



Integrated morphological and molecular characterisation of wild *Anoectochilus roxburghii* germplasm reveals genetically valuable accessions for conservation

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Abstract *Anoectochilus roxburghii* (Wall.) Lindl. is a high-value medicinal orchid threatened by over-exploitation and habitat degradation. However, information on the morphological and genetic variation of wild germplasm resources in Vietnam remains limited. This study aimed to characterise 17 wild accessions collected from Bach Ma National Park using morphological and micro-anatomical traits, DNA barcodes (*ITS*, *rbcL* and *psbA-trnH*), and simple sequence repeat (SSR) markers. Morphological and micro-anatomical analyses revealed considerable variation among accessions, and several anatomical traits were significantly associated with plant growth characteristics. DNA barcode markers successfully authenticated all accessions as *A. roxburghii* but revealed limited intraspecific variation. In contrast, SSR markers detected moderate genetic diversity and identified four genetically divergent accessions (HUIB_AR13, HUIB_AR14, HUIB_AR15 and HUIB_AR16) of potential conservation value. The integration of morphological, DNA barcode and SSR data provides a comprehensive characterisation of wild *A. roxburghii* germplasm from Bach Ma National Park. These findings provide baseline

information for the characterisation and conservation of *A. roxburghii* germplasm from Bach Ma National Park and identify candidate accessions for future conservation studies. Broader geographic sampling will be required before species-wide patterns of genetic diversity and conservation priorities can be inferred.

Keywords *Anoectochilus roxburghii* · DNA barcoding · Genetic diversity · ITS · SSR markers · Species conservation

Introduction

Anoectochilus roxburghii (Wall.) Lindl. is a highly valuable species in many Asian countries due to its ornamental, medicinal, food and cosmetic purposes (Ye et al. 2019). It has been reported to possess anti-inflammatory, antioxidant, antitumor, antiviral, and antihyperlipidemic effects (Zou et al. 2025). However, it was listed as a threatened or near-threatened species in the Convention on International Trade in Endangered Species of Wild Fauna and Flora (Wang et al. 2022) and included in the Vietnam Red Book of endangered species (Tran et al. 2022). This is due to slow growth, low natural propagation rate, specific habitat requirements, animal consumption, indiscriminate collection, and increasing human demand (Hou et al. 2025; Zhang et al. 2013). Therefore, conservation of this species is urgently required.

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The conservation and sustainable utilisation of plant genetic resources depend on an understanding of germplasm diversity. Germplasm characterisation provides essential information for identifying unique genetic resources, establishing conservation collections, supporting restoration programmes and developing breeding materials (Kumar et al. 2025). Additionally, both phenotypic and molecular variation contribute valuable information for the management of threatened species, particularly when natural populations are fragmented and vulnerable to genetic erosion (Avisé 1989; Last et al. 2014).

Although *A. roxburghii* has been studied for its phytochemical composition, medicinal applications and propagation techniques (Ye et al. 2017; Zhang et al. 2015; Zou et al. 2025), information regarding variation within natural germplasm resources remains limited. Most previous studies have focused on species identification, pharmacological potential or cultivation, whereas little attention has been paid to the characterisation of wild accessions. This gap is particularly evident in Vietnam, where information on phenotypic diversity and genetic variation within natural populations is scarce.

Integrative approaches combining morphological and molecular markers are increasingly used in germplasm research. Morphological and anatomical traits provide information on phenotypic diversity (Barwant et al. 2024). DNA barcodes facilitate species authentication (Safhi et al. 2023). Besides, simple sequence repeat (SSR) markers offer high-resolution assessments of genetic diversity among sampled germplasms (Bhattarai 2021; Kalia et al. 2011). By combining these methods, studies can achieve higher taxonomic resolution, more precise species delineation, and a broader understanding of evolutionary relationships and biodiversity (Zaman et al. 2025).

Morphological studies of *Anoectochilus* primarily relied on characteristics of leaves, stems, flowers, and roots (Bhattacharjee 2013; Ye et al. 2017; Zhang et al. 2024). However, morphological traits may be influenced by environmental conditions. Among DNA barcode markers, the internal transcribed spacer (*ITS*) is a powerful phylogenetic marker due to its broad distribution, the greater evolutionary rate and high sequence variability resulting from its non-coding nature (Letsiou et al. 2024). Hence, many studies have used *ITS* for the identification of *Anoectochilus* species (Gao et al. 2009; Hu et al. 2019; Lv

et al. 2015; Thinh et al. 2020). In addition, *psbA-trnH* and *rbcL* (ribulose biphosphate carboxylase/oxygenase large subunit) regions have also been analysed to evaluate genetic differences between *Anoectochilus* accessions (Ho et al. 2021; Lv et al. 2015; Wang et al. 2025). *psbA-trnH* is one of the most variable non-coding plastid loci, exhibiting a large level of species discrimination and high rates of insertion/deletion (Letsiou et al. 2024). Meanwhile, the *rbcL* region has been demonstrated a greater distinguishing capacity than the *matK* (maturase K) region in *Anoectochilus* (Ho et al. 2021). In addition to DNA barcoding, simple sequence repeat (SSR) markers are potential tools for analysing genetic diversity and identifying genetic resources because of their high polymorphism, codominance, high repeatability, universality and abundant information (Ding et al. 2015; Su et al. 2025; Zhao et al. 2023). Previous studies by Lu (2022) and Zhang et al. (2024) successfully applied SSR markers in transcriptome analysis to assess genetic and morphological diversity in *Anoectochilus*.

Bach Ma National Park in central Vietnam harbours natural populations of *A. roxburghii* that represent valuable genetic resources for conservation and future utilisation. However, the extent of phenotypic and genetic variation within this germplasm has not been systematically investigated. Accordingly, this study was designed to comprehensively characterise wild *A. roxburghii* germplasm from Bach Ma National Park and to identify genetically valuable accessions within the Bach Ma National Park germplasm collection that could support local conservation and provide a basis for future comparative studies. Morphological, anatomical, DNA barcode and SSR markers were integrated to achieve these objectives.

Materials and methods

Plant materials

Seventeen *Anoectochilus* accessions were utilised in this study (Table 1). These accessions were collected from Bach Ma National Park, central Vietnam in 2024 (Ho et al. 2025b). They were then cultured under controlled laboratory conditions at a temperature of 25–26 °C, humidity of 70–80% and light intensity of 2000 lux. Because *A. roxburghii* is a threatened species with fragmented natural populations,

Table 1 List of *Anoectochilus* accessions used in this study

S. N	Accession	Code	Coordinates	Place of collection
1	HUIB_AR1	AR1	16°11'36.2"N; 107°50'56.5"E	Loc Tri, Phu Loc, Hue city
2	HUIB_AR2	AR2	16°13'45.4"N; 107°50'53.2"E	Loc Tri, Phu Loc, Hue city
3	HUIB_AR3	AR3	16°11'29.4"N; 107°44'26.1"E	Huong Phu, Huong Thuy, Hue city
4	HUIB_AR4	AR4	16°11'26.7"N; 107°45'04.1"E	Huong Phu, Huong Thuy, Hue city
5	HUIB_AR5	AR5	16°11'09.4"N; 107°45'21.5"E	Huong Phu, Huong Thuy, Hue city
6	HUIB_AR6	AR6	16°11'08.1"N; 107°45'26.4"E	Huong Phu, Huong Thuy, Hue city
7	HUIB_AR7	AR7	16°04'58.1"N; 107°45'02.4"E	Thuong Lo, Dong Giang, Hue city
8	HUIB_AR8	AR8	16°04'45.9"N; 107°45'23.2"E	Thuong Lo, Dong Giang, Hue city
9	HUIB_AR9	AR9	16°08'12.4"N; 107°50'49.0"E	Thuong Lo, Dong Giang, Hue city
10	HUIB_AR10	AR10	16°07'55.4"N; 107°51'09.0"E	Thuong Lo, Dong Giang, Hue city
11	HUIB_AR11	AR11	16°07'54.2"N; 107°51'12.3"E	Thuong Lo, Dong Giang, Hue city
12	HUIB_AR12	AR12	16°07'53.1"N; 107°51'26.4"E	Thuong Lo, Dong Giang, Hue city
13	HUIB_AR13	AR13	16°11'34.5"N; 107°50'44.2"E	Loc Dien, Phu Loc, Hue city
14	HUIB_AR14	AR14	16°07'27.9"N; 107°47'16.4"E	Thuong Lo, Dong Giang, Hue city
15	HUIB_AR15	AR15	16°13'04.1"N; 107°43'53.6"E	Huong Phu, Huong Thuy, Hue city
16	HUIB_AR16	AR16	16°13'03.9"N; 107°44'50.9"E	Huong Phu, Huong Thuy, Hue city
17	HUIB_AR17	AR17	16°12'06.5"N; 107°44'50.6"E	Huong Phu, Huong Thuy, Hue city

destructive sampling was minimised and available accessions were limited.

