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Comprehensive Evaluation of Specific-Use Prunus mume Cultivars by the Analytic Hierarchy Process and Identification of Stable Reference Genes for Quantitative Real-Time PCR Normalization

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Abstract

Background: The Chinese plum (Prunus mume) is a woody ornamental of considerable cultural and horticultural importance, and while China alone holds more than 300 named cultivars, there is still a shortage of numerically defined, use-oriented frameworks for assigning germplasm to particular commercial purposes, and no validated panel of reference genes exists to support molecular investigations.

Methods: Drawing on morphological surveys, phenological records, and fruit-quality measurements, we built three AHP-based hierarchical models—one each for landscape planting, container cultivation, and combined ornamental-fruit production—and trialled them on over 80 accessions curated in a germplasm repository. Concurrently, the expression consistency of 14 candidate reference genes was assayed via qRT-PCR in leaf and petal samples from six varieties, along with various tissue types and bloom stages of the widely planted hybrid 'Meiren'. Stability was gauged through geNorm, NormFinder, BestKeeper, ΔCt, and the RefFinder meta-algorithm.

Results: Every judgment matrix across the three models satisfied the consistency condition (CR < 0.1). In the landscape-oriented system, growth vigor, decorative form, and stress tolerance received the heaviest weighting; for potted use, decorative appearance, bloom width, and petal count carried the most weight; for dual-purpose use, fruit load per tree, bloom duration, and individual fruit mass were the leading factors. From the 80-entry collection, five top-performing cultivars were pinpointed per use category. As for reference genes, GAPDH together with UBQ proved most stable in leaf tissue across varieties, GAPDH with ACT1 in petal tissue, ACT7 with UBQ across 'Meiren' tissue types, and PP2A with ACT1 through its flowering stages. When the least reliable candidates (e.g., TUB1 in leaf, ACT3 in petal, GAPDH in the tissue-type and flowering-stage series) were employed for normalization, expression profiles of four target genes (COX10, ANR, CESA1, and Exp-A20) shifted markedly, underscoring that reference-gene choice can substantially influence data interpretation.

Conclusions: The AHP framework put forward here furnishes a consistent,

adaptable instrument for purpose-driven variety selection in P. mume, and the reference-gene validations supply a tissue- and stage-informed basis for dependable qRT-PCR normalization in subsequent functional genomics and breeding investigations.

Keywords

Prunus Mume; Analytic Hierarchy Process; Germplasm Evaluation; Cultivar Screening; Reference Gene; Qrt-PCR Normalization; Gene Expression Stability

1. Introduction

Prunus mume Sieb. et Zucc., known commonly as mei or Chinese plum, occupies a singular place among East Asian woody ornamentals. Its late-winter-to-early-spring flowers, emblematic of hardiness, together with its twin function as a source of both aesthetic enjoyment and edible fruit, have earned it widespread appreciation [1,2]. Chinese cultivation records document over 300 distinct varieties, grouped by growth architecture, bloom morphology, and petal pigmentation [1,3]. Despite this richness, the sector has no broadly accepted numerical framework for deciding which accessions are best suited to a given commercial pathway—be that civic landscaping, potted and bonsai display, or dual harvest of flowers and fruit. In practice, variety deployment tends to rely on informal experience, which exposes the mei industry to inconsistent product quality and heightened market risk [4].

Multi-attribute decision techniques bring a degree of objectivity that informal expert appraisal cannot match. Among these, the Analytic Hierarchy Process (AHP) devised by Saaty [5] breaks a complicated screening problem into a tiered structure of weighted, measurable factors, with a built-in statistical test of internal coherence. AHP-style systems have lately been used to judge ornamental merit and environmental fit in genera such as Rhododendron [6] and to assess aromatic qualities in Hemerocallis [7], regularly outperforming single-trait or purely subjective approaches. To our knowledge, however, no published AHP framework has been specifically tailored to the three leading commercial use-classes of P. mume.

On a separate but equally important front, molecular research on P. mume has advanced considerably following the publication of its reference genome [2] and subsequent updates coupled with resequencing projects [8]. These resources have made it possible to conduct genome-scale surveys of gene families tied to bloom colour, scent production, cold hardiness, and plant architecture [9–12]. Quantitative real-time PCR (qRT-PCR) continues to serve as the primary tool for confirming such expression findings, but its reliability hinges on normalizing against reference genes whose transcriptional levels remain steady across the exact tissues, growth phases, and genetic backgrounds under investigation [13,14]. A gene's stability as a normalizer is not an intrinsic species-wide attribute; it shifts with tissue identity,

varietal background, and physiological condition [15]. Consequently, a calibrator verified for one experimental context cannot be assumed to work in another. Within P. mume specifically, earlier work recommended PP2A for flower-bud progression series [16] and UBQ together with UBC for cross-variety bloom comparisons and cold-exposed stems [17]. Yet no investigation has systematically tested candidate normalizers across the combined dimensions of leaf-versus-petal material, multiple-variety panels, and the flowering time course, as examined here using the horticulturally prominent Blireiana-type hybrid 'Meiren'.

The work reported here thus had two intertwined aims: (1) to design and empirically validate a set of AHP-driven hierarchical scoring models for selecting P. mume germplasm suited to landscape, potted-plant, and dual flower-and-fruit applications, and to exercise them on an 80-accession germplasm bank; and (2) to carry out a thorough stability assessment of 14 candidate reference genes in leaf and petal specimens from six P. mume varieties, plus a range of organs and bloom developmental stages of 'Meiren', applying five independent statistical techniques, with subsequent verification against four target genes. The combined findings are meant to assist both the practical horticultural rollout of elite P. mume material and the molecular studies that will drive future breeding.

2. Materials and Methods

2.1. Study I: AHP-based cultivar evaluation

2.1.1. Plant materials and study sites

More than 80 P. mume accessions preserved in the Prunus Germplasm Resource Nursery of Zhejiang A&F University (Hangzhou, Zhejiang, China) were examined over three field locations spanning the 2021 through 2023 bloom and fruiting intervals.

2.1.2. Phenological survey and selection of specific indicators

Sixteen traits covering morphology, phenology, and fruit composition were chosen with guidance from the UPOV/DUS (distinctness, uniformity, and stability) test protocol for P. mume. Bloom-related characters—petal count, corolla width, flower hue, calyx pigmentation, and scent—were evaluated on the mid-to-upper portion of the canopy at peak anthesis using visual rating, a Royal Horticultural Society colour fan, vernier callipers, and GC-MS for scent profiling. Fruit characteristics (individual fruit mass, ascorbic acid level, total soluble sugars, titratable acidity, and edible fraction) were determined on fully ripe fruit. Whole-plant features (bloom onset and duration, flower abundance, branching capacity, growth strength, tree form, and stress tolerance) were recorded across the entire growing period (Table 1). Depending on the trait, each indicator was based on 10–30 replicate specimens or structures per accession (Table S1).

