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Evaluation of ISSR Primer Applicability for Interspecific Differentiation of *Marchantia* from West Java

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Abstract. *Liverworts of the genus Marchantia play important ecological roles in humid tropical ecosystems; however, molecular marker evaluation for tropical taxa remains limited. This study aimed to assess the amplification performance and cross-species applicability of selected Inter Simple Sequence Repeat (ISSR) primers for interspecific molecular differentiation of Marchantia species from West Java, Indonesia. Four species (M. emarginata, M. geminata, M. paleacea, and M. polymorpha) were analyzed using five ISSR primers. Genomic DNA was extracted using a modified CTAB protocol and amplified through single-primer ISSR-PCR assays. Amplification profiles were scored as the presence or absence of dominant bands. Three primers (F218, F231, and F209) generated clear and reproducible multi-locus banding patterns, producing a total of 21 scorable fragments, whereas BS537 and BS3 failed to amplify detectable products. Polymorphism Information Content (PIC), interpreted as an index of band discrimination capacity across species, ranged from 0.70 to 0.91, with F209 and F218 showing the highest informativeness. The results demonstrate that the selected ISSR primers enable reliable interspecific discrimination among the taxa examined and provide a practical framework for preliminary molecular screening of tropical bryophytes. These findings support the use of validated ISSR primers in future taxonomic and comparative genetic studies of non-model liverwort species.*

Keywords: *genetic markers, inter simple sequence repeat, Marchantia, molecular fingerprinting*

INTRODUCTION

Liverworts are a major group of non-vascular plants that inhabit a variety of moisture-rich environments, including mountain forests, wetlands, and tundra. They contribute significantly to ecological functions, including soil development, nutrient cycling, and water retention, and serve as sensitive bioindicators of global climate change (Poveda, 2020; Wang et al., 2023). Within this group, the genus *Marchantia*, belonging to the family *Marchantiaceae*, has considerable ecological, biological, and phylogenetic significance (Bellini et al., 2023; Bowman et al., 2022). Previous studies have shown that *M. polymorpha* and *M. geminata* have distinct secondary metabolite profiles that may reflect functional variation under different environmental conditions (Nowaczyński et al., 2025). In addition, morphological and phytochemical variations observed in

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liverwort populations from Cibodas, West Java, suggest underlying genetic differences among local bryophyte taxa at the interspecific level. The Cibodas area, located within the Mount Gede Pangrango National Park, represents a well-known montane biodiversity hotspot characterized by high humidity, stable microclimatic conditions, and rich bryophyte diversity. These environmental conditions promote species coexistence and potential niche differentiation, making the region particularly suitable for investigating interspecific variation within the genus *Marchantia*.

The four selected species (*M. emarginata*, *M. geminata*, *M. paleacea*, and *M. polymorpha*) are among the most frequently reported and ecologically relevant *Marchantia* taxa in this region. Despite their morphological similarity and overlapping ecological distribution, previous studies have suggested potential biochemical and functional differences among these species. Therefore, evaluating their interspecific differentiation using molecular markers is crucial for clarifying taxonomic boundaries, assessing marker applicability, and providing a preliminary framework for the molecular characterization of tropical *Marchantia* species.

Baseline genetic information on *Marchantia* species is important for conservation planning and evolutionary research. Phylogenetic reconstructions have revealed two distinct monophyletic lineages that separate bryophytes from tracheophytes (Bowman et al., 2022). However, earlier assessments of *Marchantia* variation primarily relied on morphological, phenological, and biochemical traits, which are often influenced by environmental conditions and developmental stages, thereby limiting their reliability for genetic characterization. Compared to morphology-based approaches, molecular markers offer more consistent information for examining genetic variation, with reduced influence from external environmental factors (Jamil et al., 2022; Sakhravi et al., 2023).