Morphological and micro-anatomical identification

Morphological identification

Quantitative morphological traits included stem length (SL), number of stem nodes (NSN), stem node length (NL), number of leaves (NOL), leaf length (LL), leaf width (LW), number of main veins (NV), petiole diameter (PD) and petiole length (PL). Qualitative morphological traits included plant habit (PH), plant growth (PG), kinds of stem (KS), stem color (SC), texture of stem (TS), vein color on the leaf's upper surface (VUP), vein color on the leaf's abaxial surface (VUD), mesophyll color on the leaf's upper surface (MUP), mesophyll color on the leaf's abaxial surface (MUD), leaf shape (LSH), leaf surface (LSF), leaf margin (LM), petiole color (PC), leaf sheath color (LSC), root color (RC) and root habit (RH). These traits were scored at the full foliage stage at one month after transplanting.

Micro-anatomical identification

Accessions were prepared for microscopic imaging based on the method of Truong et al. (2025). Sixteen

micro-anatomical traits were studied including number of epidermal cell layers of stem (NEPS), number of collenchyma cell layers of stem (NCS), number of parenchyma cell layers of stem (NPS), number of endodermal cell layers of stem (NEDS), number of xylem bundles of stem (NXS), number of phloem bundles of stem (NPHS), Number of pith cell layers of stem (NPIS), number of epidermal cell layers of petiole (NEPP), number of collenchyma cell layers of petiole (NCP), number of parenchyma cell layers of petiole (NPP), number of xylem bundles of petiole (NXP), number of phloem bundles of petiole (NPHP), number of upper epidermal cell layers of leaf (NUEL), number of lower epidermal cell layers of leaf (NLEL), number of palisade mesophyll cell layers (NPM) and number of spongy mesophyll cell layers (NSM).

One representative plant was used per accession due to limited germplasm availability of *Anoectochilus roxburghii*. Three measurements from three leaves or anatomical sections of the same plant were treated as subsamples rather than independent replicates, and their mean was used as a single observation for statistical analysis.

The quantitative data were analysed using IBM SPSS Statistics (version 27). Mean values from accessions were compared and separated using ANOVA and Tukey's HSD test, respectively ($\alpha = 0.05$). In

addition, PAST v4.03 software was used in this analysis to cluster 17 accessions by the UPGMA algorithm and the Euclidean similarity index (Hammer et al. 2001). All quantitative traits were compiled for correlation analysis among them.

DNA extraction, PCR and DNA barcoding

Genomic DNA was extracted from adult leaves of each accession by the CTAB method (cetyl trimethyl ammonium bromide) and purified (Ho et al. 2025a). Next, they were amplified with *ITS*, *psbA-trnH* and *rbcL* primers in a PCR system (Applied Biosystems, USA) (Table 2). Each 20 µL PCR contained 50 ng DNA, 10 µL of 2X MyTaq Mix (Meridian Bioscience, US) and 0.5 µM of each primer. The thermocycling program was described in Table 2. PCR products were then sequenced in 1st BASE (Apical Scientific Sdn. Bhd., Malaysia) and analysed using BLASTn available on the NCBI. Used parameters in BLASTn included > 97% identity, 0.0 e-value and

> 85% coverage. Next, a phylogenetic tree was constructed in MEGA 12 software (Kumar et al. 2024) based on the Neighbor-Joining method with a Kimura 2-parameter model and 1000 bootstrap replicates. The sequencing data in this work were aligned with some reference sequences of *Anoectochilus* species that downloaded from BOLD (<https://www.boldsystems.org/>). Besides, DnaSP v6 software was applied to evaluate parameters of sequence, including the number of variable (polymorphic) sites, the number of mutations, nucleotide diversity, average number of nucleotide differences, number of Haplotypes and Haplotype diversity (Rozas et al. 2017).

SSR markers

The SSR analysis was carried out in 10 µL PCR containing 30 ng of DNA, 5 µL of 2X MyTaq buffer and 0.5 µM SSR primers (Table 2). The PCR products were then stained with SYBR Green, separated on 3% agarose gel (0.5X TBE buffer) and observed

Table 2 List of DNA barcode primers and simple sequence repeat (SSR) primers

Primer name		Forward sequence (5'–3')	Reverse sequence (5'–3')	PCR conditions	References
DNA barcode primers	<i>ITS</i>	AGGAGAAGTCGT AACAAGGTTTCC	GATATGCTTAAA CTCAGCGGGTC	94 °C (4 min); 30 cycles- 94 °C (30 s), 55 °C (40 s), 72 °C (40 s); 72 °C (5 min)	Thinh et al. (2020)
	<i>psbA-trnH</i>	GTTATGCATGAACGT AATGCTC	CGCGCATGGTGG ATTCACAATCC	95 °C (2 min); 35 cycles- 95 °C (30 s), 53.8 °C (30 s), 72 °C (1 min); 72 °C (5 min)	Lv et al. (2015)
	<i>rbcL</i>	TGAAAACGTGAA TTCCCAACCGTT TATGCG	GCAGCAGCTAGT TCCGGGCTCCA	95 °C (2 min); 35 cycles- 95 °C (30 s), 55 °C (30 s), 72 °C (1 min); 72 °C (5 min)	Ho et al. (2021)
SSR primers	3-4FR	CCGGATCCAGCA AACACTCT	GCCGCCGTCTAT TGGTAGAA	95 °C (3 min); 35 cycles- 95 °C (40 s), 60 °C (30 s), 72 °C (1 min); 72 °C (8 min)	Zhang et al. (2024)
	2-5FR	AGGAGGCGTTCG TGAAGAAG	GTATCATCAGAG CCGCCCAA	95 °C (3 min); 35 cycles- 95 °C (40 s), 59 °C (30 s), 72 °C (1 min); 72 °C (8 min)	
	3-2FR	GCAGCTTTTGTC GCAGCTAT	TGGAAGCTTGGG TAACAACAGA		
	3-9FR	AGAGGATGGTGC CAATGTTGT	GAGCATCGTTTTCAT CGCCC		
	4-1FR	GAGAGAGCTTCC GACGATGG	TTCCCAACAGG AATCCTCG		
	4-3FR	CTCTCCACCTCTCTC CCTCC	TCTTTCACAGCCATC GCAGT		

under UV light. SSR alleles were scored using an allelic scoring approach, in which bands of different fragment sizes were treated as distinct alleles, whereas bands of the same size were considered the same allele. The resulting allelic data were analysed using NTSYSpc 2.1 to determine the genetic distance among accessions based on simple matching coefficients (SM) of similarity and to construct the UPGMA dendrogram (Rohlf 2000). The number of alleles was used to calculate successful amplification rate, band size, observed number of alleles (na), effective number of alleles (ne), Shannon's Information index (I), polymorphism information content (PIC), expected heterozygosity (He) and observed heterozygosity (Ho) using POPGENE 1.32 and Cervus 3.0.7 (Kalinowski et al. 2007; Yeh et al. 1999). The genetic structure of the analysed germplasm were assessed by STRUCTURE v.2.3.4 (Pritchard et al. 2000). Ten hypothetical numbers of genetic clusters ($K = 1-10$) were evaluated. For each K value, 10 independent runs were performed using different random seeds. Each run consisted of a burn-in period of 100,000 iterations followed by 100,000 MCMC iterations. A web version of StructureSelector (<http://lmme.qdio.ac.cn/StructureSelector/>, accessed on 10 July 2026 (Li et al. 2018)), which implements the method developed by Evanno et al. (2005) to calculate K values for the analysed data. Additionally, a Mantel test for correlation of genetic and morphological distance was also conducted based on R v4.5.2 and RStudio v2025.09.2+418.

Results

Morphological and anatomical variation among wild *A. roxburghii* accessions

In terms of morphological characteristics, all *Anoectochilus* accessions grew erect; the stem structure was hard; the leaf veins appeared glittering on the upper surface and light green on the abaxial surface; the mesophyll on the leaf upper surface was dark green; the leaves were orbicular, smooth, and had sinusoidal margins; the petioles were green; the roots were creeping and brownish-green. The growth among accessions was heterogeneous, with most samples growing moderately to well. Only HUIB_AR6, HUIB_AR8, and HUIB_AR13 showed symptoms

consistent with fungal infection (Fig. 1). The purplish-green color of HUIB_AR10 stem was distinctly different from the green stems of the other samples. Meanwhile, the purplish-green color of the lower leaf mesophyll only appeared in HUIB_AR2 and HUIB_AR15. The green color of the leaf sheath was only present in HUIB_AR3 and HUIB_AR9 (Table 3, Fig. 1).

Although quantitative morphological traits were recorded, the limited sample size constrained robust statistical inference, reflecting the rarity of this species. Overall, there was considerable variation in these traits. The average stem length was 8.22 cm, with sample HUIB_AR7 having the greatest length (15.5 cm). The number of stem nodes and leaves showed substantial variation among accessions, ranging from 2 to 7. HUIB_AR2 and HUIB_AR7 had the longest node lengths, at 2.02 and 2.03 cm, respectively. The largest leaf size was observed in HUIB_AR4 and HUIB_AR14. On average, there were 5.31 main veins per leaf, with the highest number of veins (7 veins) observed in HUIB_AR5. Additionally, most accessions had petiole diameters exceeding 0.19 cm and petiole lengths exceeding 0.83 cm. Notably, HUIB_AR14 and HUIB_AR15 had the largest number of xylem and phloem bundles (22.33 and 20.33 bundles/stem, respectively). These anatomical characteristics may be associated with greater vascular development, although their functional significance requires experimental verification. HUIB_AR11 had a thicker petiole than the other samples with the most parenchyma layers (11.67 layers). Furthermore, the leaf cross-section of sample HUIB_AR16 was different from the others, showing 10 cell layers, including one upper epidermis, one lower epidermis, three palisade cell layers, and five spongy mesophyll cell layers (Table 4, Fig. 2). These observations indicate that several accessions possess distinct anatomical features, suggesting the existence of phenotypic diversity that may be useful for germplasm discrimination.