2.1.3. Determination of fruit-quality traits

Individual fruit mass was obtained from 30 randomly picked fruits per accession with an electronic balance (Sartorius BSA323S). Ascorbic acid was assayed by the 2,6-dichlorophenol-indophenol titration procedure: pulp was homogenized in oxalic acid solution, titrated with standardized dye, and the vitamin C level per 100 g fresh weight was back-calculated from titre readings against an ascorbic-acid calibration curve. Total soluble sugars were read with a digital refractometer (Lamberts) on 30 fruits per accession. Titratable acidity was quantified by neutralizing an aqueous fruit homogenate with standardized NaOH, with phenolphthalein as the endpoint indicator, and expressed as a percentage from the recorded NaOH volume, normality, and sample mass.

2.1.4. Construction of the AHP hierarchy and consistency testing

For each of the three target end-uses—landscape planting, container display, and combined bloom-and-fruit production—a three-tier hierarchical architecture was set up: an apex representing the overall decision goal, a criterion (element) tier clustering allied traits, and a sub-criterion (factor) tier composed of the individually measured variables. Pairwise comparison matrices were populated at each tier with Saaty's 1–9 fundamental scaling, reflecting how much each factor contributed to the use-specific objective relative to other variables within the same set of accessions. For every matrix, the principal eigenvalue (λmax) was computed, from which the consistency index $CI = (\lambda max - n)/(n - 1)$ and the consistency ratio $CR = CI/RI$ were derived (n = matrix dimension; RI = Saaty's random reference value for that dimension). A matrix was regarded as adequately consistent when CR fell below 0.1. Normalized eigenvectors from self-consistent matrices were multiplied across tiers to yield the definitive weight of each sub-criterion indicator. Every surveyed accession was rated against the five-grade rubric laid out in Table 1, and a weighted aggregate score was calculated for each use scenario; hierarchical cluster analysis (SPSS v.26) was additionally performed to verify the scoring-based groupings. The five accessions attaining the highest scores under each use model were selected as elite purpose-specific varieties.

2.2. Study II: Reference gene screening for qRT-PCR

2.2.1. Plant materials

Leaf and petal specimens were gathered from six P. mume varieties spanning distinct cultivar groupings—'Changyan Gongfen', 'Beijing Yudie', 'Meiren', 'Guhong Zhusha', 'Dan Fenghou', and 'Jiangmei Chuizhi'—all held in the same germplasm repository. Furthermore, annual and perennial stems, young and mature leaves, and fully expanded flowers were collected from 'Meiren' (Blireiana Group, a P. mume × Prunus cerasifera hybrid) to represent distinct organ categories, and flowers at four progressive opening phases—small bud (S1), large bud (S2), early bloom (S3), and full bloom (S4)—were harvested from the same variety to represent the flowering time course. Every sample was flash-frozen in liquid nitrogen and held at −80 °C;

three biological replicates were taken for each sample type.

2.2.2. RNA extraction and cDNA synthesis

Total RNA was extracted from 0.5 g tissue using the RNAprep Pure Plant Plus Kit (Tiangen, Beijing, China). First-strand cDNA was synthesized from extracted RNA using Reverse Transcriptase M-MLV (Takara, Dalian, China) and diluted ten-fold in ddH2O prior to qRT-PCR.

Total RNA was isolated from 0.5 g tissue portions with the RNAprep Pure Plant Plus Kit (Tiangen, Beijing, China). First-strand cDNA was reverse-transcribed from the purified RNA using Reverse Transcriptase M-MLV (Takara, Dalian, China) and was subsequently diluted ten-fold in nuclease-free water for qRT-PCR.

2.2.3. Candidate reference and target genes, and primer design

Fourteen candidate reference genes—EF1α, GAPDH, ACT1, ACT2, ACT3, ACT7, UBQ, PP2A, TUA, TUB1 through TUB4, and PHK—were drawn from the P. mume genome database. Gene-specific primers were generated with Primer-BLAST (NCBI), aiming for amplicons of 75–180 bp, primer length of 17–23 nucleotides, GC percentage of 40–60%, and an annealing temperature range of 58–62 °C (Table 2). Specificity of amplification was checked by conventional PCR on 2% agarose gels and by melt-curve profiling. Four target genes—Cyclooxygenase 10 (COX10), Anthocyanidin reductase (ANR), Cellulose synthase 1 (CESA1), and Expansin-A20 (Exp-A20)—served as validation targets: COX10 together with ANR in the leaf/petal varietal comparison, and CESA1 together with Exp-A20 across the 'Meiren' organ set and flowering time course.

2.2.4. Quantitative real-time PCR

qRT-PCR was carried out with TB Green Premix Ex Taq II (Takara, Dalian) on a real-time thermal cycler. Each 10-μL reaction consisted of 1.0 μL cDNA template, 0.5 μL each of forward and reverse primer (25 nM), 5.0 μL 2× TB Green Premix, and 3.0 μL nuclease-free water. The thermal regime comprised an initial 3-min denaturation at 95 °C, followed by 40 cycles of 5 s at 95 °C and 30 s at 60 °C, ending with a melt-curve acquisition step. Amplification efficiency (E) and the linear correlation coefficient (R^2) were estimated from ten-fold serial cDNA dilutions (initial concentration 500 ng/μL). Biological as well as technical triplicates were run for every reaction.

2.2.5. Expression stability analysis

Stability of expression among the 14 candidates was assessed through five complementary computational approaches: geNorm, which orders genes by pairwise variation (M-value; $M < 1.5$ is taken to indicate acceptable stability) and estimates the minimum number of reference genes required from the pairwise variation ratio Vn/Vn+1 (a value under 0.15 suggests n genes are adequate);

NormFinder, which separates within-group from among-group variance; BestKeeper, which ranks by the standard deviation (SD) and coefficient of variation of raw Cq values (SD > 1 flags instability); the comparative ΔCt technique, which ranks by the standard deviation of pairwise Ct differences; and RefFinder, a meta-tool that merges the outputs of the preceding four algorithms into a unified rank order [18–22]. Evaluations were performed independently for (i) leaf material from the six varieties, (ii) petal material from the six varieties, (iii) the panel of 'Meiren' organs, and (iv) the four 'Meiren' bloom stages.