Inter Simple Sequence Repeat (ISSR) markers have been widely used in plant genetic studies because they can detect polymorphic loci across the genome without requiring prior sequence information (Hancı & Paşazade, 2025; Tran et al., 2025). Compared with Random Amplified Polymorphic DNA (RAPD) techniques, ISSR markers are more reproducible because they use longer primers and higher annealing temperatures (Amiteye, 2021; Ho et al., 2021). These characteristics make ISSR markers suitable for assessing genetic variation and marker performance in a broad range of plant taxa (Doğan et al., 2016; Polaiah et al., 2023). In addition, ISSR primers tend to yield more consistent amplification patterns than RAPD, resulting in clearer and more reliable banding profiles (Petolescu et al., 2024; Susilo et al., 2018; Susilo & Meitiyani, 2018). ISSR analysis uses primers that target complementary repetitive regions in the genome, enhancing specificity and consistency in banding profiles (Amiteye, 2021; Tahir et al., 2023). However, the success of ISSR-based analyses is strongly influenced by primer selection and genome compatibility, making preliminary evaluation of primer performance necessary for each target taxon (Dong et al., 2020).

Previous studies have successfully developed and applied ISSR primers in species such as *M. inflexa* (Brzyski et al., 2012) and *Brachypodium sylvaticum* (Mo et al., 2013). Nevertheless, systematic evaluation of ISSR primer performance across different *Marchantia* species remains limited, particularly for taxa originating from tropical regions such as Indonesia. Therefore, this study aims to evaluate the amplification performance and cross-species applicability of selected ISSR primers in four *Marchantia* species collected from a tropical montane forest in Indonesia. This work focuses on assessing primer compatibility, reproducibility, and band informativeness for ISSR-based molecular fingerprinting, rather than estimating within-species genetic diversity or population genetic structure.

MATERIALS AND METHODS

This study was exploratory and aimed at primer evaluation rather than population genetic inference.

Sample collection and identification

Four Marchantia species were included in this study, namely *M. emarginata*, *M. geminata*, *M. paleacea*, and *M. polymorpha*. Samples were collected from Mount Gede Pangrango National Park, West Java, Indonesia (6°44'08.7"S 106°59'52.3"E), a humid montane forest ecosystem known for its high bryophyte diversity and relatively stable microclimatic conditions. Although specific environmental parameters (e.g., humidity and temperature) were not quantitatively measured during sampling, the site is well documented in previous studies as a representative habitat for diverse liverwort communities. Sampling was designed to obtain representative material for molecular marker evaluation rather than for population-level genetic analysis. Fresh thalli were collected in the field and immediately desiccated using silica gel to preserve DNA integrity before laboratory analysis (Ongom et al., 2021). For each species, samples were treated as taxonomic representatives to assess primer performance, and no assumptions were made about intraspecific genetic variation. Morphological identification was performed using established taxonomic keys for liverworts, focusing on diagnostic characters including thallus morphology, branching patterns, air-chamber structure, and reproductive features (Lücking et al., 2023). The identification procedure followed standard bryophyte taxonomy guidelines as described by Gradstein et al. (2001) and Söderström et al. (2016), and was supported by recent molecular and morphological studies on *Marchantia* (Bowman et al., 2022). Species identity was subsequently verified by comparison with authenticated herbarium specimens at the Herbarium of Biology, Universitas Muhammadiyah Prof. Dr. Hamka. Although detailed morphometric data were not quantitatively recorded, the identification procedure followed standard taxonomic practices used in bryophyte studies.

DNA Extraction

Genomic DNA was extracted from dried *Marchantia* thalli using a modified cetyltrimethylammonium bromide (CTAB) protocol (Susilo et al., 2018). Before extraction, fresh thalli were desiccated with silica gel to minimize DNA degradation (Aboul-Maaty & Oraby, 2019). Approximately 100 mg of young, clean thallus tissue was finely chopped, placed into sterile 2.0 mL microcentrifuge tubes, and ground under liquid nitrogen to obtain a fine powder.

A 700 µL volume of CTAB extraction buffer was added to each tube, and the samples were incubated at 65°C for 20 min with gentle mixing to facilitate cell lysis. After incubation, an equal volume of chloroform–isoamyl alcohol (24:1, v/v) was added, and the mixture was gently inverted, then centrifuged at 15,000 × g for 10 min at 20°C. The aqueous phase was carefully transferred to a new microtube, and DNA was precipitated by adding absolute ethanol to a final volume of 1,000 µL, followed by centrifugation at 12,000 × g for 5 min. The resulting DNA pellet was washed twice with 70% ethanol, air-dried at room temperature, and resuspended in 200 µL TE buffer. The DNA solution was incubated at 65°C for 20 min to ensure complete dissolution (Tahir et al., 2023). Extracted genomic DNA was stored at −20°C until further analysis.

