PHYLOGENY OF *Melodinus cochinchinensis* (Lour.) Merr. FROM VIETNAM BASED ON CHLOROPLAST SEQUENCES

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ABSTRACT

To support the conservation of plant resource diversity, it is necessary to prioritize the development, identification, and phylogenetic relationships of the plant for sustainable development. The genus *Melodinus* is widely distributed in tropical and subtropical regions. Due to its medicinal potential, the genus *Melodinus* is highly exploited, and it therefore provides a good example of applying scientific research to identify species and determine their genetic relationships for conservation and resource exploitation. The study using molecular data from three DNA regions (*matK*, *rbcL*, and *trnH-psbA*) supported the monophyly of *Melodinus*. The *M. cochinchinensis* from Vietnam and China are closely related. There are some genetic variations in the sequences of *Melodinus cochinchinensis* from Belgium versus Asiatic *M. cochinchinensis*, as well as the phylogenetic position of the species, suggesting the possibility of misidentification of *M. cochinchinensis* from Belgium. The Australian *Melodinus* is not a monophyletic group. Further study is necessary to resolve the phylogenetic relationship of Australian *Melodinus*.

NGHIÊN CỨU PHÁT SINH LOÀI *Melodinus cochinchinensis* (Lour.) Merr. Ở VIỆT NAM DỰA TRÊN MỘT SỐ TRÌNH TỰ GEN CỦA LỤC LẠP

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TỪ KHÓA

Phát sinh loài phân tử
Melodinus
Tự nhiên
Định danh
Tương đồng di truyền

TÓM TẮT

Nhằm hỗ trợ bảo tồn đa dạng tài nguyên thực vật, việc phát triển định danh tên khoa học và xác định mối quan hệ di truyền của các loài thực vật là cần thiết và nên được ưu tiên thực hiện cho sự phát triển bền vững. Chi *Melodinus* được phân bố rộng rãi ở vùng nhiệt đới và cận nhiệt đới. Do tiềm năng về dược tính của chúng mà chi này đã được quan tâm và khai thác, do đó nó cung cấp một ví dụ điển hình về việc áp dụng nghiên cứu khoa học nhằm định danh loài và xác định mối quan hệ di truyền của chúng phụ vụ cho công tác bảo tồn và khai thác tài nguyên. Nghiên cứu sử dụng dữ liệu phân tử của ba vùng DNA (*matK*, *rbcL* và *trnH-psbA*) ủng hộ *Melodinus* là nhóm đơn phát sinh. Loài *M. Cochinchinensis* ở Việt Nam và Trung Quốc có quan hệ di truyền gần gũi. Kết quả phân tích dữ liệu phân tử chỉ ra rằng, có một số biến thể di truyền trong trình tự của *M. Cochinchinensis* từ Bỉ so với *M. Cochinchinensis* ở châu Á, kết hợp với vị trí trên cây phát sinh loài của các cá thể *M. cochinchinensis* gợi ý rằng, có thể đã có sự nhầm lẫn trong định danh mẫu *M. cochinchinensis* ở Bỉ. *Melodinus* ở châu Úc được xác định không phải là nhóm đơn phát sinh, tuy nhiên, cần thêm các nghiên cứu để giải quyết mối quan hệ phát sinh loài của các loài *Melodinus* ở Úc.

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1. Introduction

The plant genus *Melodinus* belongs to the Apocynaceae, with about 53 species all over the world. The plants of the genus *Melodinus* are widely distributed in tropical and subtropical regions [1], [2]. Due to the potential benefits for human health, with over 100 alkaloids together with flavonoids, lignans, steroids, terpenoids and coumarins identified in the genus. *Melodinus* members have been used in folk medicine for a long time to treat meningitis in children, increase blood circulation, dispelling wind, nourish the lungs and anticancer and anti-inflammatory properties [1], [2].

Melodinus cochinchinensis (Lour.) Merr. is natively distributed in India, south China, and Southeast Asia including Vietnam (Figure 1). This species was used as traditional medicine to treat hernia, abdominal pain, and indigestion diseases [3].