2.2.6. Validation of selected reference genes

To verify that stability rankings had practical consequences for data accuracy, relative expression levels of the four target genes were calculated with the 2^(−ΔΔCt) formula, normalizing in parallel to the highest-ranked and the lowest-ranked reference gene(s) identified within each sample set, and the resultant expression patterns were contrasted.

3. Results

3.1. AHP hierarchy weighting and consistency

Judgment matrices from all three purpose-specific hierarchies cleared the consistency threshold (CR < 0.1) at each tier (Table 3), indicating that the pairwise importance assignments underpinning the weight sets were internally congruent. In the landscape-oriented model, growth vigor (weight 0.240), ornamental form (0.175), and stress tolerance (0.165) emerged as the three most heavily weighted sub-criterion indicators, jointly accounting for 58% of the overall weight; calyx hue (0.013) and floral scent (0.030) were the least influential (Table 4). Within the container-plant model, ornamental form (0.345), bloom diameter (0.154), and petal number (0.141) predominated, together capturing 64% of total weight, while growth vigor (0.018) and stress tolerance (0.019)—traits of marginal relevance to a pot-maintained specimen that undergoes regular repotting—ranked lowest (Table 4). In the combined bloom-and-fruit model, per-tree harvest (0.312), bloom duration (0.113), and individual fruit mass (0.091) led, jointly covering 52% of the total, whereas calyx hue (0.008) and titratable acidity (0.014) exerted the smallest influence (Table 5). This clear reshuffling of indicator importance from one usage scenario to another aligns with the distinct horticultural aims of each end-use and bolsters the discriminant validity of the AHP-based models.

3.2. Screening of elite specific-use cultivars

When the three weighted scoring formulas were applied to the 80-accession panel, each use category produced its own distinct rank ordering (Tables 6–8; comprehensive rankings for every accession evaluated appear in Supplementary Tables S2–S4). For landscape use, the five accessions receiving the top scores were

'Fenxia', 'Nanjinghong', 'Sanlun Zhusha', 'Qiaozhuang Yudie', and 'Dalun Lve' (composite scores ranging from 15.4 to 15.8; Table 6). For container use, the five leaders were 'Yan Zhusha', 'Chongban Dahong', 'Qianban Zhusha', 'Tieguhong', and 'Suya Lve' (scores between 15.0 and 17.3; Table 7). For dual-purpose use, the foremost five were 'Lijiang Zhaoshui Mei', 'Yulong Feixue', 'Yulong Hongfei', 'Dan Fenghou', and 'Xiaohong Zhusha' (scores ranging from 17.9 to 18.9; Table 8). Salient morphological and phenological characteristics of these fifteen standout varieties are condensed in Table 9; representative in situ photographs are displayed in Figure 1.

Looking more closely at the landscape group, 'Fenxia' and 'Nanjinghong' paired profuse, scented, reddish-to-purple blossoms with robust growth and strong hardiness, while 'Dalun Lve' stood apart for an unusual green calyx rare among the surveyed material. Within the container group, 'Yan Zhusha' earned the highest marks through its tight growth habit and brilliant, uniformly deep-red, fragrant flowers, and 'Suya Lve' drew notice for a vivid emerald-green calyx—a characteristic prized for potted exhibits. In the dual-use set, 'Lijiang Zhaoshui Mei' bloomed the earliest (mid-January to early February) while setting copious fruit, whereas 'Yulong Hongfei' bore exceptionally dense bloom clusters (1–4 flowers per node) together with dependable single-fruit set (Table 9).

3.3. Primer specificity and amplification efficiency

Every one of the 18 primer pairs—14 for reference genes and 4 for target genes—yielded a single amplification product of the predicted length (85–155 bp) in conventional PCR and generated a unique melt-curve peak, attesting to their specificity (Figure 2). The amplification efficiencies calculated for the 14 candidate reference genes fell between 91% and 107%, with R^2 exceeding 0.98 across all four specimen panels—leaf and petal samples from multiple varieties, and the organ and flowering-stage series from 'Meiren' (Table 10)—satisfying the precision requirements for dependable relative quantification.

3.4. Candidate reference gene expression profiles

Quantification cycle (Ct) readings of the 14 candidate genes extended from 21 to 35 in leaf material, 16 to 32 in petal material, 19 to 34 across 'Meiren' organ types, and 24 to 36 through its bloom progression (Figure 3), pointing to a broad span of absolute transcript levels among the candidates. In leaf tissue, GAPDH returned the lowest Ct values, signifying the highest transcript abundance; EF1α was the most abundant in the organ panel; and ACT7 gave the lowest Ct readings across the flowering stages. Within petal tissue, by contrast, all 14 genes exhibited fairly comparable Ct distributions.

3.5. Expression stability in leaf and petal tissue of different cultivars

For leaf-tissue samples, geNorm picked GAPDH together with UBQ (pairwise M =

0.247) as the most firmly anchored gene duo, whereas TUB1 (M = 2.00) was the least steady. Both NormFinder and the ΔCt procedure agreed in placing GAPDH at the top, trailed closely by UBQ and TUB2; BestKeeper diverged somewhat by favouring ACT2 and ACT3, a reflection of that tool's responsiveness to raw Cq scatter rather than to pairwise or partitioned variance (Table 11). The RefFinder composite ranking assigned first place to GAPDH and second to UBQ, with TUB1 occupying the bottom (14th) slot—a result consonant with the geNorm outcome and pointing to GAPDH plus UBQ as the most broadly trustworthy normalizers for leaf-based cross-variety expression work in P. mume.

Turning to petal tissue, geNorm flagged ACT7 partnered with PP2A (M = 0.252) as the most stable combination, whereas NormFinder and the ΔCt technique both gave the highest marks to ACT1 and GAPDH, and BestKeeper leaned toward TUB2 and PHK (Table 11). The RefFinder consensus placed GAPDH first and ACT1 second, while ACT3 sat at the bottom (14th). The greater divergence among the individual algorithms seen in petal relative to leaf tissue highlights the usefulness of a consensus-driven (RefFinder) strategy when single-method outputs conflict.