Primer Screening

Five ISSR primers previously reported in bryophyte and angiosperm studies were screened for cross-species applicability. The selected ISSR primer sequences were obtained from the National Center for Biotechnology Information (NCBI) database. Five primers (F218, F231, F209, BS537, and BS3) were selected for their documented effectiveness in prior molecular studies. Primer sequences, repeat motifs, annealing temperatures, and reference sources are summarized in Table 1.

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Table 1: ISSR primer sequences, repeat motifs, annealing temperatures (*Ta*), and expected amplification product sizes were used for molecular analysis of four *Marchantia* species.

Locus	GenBank accession no.	Primer sequences (5'–3')	Repeat motif	*Ta* (°C)	Product size (bp)	Source
F218	JQ812721	TCCAGACGGACCCGCAATTT	(CT)13	55	179	(Brzyski et al., 2012)
F231	JQ812718	CGGAGCGATTCAGGGCACACC	(CT)13	55	173	(Brzyski et al., 2012)
F209	JQ812714	CCCAGCAGCCCTCCAGAAGT	(CT)13	55	286	(Brzyski et al., 2012)
BS537	JQ307463	GGATTGATATTGGCATTGAGTT	(CAG)5	55	139-190	(Mo et al., 2013)
BS3	JQ307442	CCTCGGGTGATCGATTCTTA	(GGC)6	55	117	(Mo et al., 2013)

PCR Amplification

PCR amplification with ISSR markers was performed following established protocols used in plant genetic studies, including analyses of *Allium* (Hancı & Paşazade, 2025) and *Prunus persica* (Tran et al., 2025). PCR reactions were performed in a total volume of 15 µL containing 7.5 µL of 2× KAPA 2G Fast ReadyMix (Kapa Biosystems, USA), 1.0 µL of ISSR primer (10 µM), 4.0 µL of genomic DNA template, and 2.5 µL of nuclease-free water. The ReadyMix contains Taq DNA polymerase, dNTPs, $MgCl_2$, and reaction buffer. Amplifications were carried out in a thermal cycler with an initial denaturation at 95°C for 1 min, followed by 40 cycles of denaturation at 95°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 30 s. A final extension step was performed at 72°C for 5 min. The annealing temperature (55°C) was selected based on preliminary optimization tests to ensure consistent amplification across primers.

Amplification products were separated using 8% vertical polyacrylamide gel electrophoresis. A 100 bp DNA ladder (Thermo Scientific, USA) was used as a molecular size standard. Gels were stained with ethidium bromide (0.5 µg/mL) and visualized using a ChemiDoc Image Analyzer (Bio-Rad). Only clear and reproducible banding patterns were included in the analysis. Although several primers were originally designed from SSR-related sequence regions, each primer was used individually in single-primer PCR reactions to generate multi-locus dominant amplification profiles typical of ISSR-based assays. Only one primer was included in each PCR reaction, and no primer pairs were used. The resulting amplification patterns were therefore interpreted as dominant multi-locus ISSR profiles rather than locus-specific microsatellite amplification. Amplified fragments were treated as independent dominant loci and scored as present (1) or absent (0), following previously described ISSR-based analytical approaches (Doğan et al., 2016).

Data Analysis

ISSR amplification profiles were analyzed using GelAnalyzer and PowerMarker version 3.25 by scoring DNA bands as present (1) or absent (0) for each primer. Only distinct and clearly visible bands were included in the analysis to ensure data reliability and scoring consistency. Differences in band intensity were not considered, as ISSR analyses typically rely on the presence or absence of reproducible bands rather than quantitative signal variation (Doğan et al., 2016). The resulting binary matrix was used specifically to evaluate primer performance rather than to infer population-level genetic variation. The degree of polymorphism for each primer was determined by calculating the proportion of polymorphic bands relative to the total number of amplified bands (Brilhante et al., 2021; Sakhravi et al., 2023; Serrote et al., 2020). Polymorphic bands were defined as fragments present in at least one species and absent in at least one other species.