The hierarchical clustering analysis grouped the 17 accessions into three major clusters. Cluster 1 comprised four accessions showing relatively high similarity, while cluster 3 included five closely related accessions with low internal distances. Cluster 2 was the largest and exhibited moderate internal variation. Notably, cluster 3 was clearly separated from the other clusters at higher distance levels (> 12.0) (Fig. 3). These results suggested the presence of



Fig. 1 Morphology of 17 *Anoectochilus* accessions in this study

morphological structuring among accessions. Based on the results of correlation analysis between quantitative traits, changes in stem length and stem node size were significantly correlated with changes in leaf and petiole size. These changes were associated with an increase in the number of parenchyma cell layers, pith cell layers, and xylem and phloem vascular bundles in plant parts. Meanwhile, the number of veins per leaf appeared to be inversely proportional to petiole length, the number of xylem bundles and phloem bundles, and the number of pith cell layers in the stem (Fig. 4).

DNA barcode characterisation of wild germplasm accessions

Seventeen *Anoectochilus* accessions were successfully sequenced and published on NCBI. The *ITS*, *rbcL* and *psbA-trnH* sequences were 740 bp, 731 bp long and 857 bp, respectively. Referencing these

sequences in the Genbank database showed high genetic similarity (98.92–100%) to *A. roxburghii* and *A. setaceus* (synonymous with *A. roxburghii* (Wall.) Lindl.) (Table 5). Additionally, among the three barcode loci, *ITS* showed limited sequence variation. It was represented by a single SNP that separated the accessions into two sequence haplotypes. Meanwhile, *rbcL* was completely conserved. Variation detected in the *psbA-trnH* region was restricted to repeat-length differences and did not increase nucleotide diversity estimates. Accordingly, DNA barcode markers revealed extremely low intraspecific variation within the sampled germplasm (Table 6, Fig. 5).

Among the three barcode regions examined, *ITS* exhibited the highest level of sequence variation, although overall nucleotide diversity remained low. Two *ITS* haplotypes were detected among the 17 accessions, whereas no informative variation was observed in *rbcL* and only repeat-length variation was found in *psbA-trnH*. The two combinations (*ITS*

Table 3 Qualitative morphological traits observed in *Anoectochilus* accessions

Accessions	PH	PG	KS	SC	TS	VUP	VUD	MUP	MUD	LSH	LSF	LM	PC	LSC	RC	RH
AR1	1	3	1	1	1	1	1	1	1	1	1	1	1	2	1	1
AR2	1	3	1	1	1	1	1	1	2	1	1	1	1	2	1	1
AR3	1	3	1	1	1	1	1	1	1	1	1	1	1	1	1	1
AR4	1	3	1	1	1	1	1	1	1	1	1	1	1	2	1	1
AR5	1	2	1	1	1	1	1	1	1	1	1	1	1	2	1	1
AR6	1	1	1	1	1	1	1	1	1	1	1	1	1	2	1	1
AR7	1	3	1	1	1	1	1	1	1	1	1	1	1	2	1	1
AR8	1	1	1	1	1	1	1	1	1	1	1	1	1	2	1	1
AR9	1	2	1	1	1	1	1	1	1	1	1	1	1	1	1	1
AR10	1	3	1	2	1	1	1	1	1	1	1	1	1	2	1	1
AR11	1	3	1	1	1	1	1	1	1	1	1	1	1	2	1	1
AR12	1	3	1	1	1	1	1	1	1	1	1	1	1	2	1	1
AR13	1	1	1	1	1	1	1	1	1	1	1	1	1	2	1	1
AR14	1	3	1	1	1	1	1	1	1	1	1	1	1	2	1	1
AR15	1	3	1	1	1	1	1	1	2	1	1	1	1	2	1	1
AR16	1	3	1	1	1	1	1	1	1	1	1	1	1	2	1	1
AR17	1	3	1	1	1	1	1	1	1	1	1	1	1	2	1	1

PH; Plant habit: Erect (1), PG; Plant growth: Weak (1), Medium (2), Vigorous (3). KS; Kinds of stem: Rhizomatous (1). SC; Stem color: Green (1), Purplish green (2). TS; Texture of stem: Hard (1). VUP; Vein color on the leaf's upper surface: Glitter (1). VUD; Vein color on the leaf's abaxial surface: Light green (1). MUP; Mesophyll color on the leaf's upper surface: Dark green (1). MUD; Mesophyll color on the leaf's abaxial surface: Purple (1), Greenish purple (2). LSH; Leaf shape: Orbicular (1). LSF; Leaf surface: Glabrous (1). LM; Leaf margin: Sinuate (1). PC; Petiole color: Green (1). LSC; Leaf sheath color: Green (1), Purplish green (2). RC; Root color: Greenish brown (1). RH; Root habit: Creeping (1)

+ *psbA-trnH* and *ITS + rbcL + psbA-trnH*) had constant S, Eta, k, h and Hd values, while π tended to decrease from 0.72×10^{-3} to 0.33×10^{-3} and 0.23×10^{-3} compared to results of *ITS* (Table 6).

SSR markers reveal genetic diversity within the germplasm collection

Most SSR primers successfully amplified all accessions, except for the 3-2FR primer, with an amplification rate of 82.35%. The generated product bands ranged in size from 170 to 450 bp, and there were differences in amplified band size between accessions (Fig. 6). The mean observed number of alleles (na) and mean effective number of alleles (ne) were 4.500 and 2.689, respectively. Four primers (2-5FR, 3-2FR, 3-9FR, and 4-1FR) reported the highest I and PIC values. Additionally, expected heterozygosity (H_e) ranged from 0.373 to 0.779, with loci having expected heterozygosity of 0.5 or higher only found in 2-5FR, 3-2FR, 3-9FR, and 4-1FR. This pattern differed from the observed heterozygosity (H_o),

particularly in primers 2-5FR, 3-4FR, and 4-1FR (Table 7). This suggests that four loci (2-5FR, 3-2FR, 3-9FR and 4-1FR) may be useful for future germplasm characterisation and management of *A. roxburghii* genetic resources. Furthermore, the UPGMA tree generated from the SSR data divided the *Anoectochilus* accessions into four clusters at a coefficient of 0.7. The similarity coefficients of the accessions ranged from 0.39 to 0.93. Cluster 1 included HUIB_AR1, HUIB_AR2 and HUIB_AR7. Clusters 2 and 3 contained 8 and 5 accessions, respectively. Accessions, such as HUIB_AR13, HUIB_AR14 and HUIB_AR16 in cluster 2 and HUIB_AR17 in cluster 3, were separated from the others in the same cluster. Meanwhile, HUIB_AR15 was the sole member of cluster 4 (Fig. 7). SSR analysis revealed moderate genetic diversity (mean PIC = 0.535; mean H_e = 0.595) among germplasms, indicating that substantial allelic variation is maintained despite the restricted geographic origin of the accessions. Mean observed heterozygosity (H_o = 0.311) is substantially lower than mean expected heterozygosity (H_e = 0.595), and

Table 4 Quantitative micro-morphological features observed in *Anoetochilus* accessions