3.6. Expression stability across organs and flower-opening stages of 'Meiren'

Across the different organ types of 'Meiren'—annual and perennial stems, young and mature leaves, and flower—both geNorm and BestKeeper concurred that ACT7 plus UBQ (M = 0.412) constituted the most invariant pair; NormFinder ranked ACT7 foremost and ACT3 next, and the ΔCt method gave preference to EF1α and TUA. The RefFinder meta-ranking settled on ACT7 in first place and UBQ in second, with GAPDH ranked lowest (14th; Table 12).

When the four bloom-opening phases of 'Meiren' (S1 through S4) were analyzed, geNorm pointed to EF1α and TUA (M = 0.255) as the top candidates, while NormFinder together with the ΔCt method both endorsed ACT1 and PP2A, and BestKeeper singled out TUB3 and TUB2. The RefFinder consensus gave PP2A the leading position and ACT1 the runner-up spot, and once again GAPDH was ranked the least stable (14th; Table 12).

3.7. Validation of selected reference genes

Normalizing COX10 and ANR against the top-performing leaf-tissue genes (GAPDH, UBQ) yielded steady relative expression trajectories across the six varieties, whereas swapping in the least stable gene (TUB1) produced a distinctly different pattern, particularly pronounced for 'Beijing Yudie' (Figure 4). In petal material, normalization to GAPDH and ACT1 returned congruent COX10/ANR profiles; normalization to ACT3, however, inverted the apparent cultivar ordering for both target genes. For instance, ANR expression in 'Changyan Gongfen' seemed nearly twice as high with ACT3 normalization as with GAPDH normalization. In the same vein, normalizing CESA1 expression across 'Meiren' organ types to ACT7/UBQ

versus GAPDH, and normalizing Exp-A20 across bloom stages to PP2A/ACT1 versus GAPDH, generated qualitatively contrasting developmental trajectories (Figure 5). GAPDH-based normalization inflated Exp-A20 readings at stage S2 while deflating them at stage S4. These findings provide direct confirmation that the choice of calibrator gene tangibly shapes how target-gene expression data are interpreted in P. mume.

Table 1. Five-point scoring criteria used to evaluate specific indicators of Prunus mume cultivars, adapted from DUS testing guidelines.

Trait	20–17	16–13	12–9	8–6	5–1
Number of petals	Very heavy (>30)	Double (26–30)	Semi-double (15–25)	Compound (9–14)	Single (5–8)
Flower diameter (mm)	Maximum (≥36)	Large (27–35)	Medium (22–27)	Small (17–22)	Minimal (<17)
Flower colour	Pure/vivid, special	More pure/vivid	More vivid	Purer	General
Calyx colour	Vivid, unique, bright	Vivid, uniform	More vivid, variegated	Old, fades easily	Dull, fades readily
Fragrance	Full-bodied, special	Strong	Light	Weak	None
Single fruit weight (g)	≥30.0	25.0–29.9	20.0–24.9	15.0–19.9	<15.0
Vitamin C (mg/100 g)	≥10.0	8.0–9.9	6.0–7.9	5.0–5.9	<5.0
Total sugar (%)	≥5.0	4.0–4.9	3.0–3.9	2.0–2.9	<2.0
Total acid (%)	≥7.0	6.0–6.9	5.0–5.9	4.0–4.9	<4.0
Edible rate (%)	≥95.0	90.0–94.9	85.0–89.9	80.0–84.9	<80.0
Flowering time	Long	Longer	Medium	Shorter	Short
Flower density	Extremely dense	Denser	Medium	Sparser	Few/scattered
Branching ability	Very strong	Strong	General	Weak	Extremely weak
Tree posture	Good	Better	General	Weak	Poor
Growth potential	Excellent	Good	General	Weak	Extremely weak
Resistance	Very strong	Strong	General	Weak	Extremely weak

Sixteen indicators spanning flower, fruit, and tree-condition traits; investigation methods and replicate numbers are given in Supplementary Table S1.

Table 2. Primer sequences, annealing temperatures, and amplicon sizes for 14 candidate reference genes and 4 target genes.

Gene	Annotation	Forward / Reverse primer (5'→3')	Tm (°C)	Size (bp)
Candidate reference genes				

EF1α	Elongation factor-1α	F: TGGTGGTTTTGAAGCCGGTAT R: AGTGGTGGCATCCATCTTGTT	60.0	132
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	F: AAAACCGCAACCACCCAT R: ACCAAACGACCGATTCTTCC	59.5	116
ACT1	Actin 1	F: CTGAACCCAAAGGCTAATCGT R: CATAAGGGCATCGGTGAGG	58.6	133
ACT2	Actin 2	F: GTTTCCATTTTGAACCAACACC R: CCTCATCCCCAACATACGC	60.3	128
ACT3	Actin 3	F: ATGTCCGCCTCCCCTTCTT R: GCCTTGACCATTCCCGTTC	59.2	145
ACT7	Actin 7	F: TGAGGCTCCTCTCAACCCTA R: CAGTTGTACGACCACTGGCA	58.9	97
UBQ	Ubiquitin	F: CGTGAAAACCCTAACGGGTA R: AGGGTCGATTCCTTCTGGAT	58.6	112
PP2A	Protein phosphatase 2A	F: AGTCGGCAAATTACCCAAGTG R: GCCCCCATGCAAACAGAAAAT	59.5	126
TUA	α-Tubulin	F: TGAGGCTCCTCTCAACCCTA R: CAGTTGTACGACCACTGGCA	59.7	136
TUB1	β-Tubulin 1	F: TGGGCCAAAGGCCATTACAC R: AGTACCACCACCCAACGAAT	59.7	122
TUB2	β-Tubulin 2	F: TGTGGACCTATGCCTTGACAG R: GAACCAGTGCCACCACCAAC	59.6	102
TUB3	β-Tubulin 3	F: AGGGTCATTACACCGAAGGC R: AGCGTTCCCATTCCAGAACC	60.0	155
TUB4	β-Tubulin 4	F: ACTCCTTGGGTGGAGGAACT R: TTCTCCACAAGCTGATGCAC	59.4	128
PHK	Phosphorylase kinase	F: CGACCAAGAGGAGCGTGAGT R: CCCAAGTGAGAAGCGAGGAT	59.5	136
Target genes (validation)				
COX10	Cyclooxygenase 10	F: CTGCTGTTGATCTCGCGTCT R: AACAACCTGAGGCGAGCTTT	60.6	98
ANR	Anthocyanidin reductase	F: GCCACCCAACCCATCTCAA R: TGACGGCATAGCCCTTCTC	60.2	85
CESA1	Cellulose synthase 1	F: TGTTACTGCTTCTGGGCTTCC R: GCTTGAGACCTGCGGAAAT	60.0	86
Exp-A20	Expansin-A20	F: ATGGGGAAATACTTGCTGTCA R: GAGGCTGCCCTTTAAGATTGA	60.0	88

Primers were designed with Primer-BLAST (NCBI); annealing temperature range 58–62 °C, amplicon length 75–180 bp.