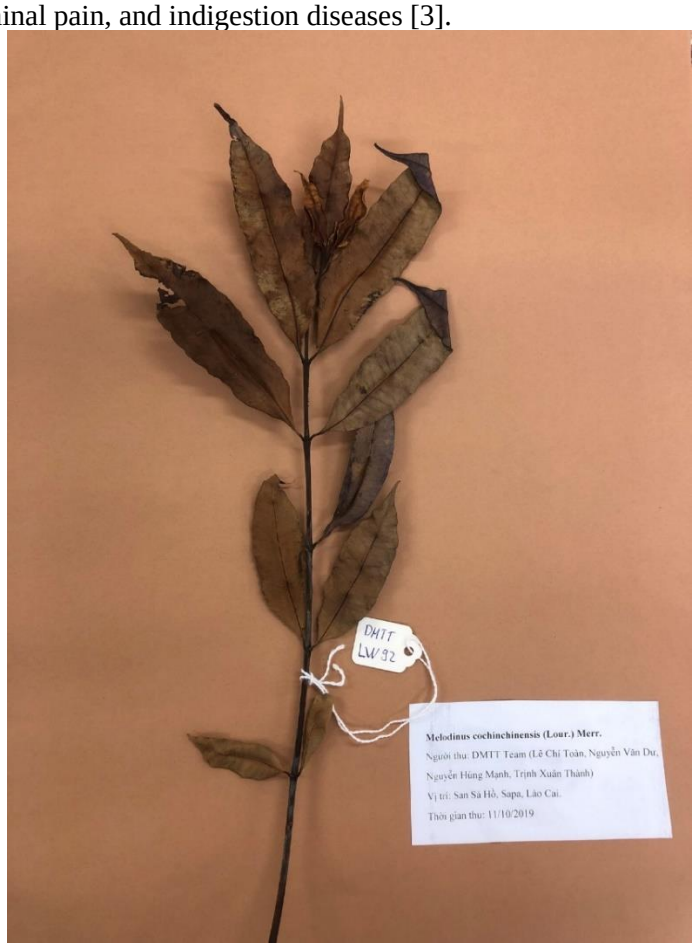

Figure 1. *The specimen of Melodinus cochinchinensis from Lao Cai, Vietnam*

Short orthologous DNA sequences, also referred to as "DNA barcodes", can be used to identify species, making them easier to study biodiversity, phylogenetic relationship of plant and animal [4]. Many chloroplast, mitochondrial and nuclear genes have been utilized for studying sequence variation at genus level. Among these genes, the ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit (*rbcL*) gene sequence has been analyzed by various workers to address plant systematics. The maturase K (*matK*) gene of chloroplast is about 1500 bp long, located within the intron of the trnK and codes for maturase like protein, which is involved in Group II intron splicing. The gene contains high substitution rates within the species and is emerging as potential candidate to study plant systematics and evolution. Additionally, the noncoding markers, such as *trnH-psbA* is universally applied based on its utility [5], [6].

Some phylogenetic studies of Apocynaceae were performed using molecular data [7]-[9]. Potgieter and Alber [7] conducted a molecular phylogeny of Apocynaceae based on *trnLF* DNA region of 146 species. The study indicated that Apocynaceae is a monophyletic group, and further taxonomic treatments were provided thereafter for Apocynaceae. Additionally, the study suggested that Apocynaceae likely originated in Gondwanan. However, the phylogenetic relationship within *Melodinus* is not mentioned.

Simões et al. [8], addressed the phylogeny of subfamily Rauvolfioideae (Apocynaceae) using both molecular and morphological data. The results supported the monophyly of Rauvolfioideae, but the tribe Melodineae is polyphyletic. *Melodinus cochinchinensis* was placed together with *M. australis*. Unfortunately, the sampling of *Melodinus* in the study was limited, thus, the phylogenetic relationship within this genus is still unclear.

A discussion on phylogenomic of Apocynaceae was performed by Fishbein et al. [9]. In this study, *Melodinus* and *Craspidospermum* were supported as sister, however, it is limited on the sampling of *Melodinus* (only *M. cambodiensis*) with lone species per genus thus, the genetic relationship of the genus needs further study.

Additionally, Lu et al. [1], and Yang et al. [2] investigated the chemical constituents from *Melodinus* genus and *M. cochinchinensis*. The studies did not discuss about the phylogeny of *Melodinus* members.

Thus, a phylogenetic analysis of *Melodinus* with extended sampling is needed to clarify the phylogenetic relationships of *Melodinus cochinchinensis* in Vietnam. This study aims to reconstruct the phylogeny and clarify the phylogenetic relationship of *Melodinus cochinchinensis* from Vietnam and its allies.