Marker informativeness was evaluated using the Polymorphism Information Content (PIC) index, followed by Serrote et al. (2020). In this study, PIC values were calculated across species treated as operational taxonomic units (OTUs) and therefore interpreted as indices of band-discrimination capacity rather than indicators of allele diversity within natural populations. PIC values were classified as highly informative when exceeding 0.50, moderately informative between 0.25 and 0.50, and low informative when below 0.25 (Serrote et al., 2020).

The effective number of bands (Ne) was calculated based on the dominant band frequency across OTUs to estimate the distribution of band presence among species (Serrote et al., 2020). Because only one representative individual per species was analyzed, Ne values should be interpreted as measures of band-frequency distributions rather than as true population-level allele richness. To provide an additional measure of marker diversity, Shannon's Information Index (I) was calculated to quantify band distribution complexity among species. This index complements PIC by accounting for the distribution of band presence and absence patterns. Overall primer efficiency was further evaluated using the Marker Index (MI), which integrates polymorphism and marker informativeness (Hancı & Paşazade, 2025; Sakhravi et al., 2023). The primer's discriminatory capacity was assessed using Resolving Power (Rp) as proposed by Buczkowska et al. (2016).

Pairwise genetic similarity among species was calculated using the Jaccard similarity coefficient based on shared presence of bands, excluding shared absences. A similarity matrix was constructed from these coefficients and used to generate a dendrogram using the Unweighted Pair Group Method with Arithmetic Mean (UPGMA). Cluster analysis was performed to visualize interspecific genetic relationships based on ISSR data. All analyses were conducted within the framework of ISSR primer evaluation and interspecific band discrimination, rather than estimating within-species genetic diversity or population genetic structure.

RESULTS AND DISCUSSION

This study evaluated the cross-species amplification performance and discriminatory capacity of selected ISSR primers in four *Marchantia* species collected from Mount Gede Pangrango National Park, West Java, Indonesia. The analysis focused on primer compatibility, amplification reliability, and band informativeness across operational taxonomic units (OTUs), rather than estimating population-level genetic diversity.

DNA Quality and ISSR Amplification Screening

Genomic DNA extracted using the CTAB method yielded measurable concentrations across all four species (Table 2). The A260/A280 ratios ranged from 1.53 to 1.64, indicating relatively low to moderate DNA purity compared to the ideal value of ~1.8 for pure DNA. Such deviations are commonly reported in plant tissues rich in secondary metabolites, particularly in bryophytes. Despite this, successful downstream PCR amplification suggests that the extracted DNA was of sufficient quality for molecular analysis. However, it should be noted that the absence of PCR inhibition was inferred from successful amplification rather than directly validated by inhibitor-specific assays.

The highest DNA yield was observed in *M. paleacea*, whereas *M. geminata* exhibited the lowest concentration. Variation in DNA yield among species may reflect differences in thallus tissue structure, secondary metabolite content, or polysaccharide levels, which are known to affect DNA extraction efficiency in bryophytes (Aboul-Maaty & Oraby, 2019; Aguado-Ramsay et al., 2025). In liverworts, high levels of phenolic and specialized metabolites are known to co-precipitate during DNA extraction and may influence spectrophotometric purity ratios. However, in the present study, phenolic content was not directly measured, and therefore, this explanation remains speculative and is supported primarily by previous reports on bryophyte DNA extraction.

It is also important to emphasize that this study included only one representative individual per species. Therefore, the results should be interpreted as preliminary and limited to interspecific marker evaluation rather than reflecting population-level genetic variation.

Table 2: Genomic DNA purity and concentration of four *Marchantia* species

Species	A260/A280 Ratio	DNA Concentration (ng/µL)
Marchantia emarginata (Me)	1.54	52.7
Marchantia geminata (Ge)	1.64	48.8
Marchantia paleacea (Pa)	1.62	172.3
Marchantia polymorpha (Po)	1.53	135.1

Note: DNA quantity and quality were measured using a NanoDrop spectrophotometer

The DNA concentration and purity values reported in Table 2 represent single measurements obtained for each species. Replicate measurements were not performed, as the analysis was intended to provide an initial assessment of DNA suitability for PCR amplification rather than a quantitative evaluation of extraction variability. This approach is consistent with the study's exploratory nature, which focuses on primer performance.