Table 3. Consistency test summary for the judgment matrices of the three use-specific AHP hierarchies (all CR < 0.1, indicating acceptable consistency).

Hierarchy	Comparison level	n	λmax	CR
Greening use	Criteria layer (A–B)	2	2.0000	0.0000
Greening use	Flower traits (B1–C)	5	5.4313	0.0963
Greening use	Tree traits (B2–C)	5	5.2788	0.0622
Potted-plant use	Criteria layer (A–B)	2	2.0000	0.0000

Potted-plant use	Flower traits (B1–C)	5	5.3958	0.0884
Potted-plant use	Tree traits (B2–C)	5	5.2601	0.0581
Dual flower-fruit use	Criteria layer (A–B)	3	3.0092	0.0089
Dual flower-fruit use	Flower traits (B1–C)	5	5.3415	0.0762
Dual flower-fruit use	Fruit traits (B2–C)	5	5.1365	0.0305
Dual flower-fruit use	Tree traits (B3–C)	6	6.4364	0.0693

n, matrix order; λmax, maximum eigenvalue; CR, consistency ratio (CR = CI/RI, CI = (λmax − n)/(n − 1)); CR < 0.10 indicates acceptable consistency [5]. Full pairwise judgment matrices are provided in Supplementary Tables S5–S7.

Table 4. Relative weights and ranks of ten shared evaluation indicators for greening-use and potted-plant-use hierarchies of Prunus mume.

Indicator	Greening weight	Rank	Potted weight	Rank
Growth potential	0.2397	1	0.0183	10
Decor (ornamental form)	0.1752	2	0.3453	1
Resistance	0.1650	3	0.0194	9
Flowering period	0.1059	4	0.0817	5
Tree posture	0.0977	5	0.0282	8
Number of petals	0.0783	6	0.1409	3
Flower density	0.0584	7	0.1024	4
Flower diameter	0.0371	8	0.1538	2
Flower fragrance	0.0302	9	0.0728	6
Calyx colour	0.0125	10	0.0372	7

Weights derived from consistent judgment matrices (Table 3); each column sums to 1.0.

Table 5. Relative weights and ranks of sixteen evaluation indicators for the dual flower-and-fruit-use hierarchy of Prunus mume.

Indicator	Weight	Rank
Yield per plant	0.3120	1
Flowering period	0.1134	2
Single fruit weight	0.0914	3
Decor (ornamental form)	0.0778	4
Flower density	0.0714	5
Growth potential	0.0677	6
Edible rate	0.0585	7
Flower diameter	0.0473	8
Resistance	0.0390	9
Tree posture	0.0292	10
Number of petals	0.0285	11
Total sugar	0.0144	12
Vitamin C content	0.0144	13
Total acid	0.0137	14

Flower fragrance	0.0135	15
Calyx colour	0.0078	16

Weights sum to 1.0.

Table 6. Top-ranked Prunus mume cultivars for greening (landscape) use.

Rank	Cultivar	Comprehensive score
1	'Fenxia' (粉霞)	15.81
2	'Nanjinghong' (南京红)	15.62
3	'Sanlun Zhusha' (三轮朱砂)	15.62
4	'Qiaozhuang Yudie' (乔妆玉蝶)	15.52
5	'Dalun Lve' (大轮绿萼)	15.39
6	'Qingzhi Yudie' (青芝玉蝶)	15.20
7	'Beijing Yudie' (北京玉蝶)	14.89
8	'Sanlun Yudie' (三轮玉蝶)	14.85
9	'Fenghou' (丰后)	14.83
10	'Nanjing Hongxu' (南京红须)	14.81

Top 10 of 50 accessions evaluated for this use category; full ranking in Supplementary Table.

Table 7. Top-ranked Prunus mume cultivars for potted-plant use.

Rank	Cultivar	Comprehensive score
1	'Yan Zhusha' (艳朱砂)	17.31
2	'Chongban Dahong' (重瓣大红)	15.72
3	'Qianban Zhusha' (千瓣朱砂)	15.64
4	'Tieguhong' (铁骨红)	15.52
5	'Suya Lve' (素雅绿萼)	15.03
6	'Kunming Xiao Tiaozhi' (昆明小跳枝)	14.98
7	'Guli Hong' (骨里红)	14.96
8	'Fufen' (傅粉)	14.85
9	'Fenxia' (粉霞)	14.73
10	'Sanlun Yudie' (三轮玉蝶)	14.65

Top 10 of 50 accessions evaluated for this use category; full ranking in Supplementary Table.

Table 8. Top-ranked Prunus mume cultivars for dual flower-and-fruit use.

Rank	Cultivar	Comprehensive score
1	'Lijiang Zhaoshui Mei' (丽江照水梅)	18.91
2	'Yulong Feixue' (玉龙绯雪)	18.69
3	'Yulong Hongfei' (玉龙红翡)	17.96
4	'Dan Fenghou' (淡丰后)	17.92
5	'Xiaohong Zhusha' (小红朱砂)	17.88

6	'Danbi Chuizhi' (单碧垂枝)	17.62
7	'Yunnan Hongmei' (云南红梅)	17.25
8	'Mi Danlv' (米单绿)	17.12
9	'Da Yu' (大羽)	16.88
10	'Pin Zi Mei' (品字梅)	16.80

Top 10 of 46 accessions evaluated for this use category; full ranking in Supplementary Table.

Table 9. Key morphological and phenological traits of the five top-ranked cultivars selected for each specific-use category.