2. Materials and methods

2.1. Taxon sampling, DNA extraction, amplification, and sequencing

The study sampled seven species of *Melodinus* including three individuals of *M. cochinchinensis*. Three species *Allamanda schottii*, *Hunteria umbellata* and *Rhazya stricta* were used as outgroups. The sample *M. cochinchinensis* from Vietnam was collected during field surveys in 2019 in Lao Cai province (Figure 1), additionally, the samples of the species from China and Belgium were assembled from NCBI (Table 1). Three plastid markers *matK*, *rbcL*, *trnH-psbA* were used for molecular analyses in this study. However, the *matK* and *rbcL* sequences of *Melodinus cochinchinensis* from Vietnam are unsuccessfully sequencing or with very low quality (Figure 2) in this study, thus they are not included in the molecular data. In total, 25 sequences including one new sequence from this study were used for the molecular analyses (Table 1).

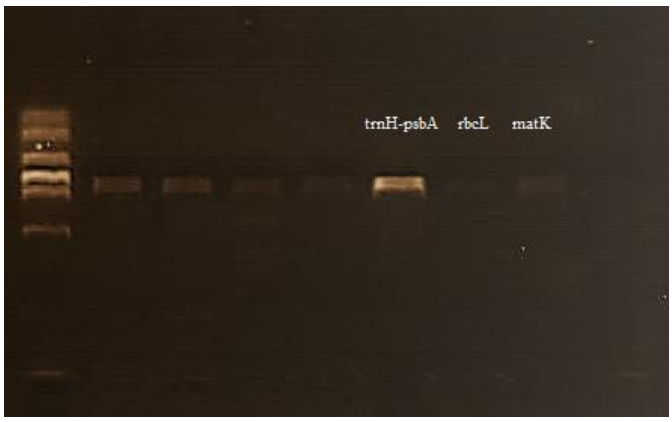

Figure 2. *The electrophoresis of Melodinus cochinchinensis samples. The three makers were noted in sample wells, respectively, the other sample wells are not Melodinus cochinchinensis*

Total genomic DNAs of samples were extracted from silica gel-dried leaves using the Plant Genomic DNA Kit (Tiangen, Beijing, China). Amplification protocol and primers for amplifying *psbA-trnH* followed Roeder et al. [10].

Table 1. *Voucher information and GenBank accession numbers for DNA sequences generated or used in this study. The sequences generated in this study begin with OR. "–" indicates missing data*

Name	Source	*rbcL*	*matK*	*trnH-psbA*
Melodinus acutiflorus	Australia	KU564826	KU564595	KU564692
Melodinus cochinchinensis	Belgium	DQ660652	DQ660525	–
Melodinus cochinchinensis	China	KF181555	HG004984	HG005102
Melodinus cochinchinensis	Vietnam	–	–	OR083241
Melodinus australis	Australia	DQ660651	DQ660524	–
Melodinus baccellianus	Australia	KF496707	–	–
Melodinus suaveolens	China	KX910872	KX526510	–
Melodinus fusiformis	China	EU916738	–	–
Melodinus monogynus	Australia	AJ419748	–	–
Rhazya stricta	–	KX602163	KX602160	KX602161
Hunteria umbellata	–	KC628608	KC627893	KC667951
Allamanda schottii	–	DQ660626	DQ660495	JX244898

The PCR amplification reactions used MasterMix of the MCLAB company (South San Francisco, California, USA). The PCR program consisted of 5 min at 95 °C, 35 cycles of 30 s at 95 °C, 35 s at 48 °C, and 1 min 30 s at 72 °C, with a final extension of 10 min at 72 °C.

PCR products were examined using electrophoresis and 1.0% agarose gels. The PCR products were purified with BioMed multifunctional DNA fragment purification recovery kits and then sequenced them using our amplification primers. The bidirectional sequencing was completed using an ABI 3730 DNA Sequencer (Applied Biosystems, Carlsbad, California, USA). The quality estimation and assembly for the newly generated sequences were performed in Geneious 8.0.5 [11]. The final sequences were aligned in MUSCLE 3.8.31 and then adjusted them manually in Geneious [11].

2.2. Phylogenetic analyses

Both the two methods, maximum likelihood (ML) and Bayesian inference (BI) were used to carry out the phylogenetic analyses of *Melodinus*. The ML analysis was conducted in RAxML 8.2.12 [12], [13] using the GTR+I+G standard nucleotide substitution model generated by jModeltest 2.1.6 [14] for each DNA region and the combined dataset applying 1,000 bootstrap replicates. The BI analysis was conducted in MrBayses 3.2.6 [15] on the CIPRES using the nucleotide substitution models that estimated separately each gene region by jModeltest 2.1.6 [10]. The MCMC algorithm was run for 10 million generations with four Markov chain Monte Carlo (MCMC) and trees were sampled every 1000 generations. To check the effective sample sizes (ESSs) of all relevant parameters (>200), we used Tracer v.1.6 [16]. With the first 25% of sampled generations (2500 trees) discarded as burn-in, a 50% majority-rule consensus tree and posterior probabilities (PP) were obtained using the remaining trees. Additionally, the proportion of variable sites in combined molecular matrices was evaluated by the maximum parsimony method in PAUP* [17].