Accessions	SL	NSN	NL	NOL	LL	LW	NV	PD	PL	NEPS	NCS	NPS	NEDS
AR1	11	6	1.60 ± 0.49ab	4	3.50 ± 0.43a-c	2.55 ± 0.32ab	6.50 ± 0.84a	0.28 ± 0.04a	0.48 ± 0.02e	1.00 ± 0.00a	1.00 ± 0.00b	13.67 ± 0.58 _{ab}	2.00 ± 0.00a
AR2	12	6	2.02 ± 0.74a	4	3.65 ± 0.11ab	2.48 ± 0.04ab	5.00 ± 0.00b	0.20 ± 0.00b-e	1.18 ± 0.04a	1.00 ± 0.00a	1.00 ± 0.00b	14.00 ± 0.00a	2.00 ± 0.00a
AR3	8.5	6	1.25 ± 0.52ab	4	3.23 ± 0.30a-d	2.48 ± 0.19ab	5.33 ± 0.52b	0.20 ± 0.00b-e	0.68 ± 0.07c-e	1.00 ± 0.00a	1.00 ± 0.00b	11.33 ± 0.58de	2.00 ± 0.00a
AR4	10	5	1.50 ± 0.65ab	3	4.43 ± 0.45a	2.88 ± 0.12ab	5.33 ± 0.52b	0.19 ± 0.02c-e	1.00 ± 0.05a-d	1.00 ± 0.00a	1.00 ± 0.00b	11.67 ± 0.58c-e	2.00 ± 0.00a
AR5	3.3	2	1.50 ± 0.14ab	2	3.25 ± 0.19a-d	2.70 ± 0.14ab	7.00 ± 0.00a	0.20 ± 0.03b-e	0.60 ± 0.04de	1.00 ± 0.00a	1.00 ± 0.00b	11.00 ± 0.00de	2.00 ± 0.00a
AR6	3	3	0.90 ± 0.25b	3	2.00 ± 0.41de	1.53 ± 0.31cd	5.00 ± 0.00b	0.10 ± 0.00g	0.60 ± 0.04de	1.00 ± 0.00a	1.00 ± 0.00b	12.33 ± 0.58b-d	2.00 ± 0.00a
AR7	15.5	7	2.03 ± 0.62a	7	3.33 ± 0.47a-d	2.57 ± 0.42ab	5.50 ± 0.55b	0.24 ± 0.02a-c	0.97 ± 0.10a-d	1.00 ± 0.00a	1.00 ± 0.00b	14.33 ± 0.58a	2.00 ± 0.00a
AR8	2.5	2	1.75 ± 0.51ab	2	3.55 ± 0.28a-c	3.00 ± 0.19a	5.00 ± 0.00b	0.25 ± 0.04ab	0.65 ± 0.03c-e	1.00 ± 0.00a	1.00 ± 0.00b	11.33 ± 0.58de	2.00 ± 0.00a
AR9	4.7	4	1.05 ± 0.36ab	4	2.25 ± 0.20c-e	1.55 ± 0.18cd	5.00 ± 0.00b	0.10 ± 0.00g	0.65 ± 0.06c-e	1.00 ± 0.00a	1.00 ± 0.00b	11.00 ± 0.00de	2.00 ± 0.00a
AR10	7.5	5	1.43 ± 0.36ab	5	3.20 ± 0.33a-e	2.37 ± 0.24a-c	5.00 ± 0.00b	0.13 ± 0.05fg	0.72 ± 0.16b-e	1.00 ± 0.00a	1.00 ± 0.00b	12.33 ± 0.58b-d	2.00 ± 0.00a
AR11	8	6	1.42 ± 0.52ab	4	2.95 ± 0.84b-e	2.03 ± 0.53b-d	5.33 ± 0.52b	0.17 ± 0.03ef	0.88 ± 0.34a-e	1.00 ± 0.00a	1.00 ± 0.00b	12.33 ± 0.58b-d	2.00 ± 0.00a
AR12	11	6	1.70 ± 0.54ab	4	3.53 ± 0.28a-c	2.77 ± 0.16ab	5.00 ± 0.00b	0.20 ± 0.00b-e	1.03 ± 0.05a-c	1.00 ± 0.00a	1.00 ± 0.00b	14.00 ± 0.00a	2.00 ± 0.00a
AR13	3.6	3	1.23 ± 0.19ab	3	1.83 ± 0.98e	1.20 ± 0.79d	5.00 ± 0.00b	0.17 ± 0.05ef	0.80 ± 0.25a-e	1.00 ± 0.00a	1.00 ± 0.00b	11.67 ± 0.58c-e	2.00 ± 0.00a
AR14	9.5	6	1.43 ± 0.47ab	5	4.33 ± 0.52a	3.20 ± 0.22a	5.00 ± 0.00b	0.25 ± 0.00ab	1.03 ± 0.15a-c	1.00 ± 0.00a	1.00 ± 0.00b	10.67 ± 0.58e	2.00 ± 0.00a
AR15	10.5	6	1.33 ± 0.43ab	3	3.53 ± 1.71a-c	2.57 ± 0.91ab	5.33 ± 0.52b	0.23 ± 0.03a-d	1.10 ± 0.42ab	1.00 ± 0.00a	1.67 ± 0.58a	10.33 ± 0.58e	2.00 ± 0.00a
AR16	8.7	5	1.53 ± 0.39ab	4	3.50 ± 0.45a-c	2.55 ± 0.39ab	5.00 ± 0.00b	0.18 ± 0.03d-f	0.88 ± 0.20a-e	1.00 ± 0.00a	1.00 ± 0.00b	13.00 ± 1.00a-c	2.00 ± 0.00a
AR17	10.5	5	1.88 ± 0.78ab	3	3.20 ± 1.14a-e	2.07 ± 0.74b-d	5.00 ± 0.00b	0.18 ± 0.03d-f	0.83 ± 0.26a-e	1.00 ± 0.00a	1.00 ± 0.00b	11.67 ± 0.58c-e	2.00 ± 0.00a
Mean	8.22	4.88	1.50 ± 0.55	3.76	3.25 ± 0.91	2.38 ± 0.66	5.31 ± 0.65	0.19 ± 0.06	0.83 ± 0.27	1.00 ± 0.00	1.04 ± 0.20	12.16 ± 1.30	2.00 ± 0.00
Accessions	NXS	NPIS	NEPP	NCP	NPP	NXP	NPHP	NUEL	NLEL	NPM	NSM		
AR1	11.67 ± 0.58ef	12.00 ± 0.00gh	6.33 ± 0.58cd	1.00 ± 0.00a	9.33 ± 0.58b-d	5.00 ± 0.00a	5.00 ± 0.00a	1.00 ± 0.00a	1.00 ± 0.00a	2.67 ± 0.58a	3.33 ± 0.58c		
AR2	15.33 ± 1.16cd	15.67 ± 0.58de	8.67 ± 0.58a	1.00 ± 0.00a	6.67 ± 0.58 fg	5.00 ± 0.00a	5.00 ± 0.00a	1.00 ± 0.00a	1.00 ± 0.00a	2.33 ± 0.58a	3.00 ± 0.00c		

Table 4 (continued)

Accessions	NXS	NPHS	NPIS	NEPP	NCP	NPP	NXP	NPHP	NUEL	NLEL	NPM	NSM
AR3	13.67 ± 0.58de	14.33 ± 0.58ef	6.00 ± 0.00d	1.00 ± 0.00a	1.00 ± 0.00a	10.00 ± 0.00b	5.00 ± 0.00a	5.00 ± 0.00a	1.00 ± 0.00a	1.00 ± 0.00a	2.00 ± 0.00a	3.67 ± 0.58bc
AR4	17.33 ± 0.58bc	16.67 ± 0.58cd	6.33 ± 0.58cd	1.00 ± 0.00a	1.00 ± 0.00a	9.67 ± 0.58bc	5.00 ± 0.00a	5.00 ± 0.00a	1.00 ± 0.00a	1.00 ± 0.00a	2.00 ± 0.00a	4.00 ± 0.00a-c
AR5	11.00 ± 0.00f	10.67 ± 0.58h	6.00 ± 0.00d	1.00 ± 0.00a	1.00 ± 0.00a	8.33 ± 0.58c-e	5.00 ± 0.00a	5.00 ± 0.00a	1.00 ± 0.00a	1.00 ± 0.00a	2.00 ± 0.00a	3.67 ± 0.58bc
AR6	10.33 ± 0.58f	11.33 ± 0.58h	6.00 ± 0.00d	1.00 ± 0.00a	1.00 ± 0.00a	8.33 ± 0.58c-e	5.00 ± 0.00a	5.00 ± 0.00a	1.00 ± 0.00a	1.00 ± 0.00a	2.00 ± 0.00a	3.67 ± 0.58bc
AR7	18.00 ± 1.00b	18.00 ± 1.00c	7.33 ± 0.58bc	1.00 ± 0.00a	1.00 ± 0.00a	8.00 ± 0.00d-f	5.00 ± 0.00a	5.00 ± 0.00a	1.00 ± 0.00a	1.00 ± 0.00a	2.33 ± 0.58a	4.00 ± 0.00a-c
AR8	11.33 ± 1.16f	11.67 ± 0.58h	6.67 ± 0.58cd	1.00 ± 0.00a	1.00 ± 0.00a	7.33 ± 0.58ef	5.00 ± 0.00a	5.00 ± 0.00a	1.00 ± 0.00a	1.00 ± 0.00a	2.00 ± 0.00b	3.00 ± 0.00c
AR9	10.67 ± 0.58f	11.00 ± 0.00h	6.00 ± 0.00d	1.00 ± 0.00a	1.00 ± 0.00a	8.33 ± 0.58c-e	5.00 ± 0.00a	5.00 ± 0.00a	1.00 ± 0.00a	1.00 ± 0.00a	2.00 ± 0.00a	3.33 ± 0.58c
AR10	16.67 ± 0.58bc	16.67 ± 0.58cd	6.00 ± 0.00d	1.00 ± 0.00a	1.00 ± 0.00a	8.00 ± 0.00d-f	5.00 ± 0.00a	5.00 ± 0.00a	1.00 ± 0.00a	1.00 ± 0.00a	2.33 ± 0.58a	3.00 ± 0.00c
AR11	13.67 ± 0.58de	13.67 ± 0.58fg	6.33 ± 0.58cd	1.00 ± 0.00a	1.00 ± 0.00a	11.67 ± 0.58a	5.00 ± 0.00a	5.00 ± 0.00a	1.00 ± 0.00a	1.00 ± 0.00a	3.00 ± 0.00a	3.67 ± 0.58bc
AR12	11.67 ± 0.58ef	11.67 ± 0.58h	6.00 ± 0.00d	1.00 ± 0.00a	1.00 ± 0.00a	10.33 ± 0.58ab	5.00 ± 0.00a	5.00 ± 0.00a	1.00 ± 0.00a	1.00 ± 0.00a	2.67 ± 0.58a	4.00 ± 0.00a-c
AR13	11.00 ± 0.00f	10.67 ± 0.58h	6.00 ± 0.00d	1.00 ± 0.00a	1.00 ± 0.00a	5.33 ± 0.58 g	3.33 ± 0.58b	3.33 ± 0.58b	1.00 ± 0.00a	1.00 ± 0.00a	2.00 ± 0.00a	3.00 ± 0.00c
AR14	22.33 ± 0.58a	22.33 ± 0.58a	8.33 ± 0.58ab	1.00 ± 0.00a	1.00 ± 0.00a	7.67 ± 0.58ef	5.00 ± 0.00a	5.00 ± 0.00a	1.00 ± 0.00a	1.00 ± 0.00a	2.00 ± 0.00b	4.67 ± 0.58ab
AR15	20.33 ± 0.58a	20.33 ± 0.58b	8.00 ± 0.00ab	1.00 ± 0.00a	1.00 ± 0.00a	7.33 ± 0.58ef	5.00 ± 0.00a	5.00 ± 0.00a	1.00 ± 0.00a	1.00 ± 0.00a	2.00 ± 0.00a	5.00 ± 0.00a
AR16	15.67 ± 0.58cd	15.33 ± 1.16d-f	8.67 ± 0.58a	1.00 ± 0.00a	1.00 ± 0.00a	7.00 ± 0.00ef	5.00 ± 0.00a	5.00 ± 0.00a	1.00 ± 0.00a	1.00 ± 0.00a	2.67 ± 0.58a	5.00 ± 0.00a
AR17	16.00 ± 0.00bc	15.67 ± 0.58de	8.33 ± 0.58ab	1.00 ± 0.00a	1.00 ± 0.00a	10.33 ± 0.58ab	5.00 ± 0.00a	5.00 ± 0.00a	1.00 ± 0.00a	1.00 ± 0.00a	2.33 ± 0.58a	3.67 ± 0.00bc
Mean	14.51 ± 3.58	14.57 ± 3.46	6.88 ± 1.11	1.00 ± 0.00	1.00 ± 0.00	8.45 ± 1.62	4.90 ± 0.41	4.88	1.00 ± 0.00	1.00 ± 0.00	2.25 ± 0.44	3.75 ± 0.72