Use	Cultivar	Petals	Diam. (cm)	Flower colour	Fragrance	Notable feature
Greening	'Fenxia'	15–19	NR	Light reddish-purple (front)	Fragrant	Good growth, strong resistance
Greening	'Nanjinghong'	21–28	2.1–2.8	Light–strong violet	NR	Extremely dense flowering
Greening	'Sanlun Zhusha'	11–20	2.8–3.2	Pale–strong violet	Light	Long flowering period
Greening	'Qiaozhuang Yudie'	12–17	2.5–3.1	White	NR	Excellent growth, strong resistance
Greening	'Dalun Lve'	22–26	2.2–2.7	White, green calyx	NR	Distinct green calyx
Potted	'Yan Zhusha'	Round, numerous	NR	Dark red (both sides)	Typical plum	Compact; excellent ornamental form
Potted	'Chongban Dahong'	23	2.2–3.0	Light purplish-red	NR	Well-developed pistils (3–4)
Potted	'Qianban Zhusha'	41–51	2.5	Thick pansy-purple	NR	Multi-petaled ("thousand-petal")
Potted	'Tieguhong'	3 layers	2.1–2.6	Pale–intense purple	NR	Saucer-type flower
Potted	'Suya Lve'	Round	NR	White, emerald calyx	Typical plum	Distinct emerald-green calyx
Dual-use	'Lijiang Zhaoshui Mei'	5–6	1.6–2.2	Very light reddish-purple	Fragrant	Earliest flowering (mid-Jan–early Feb)
Dual-use	'Yulong Feixue'	5 (single)	2.2–2.7	White, purple-red tip	NR	Open tree habit; reliable fruiting
Dual-use	'Yulong Hongfei'	5 (single)	2.3–2.9	White	NR	Extremely dense flowering (1–4/node)
Dual-use	'Dan Fenghou'	5–6	2.3–2.5	Light–intense purple	Unscented	Bears fruit readily
Dual-use	'Xiaohong Zhusha'	14–21	2.3–2.8	Very light–pansy purple	Sweet	Latest flowering (March)

NR, not explicitly reported/measured in the source dataset for this trait; Diam., flower diameter.




greening




potted-plant

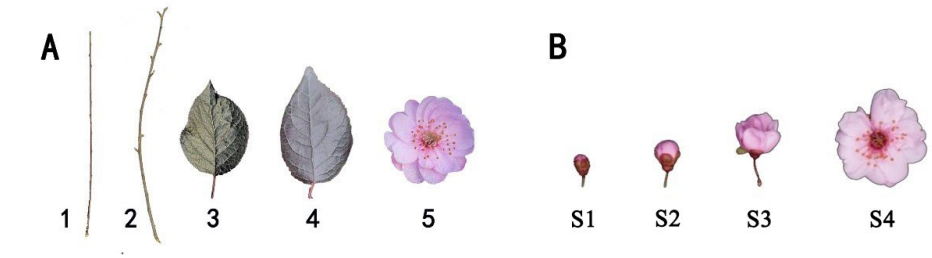



dual flower-and-fruit use

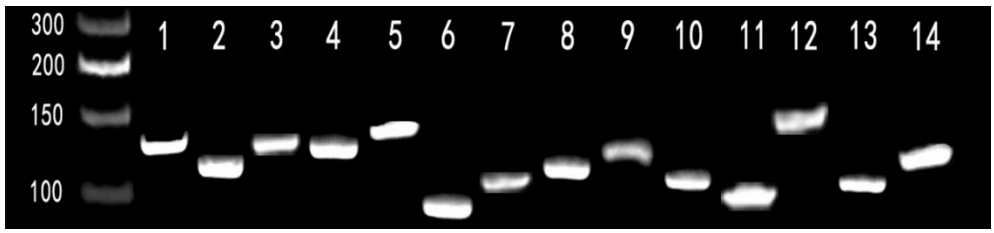
Figure 1. Representative field photographs of superior Prunus mume cultivars selected for greening, potted-plant, and dual flower-and-fruit use, with corresponding whole-tree habit.



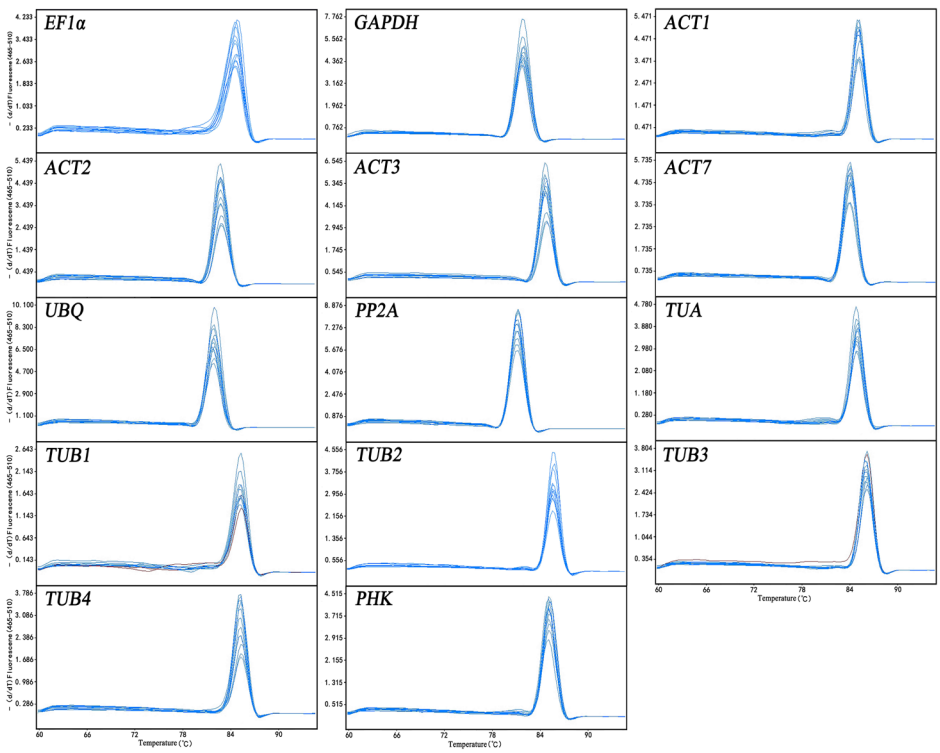
a



b



c



d

Figure 2. (A) The six P. mume cultivars and (B) the different tissues and flower-opening stages of 'Meiren' used for reference gene screening; (C) amplification products of the 14 candidate reference genes resolved on a 2% agarose gel; (D) qRT-PCR melting curves confirming single, specific amplicons for each candidate gene.

Table 10. Amplification efficiency (E) and linear correlation coefficient (R^2) of the 14 candidate reference genes across the four sample sets.