Molecular studies on bryophytes often face challenges related to the quality of DNA extraction due to the presence of complex secondary metabolites, including phenolic compounds and terpenoid derivatives. These compounds may co-precipitate with nucleic acids, affecting spectrophotometric purity ratios. Nevertheless, previous studies have shown that moderate A260/A280 values can still yield reliable PCR amplification when DNA integrity is preserved, and inhibitor levels remain below critical thresholds. In liverworts, cellular polysaccharides and phenolic constituents may interact with extraction buffers, thereby influencing DNA yield and purity (Aboul-Maaty & Oraby, 2019; Aguado-Ramsay et al., 2025). Therefore, the successful amplification observed in the present study indicates that the modified CTAB protocol was sufficiently effective in obtaining amplifiable genomic DNA from *Marchantia* tissues despite potential interference from secondary metabolites.

Among the five primers tested, three primers (F218, F231, and F209) generated clear, reproducible multi-locus banding profiles, whereas BS537 and BS3 failed to produce detectable amplification products under the PCR conditions used. The effective primers collectively produced 21 polymorphic bands across the four species. Representative amplification profiles are shown in Figure 1.

The failure of BS537 and BS3 suggests limited primer–genome compatibility. ISSR amplification success depends strongly on the presence and distribution of complementary microsatellite motifs within the genome (Dos Santos et al., 2011). Similar primer-dependent amplification variability has been reported across diverse plant taxa (Doğan et al., 2016; Petolescu et al., 2024), emphasizing that successful cross-species transferability is not universal and must be empirically validated.

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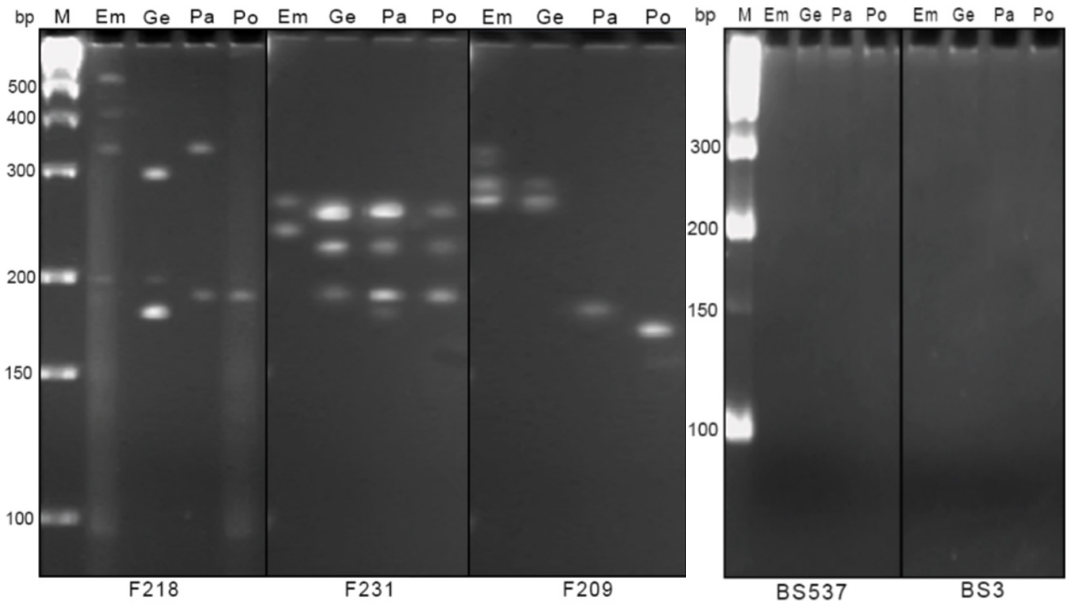

Figure 1: DNA amplification profiles generated using five ISSR primers across four *Marchantia* species: Me: *M. emarginata*; Ge: *M. geminata*; Pa: *M. paleacea*; Po: *M. polymorpha*

Differences in amplification success among ISSR primers are often associated with the abundance and distribution of microsatellite motifs within the genome. ISSR primers target regions located between simple sequence repeats, and amplification efficiency therefore depends on the presence of complementary repeat motifs and suitable distances between them. Genomes containing higher densities of compatible repeat motifs typically produce more stable and polymorphic ISSR profiles. In contrast, primers targeting rare or unevenly distributed motifs may fail to amplify. Such motif-dependent variability has been widely reported in ISSR studies across angiosperms and non-model plant taxa (Araújo et al., 2023; Peng et al., 2023). Consequently, empirical screening of multiple primers remains an essential step in molecular marker development, particularly for bryophytes, whose genomic architecture is relatively underexplored.