Within a column, mean values having a letter in common are not significantly different according to Tukey's HSD test ($p > 0.05$). Values are mean ± SD of three repeated measurements from one plant per accession; accession means were used for statistical analysis. SL: Stem length (cm), NSN: Number of stem nodes (node/stem), NL: Stem node length (cm), NOL: Number of leaves (leaf/plant), LL: Leaf length (cm), LW: Leaf width (cm), NV: Number of main veins (vein/leaf), PD: Petiole diameter (cm), PL: Petiole length (cm), NEPS: Number of epidermal cell layers of stem, NCS: Number of collenchyma cell layers of stem, NPS: Number of parenchyma cell layers of stem, NEDS: Number of endodermal cell layers of stem, NXS: number of xylem bundles of stem, NPIS: number of phloem bundles of stem, NPHS: Number of pith cell layers of stem, NEPP: Number of epidermal cell layers of petiole, NCP: Number of collenchyma cell layers of petiole, NPP: Number of parenchyma cell layers of petiole, NXP: number of xylem bundles of petiole, NPHP: number of phloem bundles of petiole, NUEL: Number of upper epidermal cell layers of petiole, NLEL: Number of lower epidermal cell layers of leaf, NPM: Number of palisade mesophyll cell layers, NSM: Number of spongy mesophyll cell layers

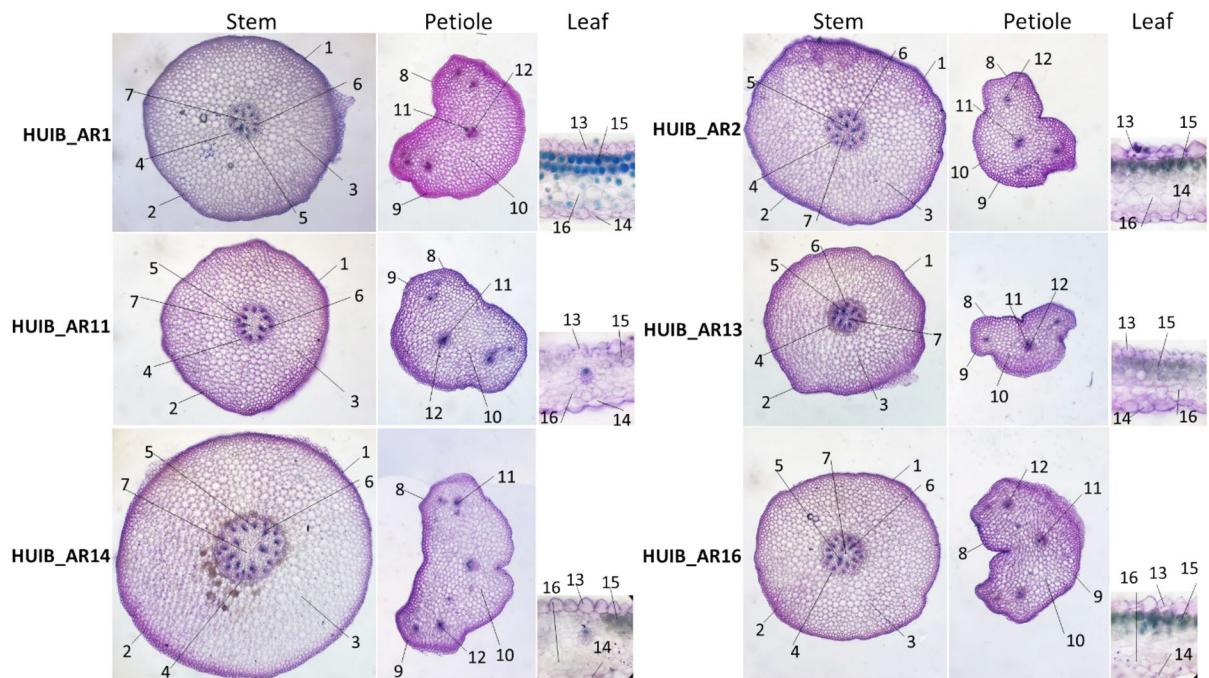
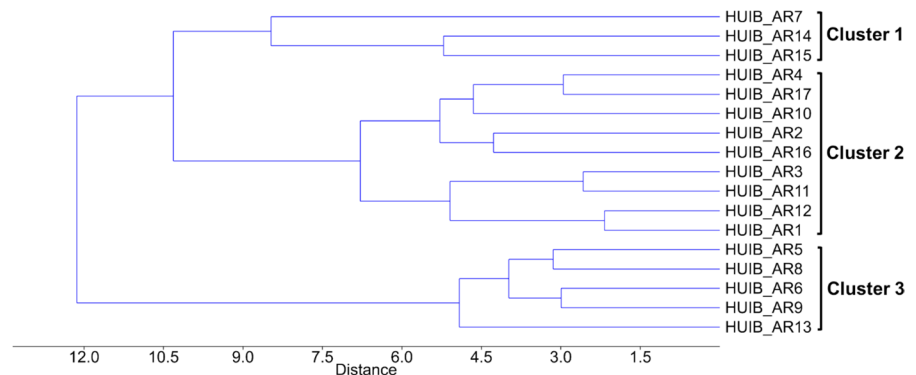


Fig. 2 Micro-anatomical features of HUIB_AR1, HUIB_AR2, HUIB_AR11, HUIB_AR13, HUIB_AR14 and HUIB_AR16. 1. Stem epidermis, 2. Stem collenchyma, 3. Stem parenchyma, 4. Stem endodermis, 5. Stem xylem, 6. Stem phloem, 7. Stem

pith, 8. Petiole epidermis, 9. Petiole collenchyma, 10. Petiole parenchyma, 11. Petiole xylem, 12. Petiole phloem, 13. Upper epidermis in leaf, 14. Lower epidermis in leaf, 15. Palisade mesophyll in leaf, 16. Spongy mesophyll in leaf

Fig. 3 Multivariate clustering of different *Anoectochilus* accessions based on micro-morphological traits



several loci show a large deficit (e.g., 2-5FR: $H_o = 0.000$ and $H_e = 0.656$; 4-1FR: $H_o = 0.294$ and $H_e = 0.756$).