Gene	Leaf (cultivars) E% / R^2	Petal (cultivars) E% / R^2	'Meiren' organs E% / R^2	'Meiren' flowering E% / R^2
EF1α	97.70 / 0.9974	104.5 / 0.9934	95.42 / 0.9921	103.49 / 0.9860
GAPDH	98.75 / 0.9908	93.44 / 0.9957	98.75 / 0.9908	104.41 / 0.9892
ACT1	104.16 / 0.9914	100.72 / 0.9970	96.72 / 0.9915	96.82 / 0.9914
ACT2	103.79 / 0.9895	92.41 / 0.9947	96.23 / 0.9916	98.47 / 0.9909
ACT3	104.54 / 0.9891	98.12 / 0.9973	95.68 / 0.9919	95.15 / 0.9922
ACT7	98.79 / 0.9909	95.94 / 0.9947	95.64 / 0.9920	95.64 / 0.9921
UBQ	101.81 / 0.9897	93.93 / 0.9980	98.79 / 0.9907	95.11 / 0.9922
PP2A	105.11 / 0.9894	94.67 / 0.9990	97.93 / 0.9911	105.06 / 0.9894
TUA	103.94 / 0.9895	94.82 / 0.9990	99.11 / 0.9906	106.13 / 0.9891
TUB1	95.66 / 0.9924	93.58 / 0.9977	101.72 / 0.9904	95.02 / 0.9925
TUB2	97.75 / 0.9921	93.82 / 0.9983	102.81 / 0.9902	98.21 / 0.9910
TUB3	100.62 / 0.9906	96.52 / 0.9918	95.91 / 0.9914	105.11 / 0.9894
TUB4	105.14 / 0.9897	102.5 / 0.9899	101.92 / 0.9897	103.01 / 0.9895
PHK	99.21 / 0.9906	98.76 / 0.9912	102.24 / 0.9902	96.31 / 0.9916

E, amplification efficiency; R^2, linear correlation coefficient of the standard curve.

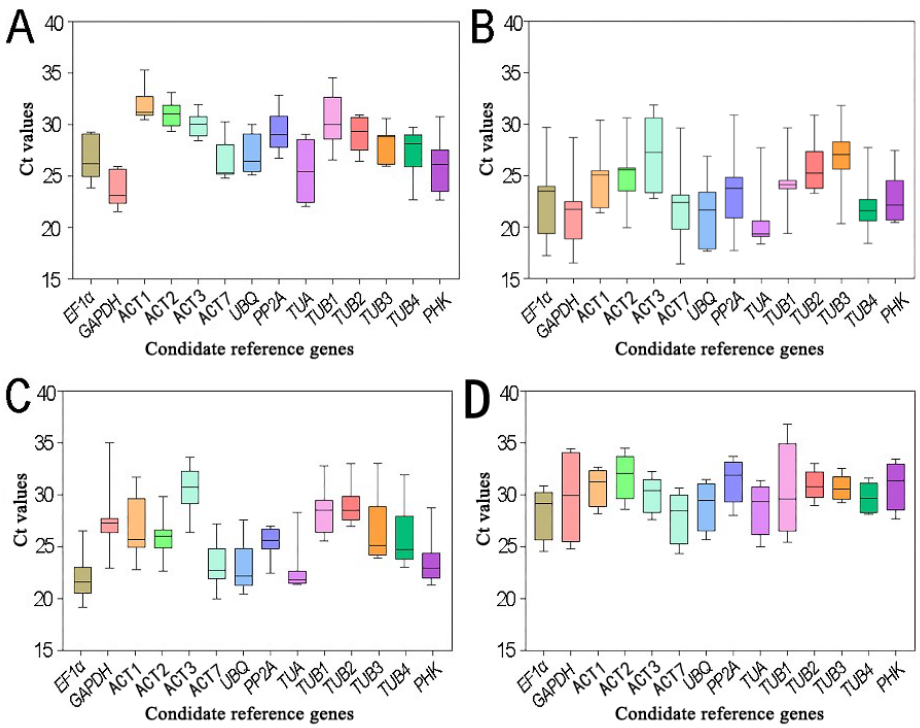


Figure 3. Box plots of quantification cycle (Ct) values for the 14 candidate reference genes in (A) leaf tissue and (B) petal tissue of six P. mume cultivars, and in (C) different organs and (D) different flower-opening stages of 'Meiren'.

Table 11. Expression-stability ranking of 14 candidate reference genes in leaf and petal tissue of six Prunus mume cultivars, by NormFinder, BestKeeper, the ΔCt method, and the

RefFinder consensus.

Gene	NormFinder M (rank)	BestKeeper M (rank)	ΔCt M (rank)	RefFinder rank
EF1α	0.930 (7)	0.648 (11)	0.95 (7)	8
GAPDH	0.374 (1)	0.312 (5)	0.78 (1)	1
ACT1	1.174 (12)	0.298 (3)	1.29 (12)	9
ACT2	1.081 (11)	0.121 (1)	1.12 (10)	7
ACT3	0.572 (5)	0.249 (2)	0.92 (5)	4
ACT7	1.305 (13)	0.397 (8)	1.58 (13)	13
UBQ	0.560 (4)	0.389 (7)	0.85 (2)	2
PP2A	0.686 (6)	0.456 (9)	0.91 (4)	6
TUA	1.005 (8)	0.869 (13)	0.98 (8)	10
TUB1	2.383 (14)	0.918 (14)	1.82 (14)	14
TUB2	0.439 (2)	0.356 (6)	0.89 (3)	3
TUB3	0.515 (3)	0.301 (4)	0.94 (6)	5
TUB4	1.080 (10)	0.555 (10)	1.23 (11)	12
PHK	1.065 (9)	0.657 (12)	0.99 (9)	11

M, stability value from each algorithm (lower = more stable); rank 1 = most stable. geNorm results (pairwise variation, M) are reported in the text and Figure 4; RefFinder integrates rankings from geNorm, NormFinder, BestKeeper and ΔCt.

(a) Leaf tissue

Table 11 (cont.). (b) Petal tissue — expression-stability ranking of the same 14 candidate reference genes.