Marker Efficiency and Band Informativeness

Quantitative assessment of amplification performance (Table 3) was conducted using standard ISSR efficiency parameters, including number of amplified bands (Na), effective band number (Ne), polymorphism information content (PIC), percentage of polymorphism (%P), marker index (MI), and resolving power (Rp). The three effective primers collectively generated 21 polymorphic bands, with 100% polymorphism observed for each primer. All effective primers showed 100% polymorphism; the overall mean decreased when non-amplifying primers were included. PIC values ranged from 0.70 to 0.81, classifying all successful primers as highly informative according to Serrote et al. (2020). Primer F209 exhibited the highest PIC (0.81) and marker index (5.67), indicating superior discriminatory potential. F218 also demonstrated strong resolving power (Rp = 3.86), while F231 maintained high informativeness despite slightly lower values.

It is critical to emphasize that PIC and Ne values in this study represent interspecific band discrimination among four OTUs rather than population-level allele diversity. Because only one representative per species was analyzed, Ne reflects band frequency distribution across species, not allelic richness within populations. This interpretation aligns with methodological considerations for dominant multi-locus markers (Serrote et al., 2020).

Table 3: ISSR primer amplification performance and marker efficiency parameters across four *Marchantia* species

Locus	Size range (bp)	Na	Ne	PIC	Np	%P	MI	Rp
F218	172–512	7	2.10	0.79	7	100	5.53	3.86
F231	187–275	7	1.95	0.7	7	100	4.9	3.14
F209	170–320	7	2.15	0.81	7	100	5.67	4
BS537	–	0	0	0	0	0	0	0
BS3	–	0	0	0	0	0	0	0
Mean	–	4.2	1.24	0.77	4.2	60	3.22	2.2

Abbreviation: Na: number of bands; Ne: effective band number; PIC: polymorphism information content; Np: number of polymorphic bands; %P: percentage of polymorphism; MI: marker index; Rp: resolving power.

To further evaluate primer discriminatory capacity, band distribution was classified into species-specific and shared fragments. A total of 21 polymorphic bands were detected across three effective ISSR primers (Table 4). Nine bands were species-specific, while 12 bands were shared among at least two species. The presence of diagnostic fragments supports the discriminatory capability of selected ISSR primers for interspecific differentiation. Such diagnostic bands are particularly valuable in genera where morphological characters may overlap or exhibit environmental plasticity, as frequently observed in bryophytes.

The observed 100% polymorphism across effective primers is particularly notable (Dong et al., 2020). Comparable levels of polymorphism have been reported in ISSR-based analyses of other plant genera (Peng et al., 2023; Sakhravi et al., 2023), suggesting that ISSR markers are particularly suitable for detecting genomic divergence at the interspecific scale.

Table 4: Distribution of polymorphic and species-specific ISSR bands among four *Marchantia* species

Primer	Total Bands	Species-Specific Bands	Shared Bands
F218	7	3	4
F231	7	3	4
F209	7	3	4
BS537	0	0	0
BS3	0	0	0
Total (effective primers)	**21**	**9**	**12**

Note: Species-specific bands refer to fragments uniquely present in only one species, while shared bands occur in two or more species.

Species-specific ISSR fragments may function as diagnostic molecular markers useful for taxonomic verification and rapid species identification. These species-specific fragments could be further developed into diagnostic markers after validation across multiple individuals per species. Such fragments represent genomic regions that are unique to particular taxa and therefore provide reliable molecular signatures that complement morphological identification. In bryophytes, where morphological plasticity and environmental influences can complicate species delimitation, molecular markers offer an additional layer of taxonomic resolution. Similar diagnostic ISSR bands have been reported in other plant groups and have proven valuable for distinguishing closely related species or populations (Dong et al., 2020; Sakhravi et al., 2023). Although the present study was not designed to construct species-specific markers for routine identification, the detection of several unique fragments indicates that ISSR markers have potential applications in future molecular taxonomy studies of *Marchantia*.