Bayesian clustering indicates heterogeneous genetic composition

Bayesian clustering was performed as an exploratory analysis to visualise genetic heterogeneity

within accessions. Although the Evanno method identified the highest ΔK value at $K = 4$ ($\Delta K = 1.68642$), this result should not be interpreted as evidence of four populations because all accessions originated from a single protected area (Bach Ma National Park) and only six SSR loci were analysed. Instead, the STRUCTURE bar plot illustrates heterogeneous ancestry patterns among accessions, with most individuals exhibiting mixed membership

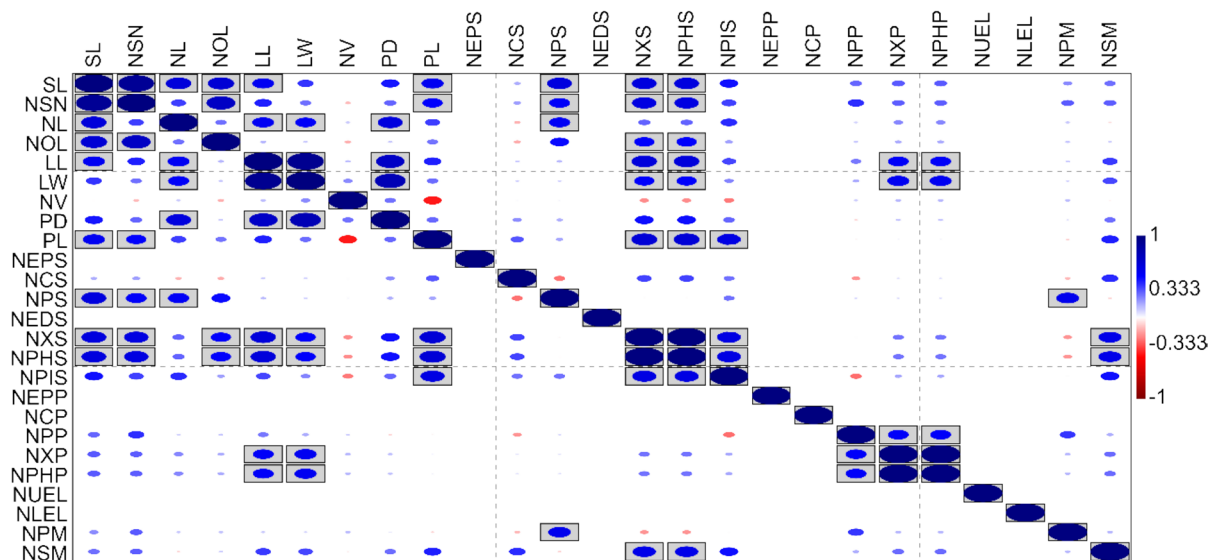


Fig. 4 Correlation among quantitative micro-morphological traits of *Anoectochilus* accessions. The color denotes the correlation level between two traits, where 1 represents a complete positive correlation (dark blue), and -1 represents a complete

negative correlation (dark red). A large circle denotes a strong correlation, and a small circle shows a weaker correlation. Squares indicate statistically significant correlations ($p < 0.05$)

coefficients rather than forming discrete genetic groups.

Among the analysed accessions, HUIB_AR13, HUIB_AR14 and HUIB_AR16 displayed higher membership coefficients for a predominant ancestry component (Fig. 8). This pattern was broadly consistent with their differentiated positions in the SSR clustering analysis. Besides, it also suggests that these accessions represent relatively distinct elements within the sampled germplasm.

No clear relationship was observed between genetic composition and geographic origin, as accessions collected from different localities frequently shared similar ancestry patterns. Overall, the STRU CTURE analysis supports the existence of heterogeneous genetic variation within the wild *A. roxburghii* germplasm rather than distinct population boundaries.

Morphological and genetic distances are not significantly correlated

The Mantel test showed a weak negative correlation between genetic distance based on SSR markers and morphological distance. However, this relationship was not statistically significant ($r = -0.038$, $p = 0.567$, 999 permutations), indicating no detectable

association between two datasets (Fig. 9). This was entirely consistent with the differences between the two clustering patterns of morphological and SSR. While morphological polymorphism split accessions into 3 clusters, 4 clusters were generated from the SSR data (Fig. 3 and Fig. 7).

Discussion

Seventeen *Anoectochilus* accessions were investigated and collected in Bach Ma National Park, Vietnam, starting in 2024 (Ho et al. 2025b). Nevertheless, information on micro-morphological and molecular characteristics was lacking for these accessions. This study provides a comprehensive characterisation of wild *Anoectochilus roxburghii* germplasm from Bach Ma National Park. Rather than attempting to infer population genetic structure across the species distribution, this study aimed to establish the first integrated germplasm characterisation of *A. roxburghii* accessions from Bach Ma National Park. Accordingly, the results should be interpreted as a baseline resource for germplasm conservation and management than as evidence of species-wide population genetic patterns.

Table 6 Evaluation indices of *Anoectochilus* population based on of the *ITS*, *rbcL* and *psbA-trnH* region

Indices	<i>ITS</i>	<i>rbcL</i>	<i>psbA-trnH</i>	<i>ITS + psbA-trnH</i>	<i>ITS + rbcL + psbA-trnH</i>
No. of variable sites, S	1	0	0	1	1
No. of mutations, Eta	1	0	0	1	1
Nucleotide diversity (per site), π ($\times 10^{-3}$)	0.72	0	0	0.33	0.23
Average number of nucleotide differences, k	0.52941	0	0	0.52941	0.52941
Number of haplotypes, h	2	1	1	2	2
Haplotype diversity, Hd	0.529	0	0	0.529	0.529

The *psbA-trnH* alignment contains a single 8-bp tandem repeat insertion/deletion (TTAGTATA). Because DnaSP treated gap positions as missing data, this repeat-length polymorphism was not included in the estimates of S, Eta or π

Such variation is valuable for germplasm characterisation because morphological traits remain important descriptors for accession identification, conservation management, and the selection of materials for cultivation and breeding programmes (Engels and Visser 2003; Rao et al. 2006). Qualitative morphological traits among the accessions are quite similar and comparable to *Anoectochilus roxburghii* in the study of Nguyen et al. (2024). Meanwhile, quantitative traits differ due to differences in their growth abilities. Specifically, HUIB_AR5, HUIB_AR6, HUIB_AR8, HUIB_AR9, and HUIB_AR13 exhibit weak to moderate growth, resulting in lower stem, node, and leaf sizes compared to others. Significant correlations are observed among quantitative traits. However, floral characteristics are lacking to accurately identify *Anoectochilus roxburghii* based on morphology (Bhattacharjee 2013). Overall, micro-anatomical traits provide relatively stable descriptors because they are generally less affected by short-term environmental fluctuations. Although the functional consequences of the observed anatomical variation were not directly evaluated in this study, differences in vascular bundle number, mesophyll organisation and parenchyma development may reflect variation in resource transport capacity, tissue organisation and growth performance among accessions. Consequently, these anatomical descriptors increase the discriminatory power of germplasm evaluation when combined with molecular markers.

Nair et al. (2024), Raj et al. (2025) and Rani et al. (2026) reported that DNA barcode analysis primarily served to authenticate species identity rather than to resolve intraspecific diversity. In this work, all accessions were confirmed as *A. roxburghii*, and only

limited variation was detected among barcode loci. This finding agrees with previous studies showing that standard plant DNA barcodes are highly effective for species identification (Thin et al. 2020; Lv et al. 2015). However, these analyses generally provide insufficient resolution for assessing genetic variation within species (Liu et al. 2022). In *psbA-trnH* results, the only difference among sequences is a single 8-bp insertion/deletion corresponding to one additional TTAGTATA tandem repeat. Thus, the observed polymorphism is a repeat-length indel rather than nucleotide substitution. Under the DnaSP settings used in this study, the zero values resulted from gap treatment and did not indicate complete absence of variation. Consequently, DNA barcode markers complemented the SSR analysis by confirming taxonomic identity, whereas SSR markers provided the discriminatory power necessary for germplasm characterisation (Vieira et al. 2016; Su et al. 2025).

The six SSR loci employed in this study revealed moderate genetic diversity (mean PIC = 0.535; mean H_e = 0.595), providing sufficient resolution to discriminate among the analysed accessions (Botstein et al. 1980; Kalinowski et al. 2007). Four loci (2-5FR, 3-2FR, 3-9FR and 4-1FR) exhibited comparatively high PIC and expected heterozygosity values, indicating that they are informative markers for future germplasm characterisation and fingerprinting of *A. roxburghii*. Notably, the mean observed heterozygosity (H_o = 0.311) was lower than the mean expected heterozygosity (H_e = 0.595), particularly at loci 2-5FR (H_o = 0.000; H_e = 0.656) and 4-1FR (H_o = 0.294; H_e = 0.756). This heterozygote deficiency may reflect biological factors such as inbreeding or population subdivision, as well as technical factors including

