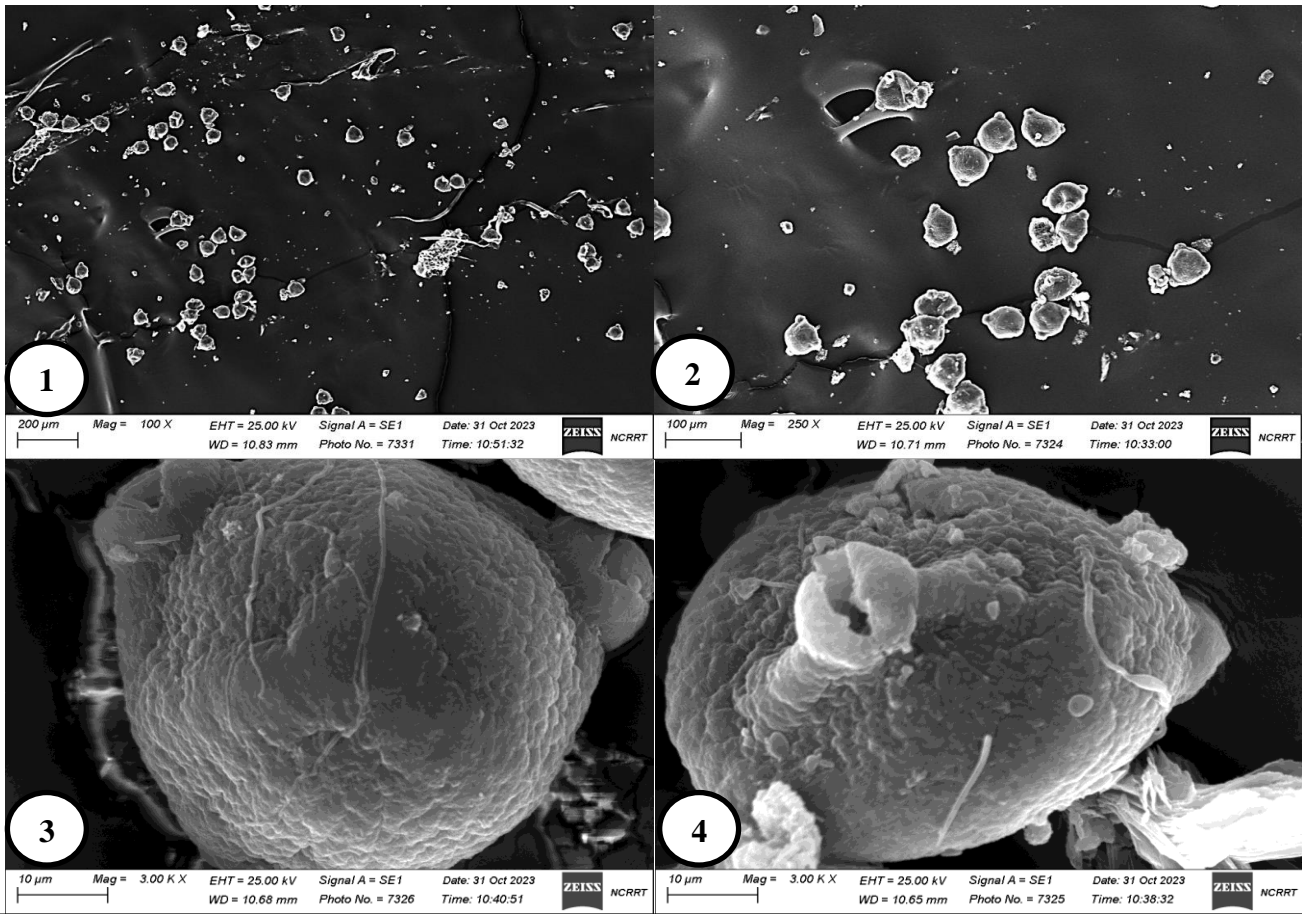


Plate 2. *Ludwigia adscendens* pollen grains on scanning electron microscope at 100- 300x magnification power. Figs. (1, 2) showing pollen grains monads, heteropolar, radiosymmetric, poller / equatorial views, oblate in shape, trizonoporate. Figs (3, 4) displaying surface ornamentation and pore

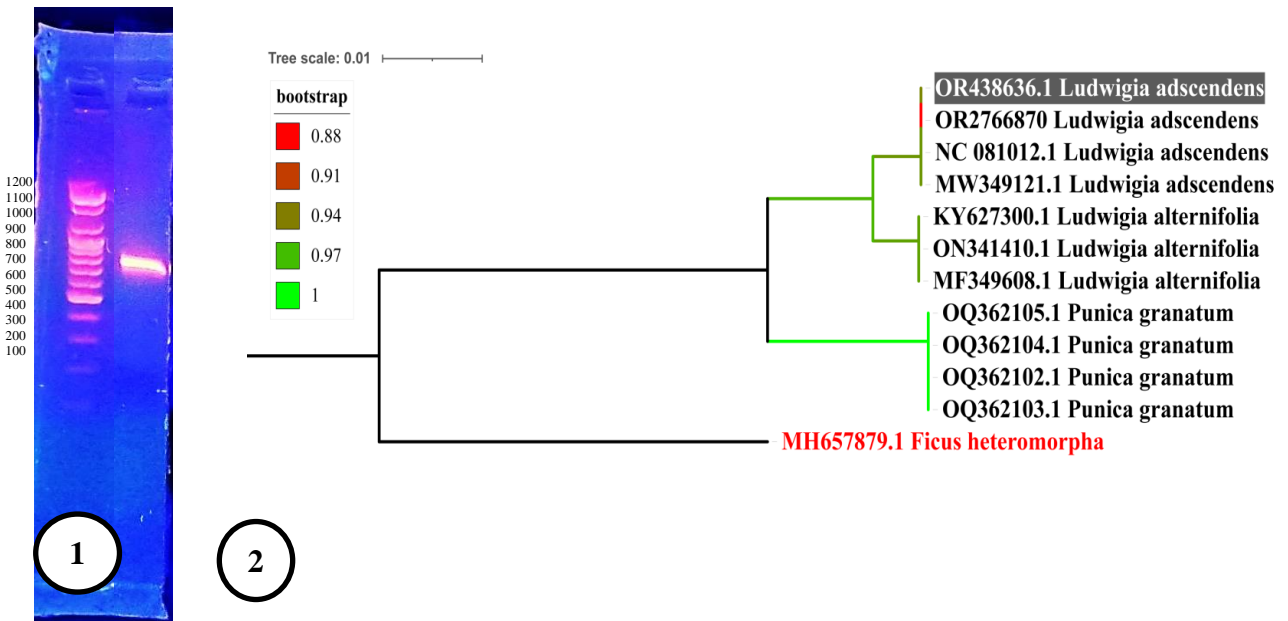


Plate 3. Fig. (1) *Ludwigia adscendens* DNA gel documentation under UV showing resulted band around 600 bp. Fig. (2) Neighbor-joining phylogenetic evolutionary tree

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