Shared bands likely represent conserved genomic regions within the genus. Because ISSR primers target regions between microsatellite motifs, amplification success depends on motif abundance and distribution (Dos Santos et al., 2011; Mint Abdelaziz et al., 2020). Therefore, interspecific differences in band number and distribution are more likely to reflect genomic organization and primer-binding site availability than differences in overall genome size or complexity.

Genetic Similarity and Cluster Analysis

Genetic similarity analysis based on the binary matrix provided additional insight into interspecific relationships. Pairwise genetic similarity based on Jaccard coefficients was calculated from the binary matrix derived from 21 polymorphic bands (Table 5).

Table 5 shows that the Jaccard similarity coefficients ranged from 0.38 to 0.61, indicating moderate genetic similarity among species. The highest similarity was observed between *M. paleacea* and *M. polymorpha* (0.61), whereas the lowest similarity occurred between *M. emarginata* and *M. polymorpha* (0.38). These values demonstrate clear genetic differentiation among the four taxa and indicate that ISSR markers can capture interspecific genomic variation. Hierarchical clustering using the UPGMA algorithm produced a dendrogram consistent with the similarity matrix. The clustering reflects interspecific band-sharing patterns among OTUs and should not be interpreted as phylogeographic structure.

Table 5: Pairwise genetic similarity (Jaccard coefficient) among four *Marchantia* species based on 21 polymorphic ISSR markers

Species	*M. emarginata*	*M. geminata*	*M. paleacea*	*M. polymorpha*
M. emarginata	1.00	0.57	0.43	0.38
M. geminata	0.57	1.00	0.48	0.42
M. paleacea	0.43	0.48	1.00	0.61
M. polymorpha	0.38	0.42	0.61	1.00

The UPGMA dendrogram (Figure 2) revealed two major clusters. Pa and Po formed the closest cluster, consistent with the highest similarity value (0.61). The Me and Ge were grouped separately with moderate similarity (0.57). The concordance between similarity coefficients and the dendrogram topology strengthens the dataset's reliability and supports the discriminative effectiveness of primers F218, F231, and F209. The clustering pattern supports the discriminatory capability of ISSR markers for interspecific differentiation within the genus *Marchantia*. This clustering pattern mirrors the values in the similarity matrix and confirms the robustness of the ISSR dataset. Comparable clustering reliability has been reported in ISSR-based phylogenetic analyses of other plant genera (Peng et al., 2023; Petolescu et al., 2024).

The observed clustering pattern also provides a preliminary indication of consistency between ISSR-based genetic relationships and existing morphological classifications within the genus *Marchantia*. Previous taxonomic studies have shown that species such as *M. paleacea* and *M. polymorpha* share several morphological characteristics, including thallus structure and reproductive features, which may reflect closer evolutionary relationships (Bowman et al., 2022; Gradstein et al., 2001). The clustering of these two species in the present study therefore agrees with their morphological similarity.

However, it is important to note that the current analysis is based on a limited number of samples and dominant molecular markers, and thus does not represent a comprehensive phylogenetic reconstruction. While the results suggest that ISSR markers can capture patterns broadly consistent with classical taxonomy, further studies incorporating multiple individuals per species and additional molecular markers would be necessary to confirm these relationships in greater detail.

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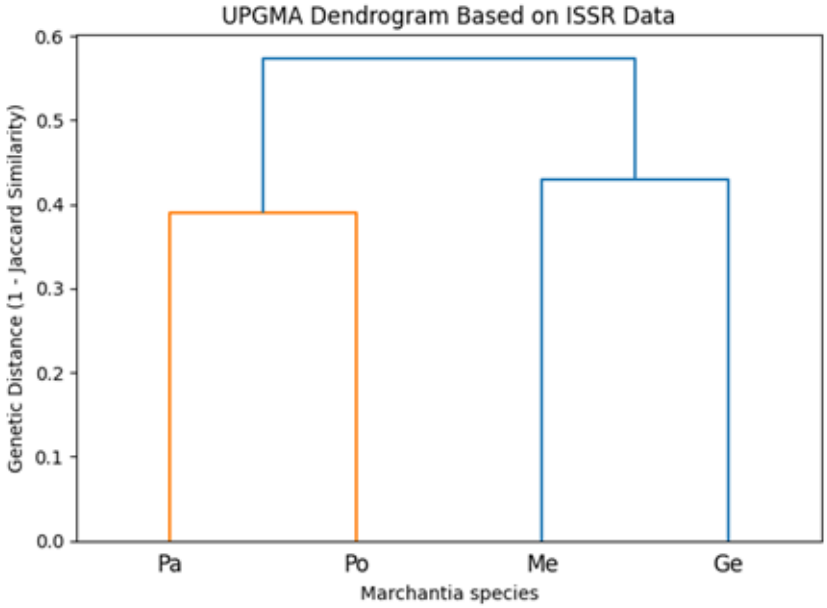