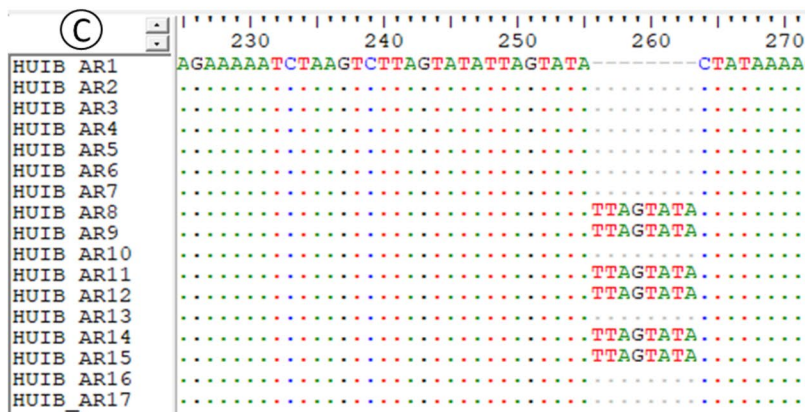
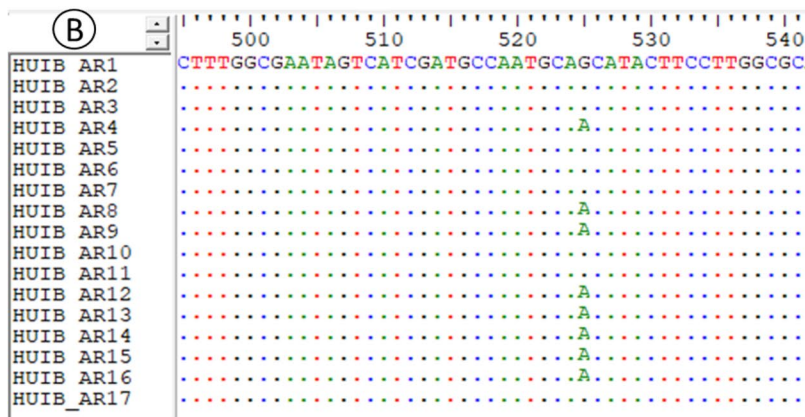
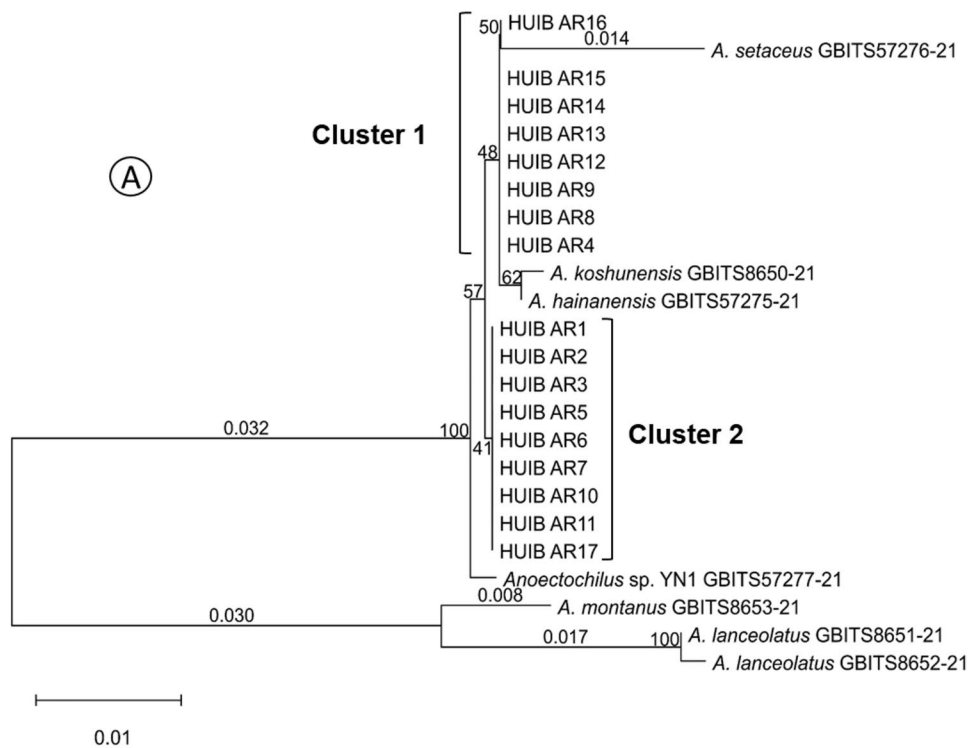


Fig. 5 The phylogenetic tree of the *ITS* region using the Neighbor-Joining method (bootstrap ≥ 50 shown) (**A**) and nucleotide differences between accessions in the *ITS* (**B**) and *psbA-trnH* region (**C**)

null alleles or scoring effects. Besides, the SSR-based UPGMA analysis further showed that several accessions, particularly HUIB_AR13, HUIB_AR14, HUIB_AR15 and HUIB_AR16, occupied relatively distinct genetic positions, suggesting heterogeneous genetic variation within the sampled germplasm. However, because only six SSR loci and a limited number of accessions from a single protected area (Bach Ma National Park) were analysed, these data should be interpreted as supporting accession-level germplasm characterisation rather than detailed population genetic inference.

The Bayesian clustering analysis should be interpreted with caution because it was based on only six SSR loci and 17 accessions, a dataset that provides limited statistical power for robust inference of population structure. Under these conditions, STRUCTURE is more appropriately regarded as an exploratory tool for visualising patterns of genetic heterogeneity rather than evidence of discrete population structure. The widespread admixture observed among individuals suggests that accessions represent a heterogeneous gene pool with no clear genetic subdivision. Likewise, the absence of an association between ancestry patterns and collection localities indicates that geographic origin was not a major determinant of genetic differentiation within the sampled germplasm. Similar patterns have been reported in other orchid species, where extensive seed dispersal and gene flow contribute to high levels of within-population genetic variation and weak geographic structure (Jacquemyn et al. 2009; Zhang et al. 2019; Xie et al. 2025). Therefore, the STRUCTURE analysis should be interpreted as supporting the description of genetic heterogeneity among accessions rather than inferring population structure across the species.

Although several accessions displayed distinct anatomical characteristics, the present dataset does not establish a causal relationship between phenotypic and genetic variation. The non-significant Mantel test further supports the absence of a detectable association between morphological and molecular variation. Therefore, the anatomical differences should be regarded as descriptive traits than evidence

of adaptive divergence. Future studies integrating broader geographic sampling, ecological variables and genome-wide markers will be necessary to determine whether these phenotypic differences reflect local adaptation or neutral intraspecific variation.

The identification of genetically differentiated accessions has important implications for germplasm conservation and utilisation (Kumari et al. 2023). Among the analysed materials, HUIB_AR13, HUIB_AR14, HUIB_AR15 and HUIB_AR16 consistently occupied relatively distinct positions in both the SSR clustering and Bayesian analyses, indicating that they represent valuable components of the detected genetic diversity. Accordingly, these accessions should be prioritised for ex situ conservation, the establishment of living germplasm collections and long-term genetic resource management because conserving genetically differentiated materials helps maintain overall allelic diversity and reduces the risk of losing rare variants. In addition, they may serve as valuable founder materials for future breeding programmes and as representative genotypes for high-resolution genomic studies. Nevertheless, the distinctiveness of four accessions was based on screening results from a currently limited dataset; therefore, the genetic or breeding value of these accessions requires further validation through phenotypic evaluation on larger fields and comparison with a broader genetic background.

The Mantel test revealed no significant correlation between morphological and neutral genetic distances ($r = -0.038$, $P = 0.567$). However, this result should be interpreted with caution because the relatively small number of accessions and the limited number of SSR loci substantially reduced the statistical power to detect weak genotype–phenotype associations. Consequently, the absence of a significant Mantel correlation should not be interpreted as inconsistency between the two datasets. This is biologically plausible because morphological traits are influenced by both genetic background and environmental conditions, resulting in phenotypic plasticity that does not necessarily correspond to patterns of neutral molecular variation (Mafakheri et al. 2022; Morales et al. 2010). In contrast, SSR markers generally reflect selectively neutral genetic variation and therefore may not be directly associated with genes controlling morphological traits (Manco et al. 2020; Zimmerman et al. 2020). Therefore, morphological and molecular

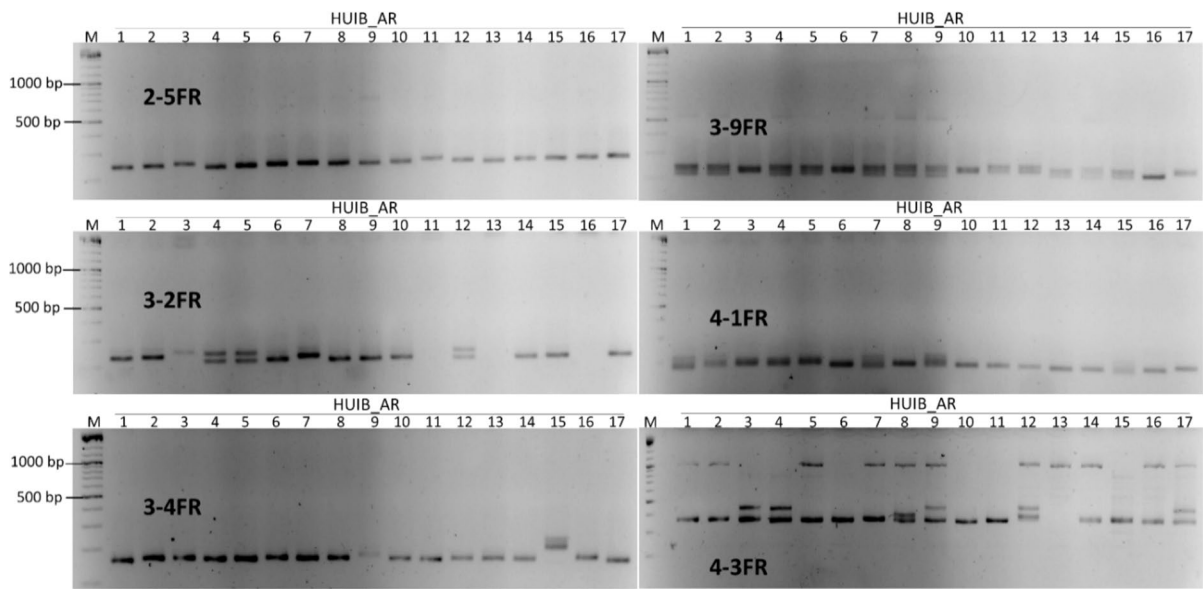


Fig. 6 Electrophoresis results showing polymerase chain reaction (PCR) products obtained using six pairs of simple sequence repeat (SSR) primers. M: GeneRuler 1 kb DNA Ladder