3. Results and discussion

3.1. Molecular data

The study generated sequence of *trnH-psbA* for *Melodinus cochinchinensis* from Vietnam (Table 1). The lengths of individuals data sets of *matK, rbcL, trnH-psbA* are 1514, 1363 and 722 base pairs, respectively. The combined dataset included 3599 aligned positions for the ingroups and outgroups.

The combined dataset includes only 247 variable sites (6.86%). Manzanilla et al. [18] and Le [19] noted that the proportion of variable sites is likely an inverse ratio to molecular relationship, thus the low proportion of variable sites in combined dataset of *Melodinus* in this study indicates the close relationship of *Melodinus* species.

3.2. Phylogenetic relationship

The phylogenetic results from ML and BI trees of *Melodinus* based on the combined dataset were highly congruent, the few differences had low support. Thus, the phylogeny of *Melodinus* was combined in BI tree with BS and PP values, and presented in Figure 3.

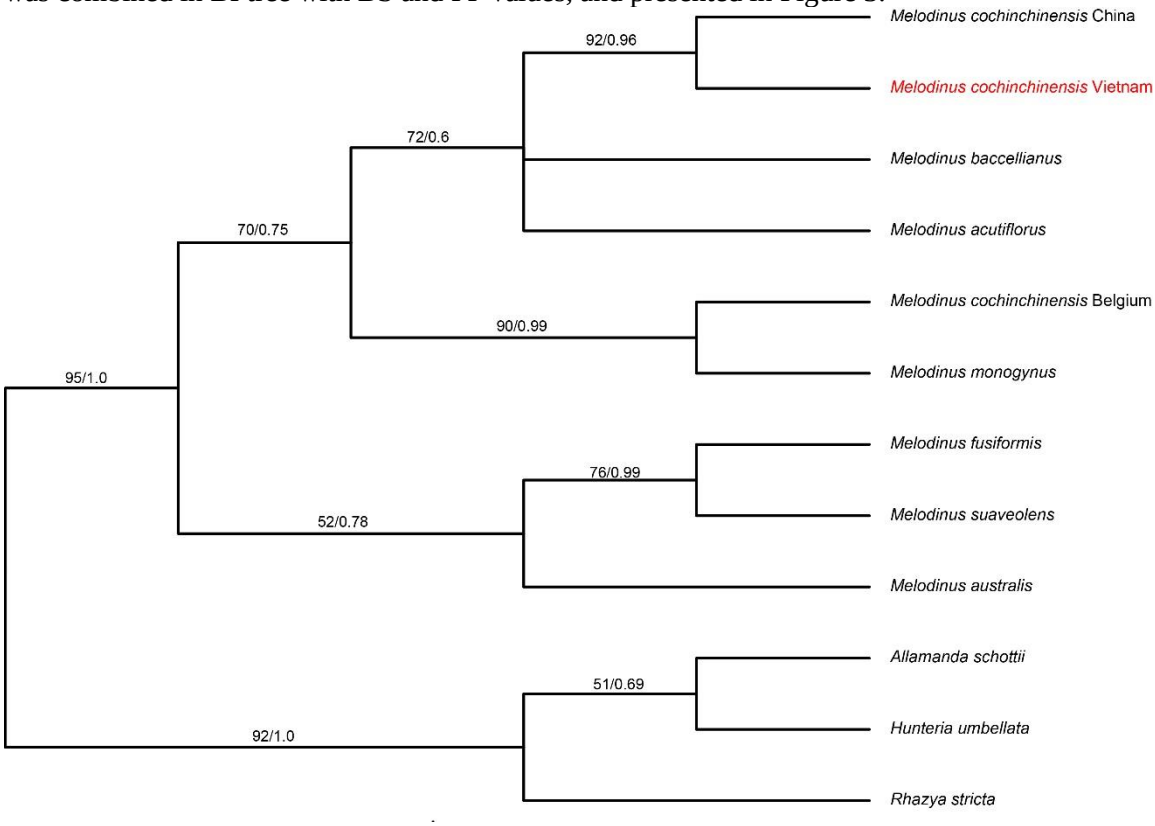

Figure 3. *Majority rule consensus tree of Melodinus based on the combined dataset of three genes (matK, rbcL and psbA-trnH) representing Melodinus and outgroups. ML bootstrap values and posterior probabilities (PP) of the BI analysis are presented above the branches*