Figure 2: UPGMA dendrogram showing genetic relationships among four *Marchantia* species based on 21 polymorphic ISSR data. Genetic distance was calculated using the Jaccard coefficient, and the clusters were clustered using the unweighted pair group method with arithmetic mean (UPGMA).

Differences in amplification performance among primers underscore the importance of genome–primer compatibility (Araújo et al., 2023). ISSR markers amplify regions flanked by microsatellite repeats; therefore, amplification efficiency depends on motif distribution and genomic architecture (Dos Santos et al., 2011). In non-model taxa such as bryophytes, genome composition remains less characterized, making primer validation particularly critical (Wu et al., 2024). The failure of BS537 and BS3 in this study, despite prior success in other taxa (Mo et al., 2013), illustrates the taxon-specific nature of ISSR transferability.

The present findings also highlight the broader applicability of ISSR markers for preliminary genetic assessments in non-model plant groups. Bryophytes remain relatively underrepresented in molecular genetic research compared with vascular plants, partly due to limited genomic resources and methodological constraints. In such taxa, ISSR markers provide a practical and cost-effective approach for generating multi-locus genomic fingerprints without requiring prior sequence information. The high levels of polymorphism and consistent amplification patterns observed in the present dataset demonstrate that ISSR markers can effectively capture interspecific genomic variation within *Marchantia*. These results contribute to the growing body of molecular studies on bryophytes and provide a methodological foundation for future investigations exploring phylogenetic relationships, population structure, and evolutionary diversification within the genus.

Because this study was intentionally designed for primer evaluation rather than population-level inference, the findings should be interpreted within that scope. Nevertheless, the detection of species-specific bands, high PIC values, consistent polymorphism levels, and coherent clustering patterns collectively demonstrates that selected ISSR primers are suitable for interspecific molecular differentiation within *Marchantia*. Future studies incorporating multiple individuals per species and additional molecular markers would enable expansion from primer evaluation to population genetic and phylogeographic analyses.

CONCLUSION

This study evaluated the applicability of five ISSR primers for molecular differentiation of four *Marchantia* species collected from a tropical montane forest in West Java, Indonesia. Among the primers tested, F218, F231, and F209 generated clear and reproducible polymorphic amplification profiles, whereas primers BS537 and BS3 failed to produce detectable products under the PCR conditions used. High Polymorphism Information Content (PIC) values obtained for primers F209 (0.81) and F218 (0.79) indicate strong marker informativeness and reliable discriminatory power among the examined species. Primer F231 also showed consistent amplification and moderate informativeness, supporting its suitability for interspecific molecular analysis. The detection of complete polymorphism and several species-specific fragments demonstrates that selected ISSR primers are effective for distinguishing closely related *Marchantia* taxa. These findings confirm that validated ISSR primers can serve as practical molecular tools for preliminary genetic screening and interspecific differentiation in bryophytes. The primer set identified in this study provides a useful foundation for future investigations exploring genetic diversity, phylogenetic relationships, and molecular taxonomy within the genus *Marchantia* and related liverwort groups.

AUTHOR CONTRIBUTION

Conceptualization, **S.S.** and **M.S.**; methodology, **J.A.**; validation, **S.S.**, **M.S.**, and **J.A.**; formal analysis, **S.L.**; investigation, **S.L.**; resources, **J.A.**; data curation, **M.S.**; writing original draft preparation, **S.S.**; writing review and editing, **J.A.**; visualization, funding acquisition, **S.S.** All authors have read and agreed to the published version of the manuscript.

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CONFLICT OF INTEREST

The authors declare no conflict of interest

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