Table 7 Genetic diversity at various loci in *Anoectochilus* accessions using six simple sequence repeat (SSR) primers

Primer	Successful amplification rate (%)	Band size (bp)	na	ne	I	PIC	Ho	He
2-5FR	100.00	240–270	3.000	2.752	1.055	0.564	0.000	0.656
3-2FR	82.35	220–260	4.000	2.361	1.089	0.534	0.429	0.598
3-4FR	100.00	170–240	5.000	1.656	0.831	0.374	0.059	0.408
3-9FR	100.00	220–270	5.000	4.099	1.491	0.717	0.706	0.779
4-1FR	100.00	190–250	7.000	3.753	1.557	0.696	0.294	0.756
4-3FR	100.00	370–1000	3.000	1.566	0.656	0.327	0.375	0.373
Mean			4.500	2.689	1.113	0.535	0.311	0.595
Standard deviation			1.517	1.055	0.356	0.161	0.259	0.172

na: Observed number of alleles, ne: Effective number of alleles, I: Shannon's Information index, PIC: Polymorphism information content, Ho: Observed heterozygosity, He: Expected heterozygosity

markers should be regarded as complementary tools for germplasm characterisation. In addition, broader sampling and higher-resolution genomic markers will be required to further evaluate genotype–phenotype relationships.

Overall, this study provides a comprehensive characterisation of wild *A. roxburghii* germplasm collected from Bach Ma National Park, representing a preliminary assessment of genetic and phenotypic variation within this protected area rather than across the species' distribution. The integration of micro-morphological, DNA barcode and SSR

analyses provided a more comprehensive characterisation of wild *Anoectochilus roxburghii* germplasm than any single approach alone. DNA barcodes reliably confirmed species identity, whereas SSR markers resolved genetic variation among accessions and micro-morphological traits supplied complementary phenotypic descriptors (Nair et al. 2024; Noshita et al. 2022; Zhu et al. 2023). Together, these datasets provide a valuable baseline for germplasm characterisation, conservation planning and the management of genetic resources in *A. roxburghii*. Future studies incorporating broader geographic

Fig. 7 UPGMA clustering of 17 accessions based on simple sequence repeat (SSR) data

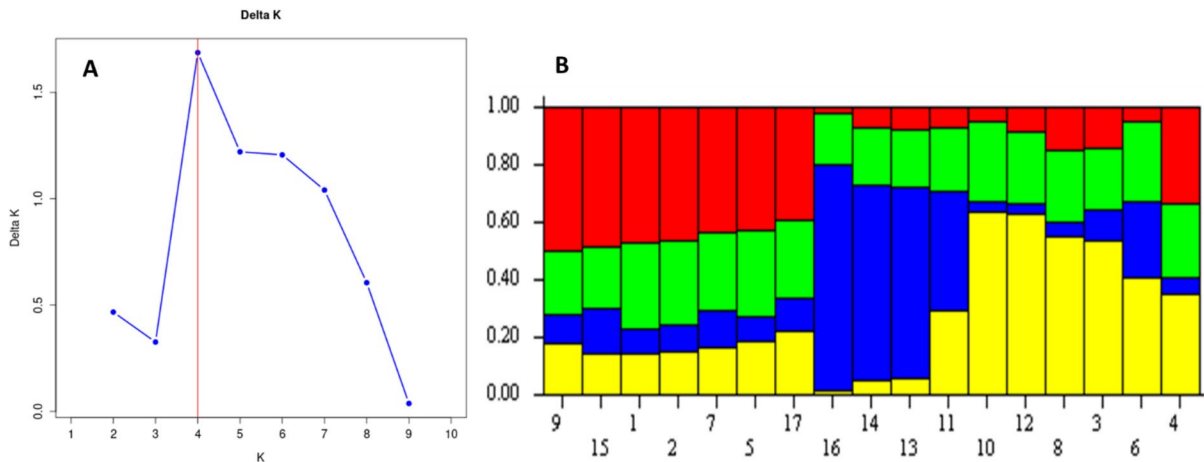
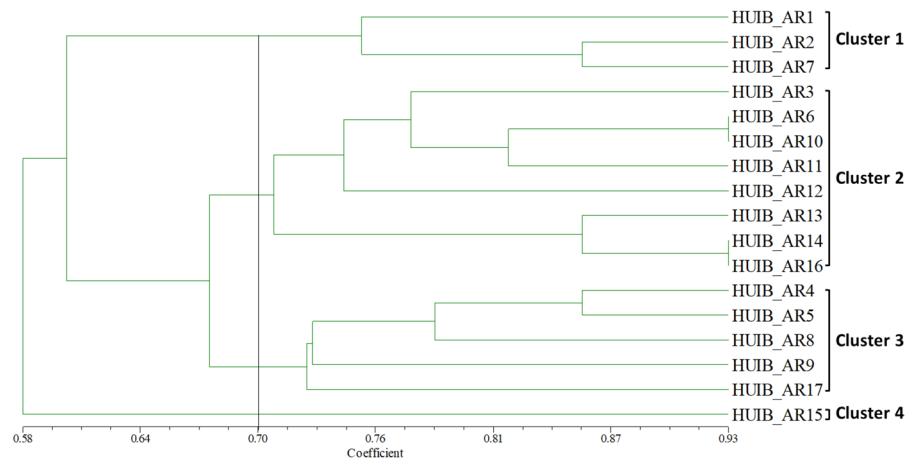


Fig. 8 Bayesian clustering of *Anoectochilus roxburghii* accessions. **A** ΔK values calculated using the Evanno method implemented in StructureSelector, showing the highest ΔK at $K = 4$ ($\Delta K = 1.68642$). **B** Admixture plot of the 17 *A. roxburghii* accessions at $K = 4$. Each vertical line represents an

individual genotype of accessions, and the colors within each line indicate the estimated membership coefficients (Q values) of the accession in each of the 4 clusters ($K = 4$, $\Delta K = 1.68642$). The ΔK value is calculated by Evanno method

sampling and genome-wide molecular markers will further improve the resolution of genetic diversity and strengthen conservation and breeding strategies.

Conclusion

This study provides an integrated characterisation of 17 wild *Anoectochilus roxburghii* accessions collected from Bach Ma National Park, Vietnam, using micro-morphological, DNA barcode and SSR markers. The accessions exhibited considerable variation in

morphological and anatomical traits, providing useful phenotypic descriptors for accession discrimination. DNA barcode analysis confirmed the identity of the analysed materials as *A. roxburghii* but revealed limited intraspecific sequence variation, with *ITS* showing two haplotypes, whereas *rbcL* was conserved and *psbA-trnH* variation was restricted to a repeat-length indel. In contrast, the six SSR loci revealed moderate genetic diversity (mean $PIC = 0.535$; mean $H_e = 0.595$) and differentiated the accessions at the germplasm level. Several accessions, particularly

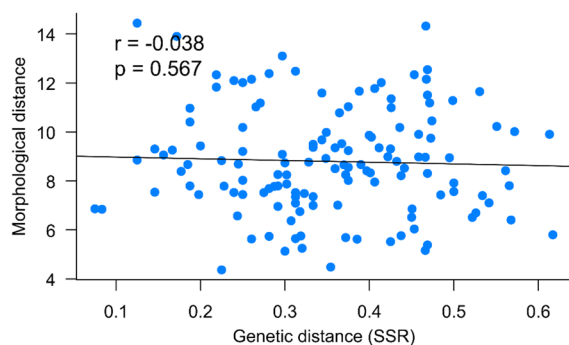


Fig. 9 Correlation between morphological and simple sequence repeat (SSR) analysis based on Mantel test (95% confidence interval = 0.210)

HUIB_AR13, HUIB_AR14, HUIB_AR15 and HUIB_AR16, occupied relatively distinct positions in the SSR markers and may therefore warrant priority consideration for ex situ conservation and further evaluation. However, these findings should be regarded as a preliminary assessment of genetic and phenotypic variation within the sampled germplasm rather than evidence of species-wide population structure, given the limited sample size and single geographic origin of the accessions. Further studies involving broader geographic sampling, additional molecular markers and larger sample sizes are needed to clarify the distribution and conservation of genetic diversity in *A. roxburghii*.

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Data availability The datasets generated and analysed during the current study are available from the corresponding author on reasonable request.

Sample availability The *Anoectochilus* germplasm is available at Institute of Biotechnology, Hue University, Hue city, Viet Nam upon reasonable request.

Declarations

Conflict of interest The authors declare no conflicts of interest.

Institutional review board statement Not applicable for studies not involving humans or animals

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