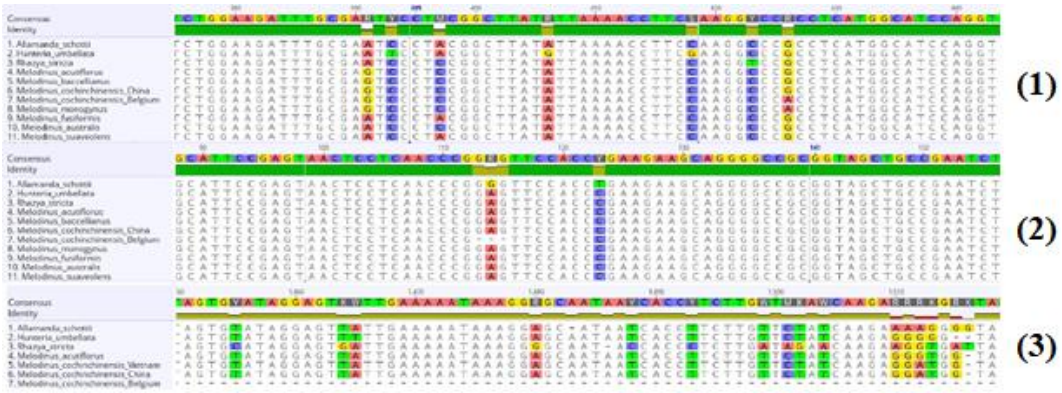

Figure 4. Some *differences in gene sequences of Melodinus cochinchinensis from Belgium and Asia. (1), (2): rbcL, (3): trnH-psbA*

The molecular results identified that *Melodinus* was strongly supported as monophyletic (BS = 95% and PP = 1.0; Figure 3), the two major clades within *Melodinus* were recognized (Figure 3). First clade includes three species *M. australis*, *M. suaveolens* and *M. fusiformis*. The second clade includes the two subclades, *M. monogynus and M. cochinchinensis* from Belgium formed a subclade, while *M. acutiflorus*, *M. baccellianus* and two individuals of *M. cochinchinensis* formed a subclade.

The *M. cochinchinensis* was addressed as non-monophyletic, three individuals of *M. cochinchinensis* are placed into two subclades (Figure 3). The molecular data strongly supported a close relationship of *M. cochinchinensis* from Belgium and *M. monogynus*, but distantly related to *M. cochinchinensis* from Vietnam and China. However, the native *M. cochinchinensis* is not distributed in Belgium, and the sample of *M. cochinchinensis* from Belgium was confirmed as a cultivated species. Moreover, there are some genetic variations in sequences of *M. cochinchinensis* in Belgium versus Asiatic *M. cochinchinensis* (Figure 4). This result suggests that there is likely a misidentification of *M. cochinchinensis* in Belgium. In addition, the *M. cochinchinensis* from Vietnam and China are closely related and highly consistent in genetics.

In addition, molecular data has been applied in taxonomic and plant systematic studies for a long time, and it plays an important role and significant contribution [20]. In the case of *M. cochinchinensis*, the molecular data was supported to determine the phylogenetic position of the species, and suggest the problem in identification of *M. cochinchinensis* in Belgium that likely has confusion due to similarity in morphological characters. The *Melodinus cochinchinensis* species contains several significant biochemical compounds such as anthraquinone, flavanone, triterpenes, indole alkaloids, and we can use most parts of the species like twigs and leaves as traditional medicine [2]. However, its morphological characters are quite similar to other *Melodinus* specie with climbing woody vine, glabrous, inflorescences cymes paniculate in terminal, flowers white. Thus, identifying this species is significant to provide the material for other study or resource exploitation.

The molecular data in this study supports Australian *Melodinus* as non monophyletic group. The positions of endemic Australian species *M. acutiflorus*, *M. australis*, *M. baccellianus* are unstable (Figure 3). *M. acutiflorus* and *M. baccellianus* are close to Asian *M. cochinchinensis*. On the other hand, *M. australis* is close to *M. suaveolens* and *M. fusiformis.* The results could be from the missing data of remaining *Melodinus* species. Thus it is significant to resolve the phylogenetic relationship of Australian *Melodinus* species in future studies.

4. Conclusions

The study supported the monophyly of *Melodinus* based on molecular data from chloroplast sequences. The *M. cochinchinensis* from Vietnam and China are closely related, while it is likely a misidentification of *M. cochinchinensis* from Belgium. The Australian *Melodinus* is not monophyletic. Further study is necessary to resolve the phylogenetic relationship of the Australian *Melodinus.*

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