Gene	NormFinder M (rank)	BestKeeper M (rank)	ΔCt M (rank)	RefFinder rank
EF1α	1.683 (10)	0.615 (13)	1.77 (10)	11
GAPDH	0.485 (2)	0.464 (10)	0.54 (1)	1
ACT1	0.305 (1)	0.374 (5)	0.63 (2)	2
ACT2	0.597 (4)	0.395 (6)	0.93 (4)	3
ACT3	2.430 (14)	0.689 (14)	2.34 (14)	14
ACT7	1.174 (7)	0.575 (11)	1.18 (6)	5
UBQ	0.546 (3)	0.454 (9)	0.86 (3)	4
PP2A	1.182 (8)	0.585 (12)	1.04 (5)	6
TUA	0.674 (6)	0.304 (3)	1.56 (8)	8
TUB1	2.032 (12)	0.402 (7)	1.89 (12)	13
TUB2	1.318 (9)	0.283 (1)	1.72 (9)	7
TUB3	0.621 (5)	0.369 (4)	1.32 (7)	9
TUB4	1.935 (11)	0.439 (8)	1.79 (11)	12
PHK	2.082 (13)	0.295 (2)	2.09 (13)	10

M, stability value from each algorithm (lower = more stable); rank 1 = most stable. geNorm results (pairwise variation, M) are reported in the text and Figure 4; RefFinder integrates rankings from geNorm, NormFinder, BestKeeper and ΔCt.

Table 12. Expression-stability ranking of 14 candidate reference genes across different organs and different flower-opening stages of Prunus mume 'Meiren', by NormFinder, BestKeeper, the ΔCt method, and the RefFinder consensus.

Gene	NormFinder M (rank)	BestKeeper M (rank)	ΔCt M (rank)	RefFinder rank
EF1α	0.596 (6)	0.341 (6)	1.01 (1)	4
GAPDH	3.581 (14)	0.951 (14)	1.76 (11)	14
ACT1	0.862 (7)	0.375 (8)	1.64 (9)	8
ACT2	1.042 (8)	0.361 (7)	1.51 (7)	7
ACT3	0.391 (2)	0.286 (4)	2.21 (14)	5
ACT7	0.135 (1)	0.173 (1)	1.15 (3)	1
UBQ	0.417 (3)	0.192 (2)	1.34 (4)	2
PP2A	0.571 (5)	0.312 (5)	1.35 (6)	6
TUA	1.589 (12)	0.679 (12)	1.12 (2)	13
TUB1	2.407 (13)	0.754 (13)	1.79 (12)	12
TUB2	0.445 (4)	0.215 (3)	1.93 (13)	3
TUB3	1.325 (11)	0.541 (10)	1.65 (10)	11
TUB4	1.147 (9)	0.391 (9)	1.58 (8)	9
PHK	1.266 (10)	0.597 (11)	1.35 (5)	10

M, stability value from each algorithm (lower = more stable); rank 1 = most stable. geNorm results (pairwise variation, M) are reported in the text and Figure 4; RefFinder integrates rankings from geNorm, NormFinder, BestKeeper and ΔCt.

(a) Different organs of 'Meiren'

Table 12 (cont.). (b) Different flower-opening stages of 'Meiren' — expression-stability ranking of the same 14 candidate reference genes.

Gene	NormFinder M (rank)	BestKeeper M (rank)	ΔCt M (rank)	RefFinder rank
EF1α	1.247 (10)	0.527 (10)	1.78 (5)	5
GAPDH	3.866 (14)	0.996 (14)	3.83 (14)	14
ACT1	0.389 (1)	0.313 (5)	1.16 (2)	2
ACT2	0.700 (3)	0.361 (7)	1.36 (3)	3
ACT3	2.044 (12)	0.278 (3)	2.34 (12)	12
ACT7	0.980 (5)	0.756 (12)	1.85 (7)	11
UBQ	1.218 (7)	0.372 (8)	1.46 (4)	6
PP2A	0.588 (2)	0.357 (6)	1.03 (1)	1
TUA	1.277 (11)	0.389 (9)	2.21 (9)	7
TUB1	3.324 (13)	0.789 (13)	2.54 (13)	13
TUB2	0.900 (4)	0.251 (2)	1.82 (6)	4
TUB3	1.235 (9)	0.218 (1)	2.31 (11)	8
TUB4	1.115 (6)	0.295 (4)	2.28 (10)	9
PHK	1.222 (8)	0.639 (11)	2.08 (8)	10

M, stability value from each algorithm (lower = more stable); rank 1 = most stable. geNorm results (pairwise

variation, M) are reported in the text and Figure 4; RefFinder integrates rankings from geNorm, NormFinder, BestKeeper and ΔCt.

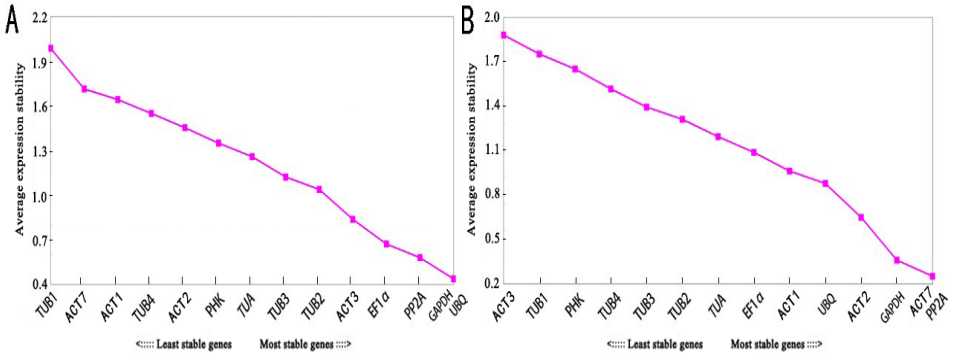


Figure 4. geNorm average expression-stability (M) ranking of the 14 candidate reference genes in (A) leaf tissue and (B) petal tissue of six P. mume cultivars, ordered from least to most stable.

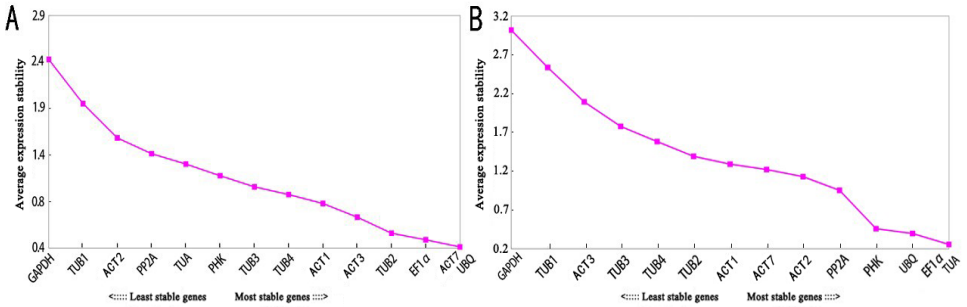


Figure 5. geNorm average expression-stability (M) ranking of the 14 candidate reference genes across (A) different organs and (B) different flower-opening stages of 'Meiren', ordered from least to most stable.

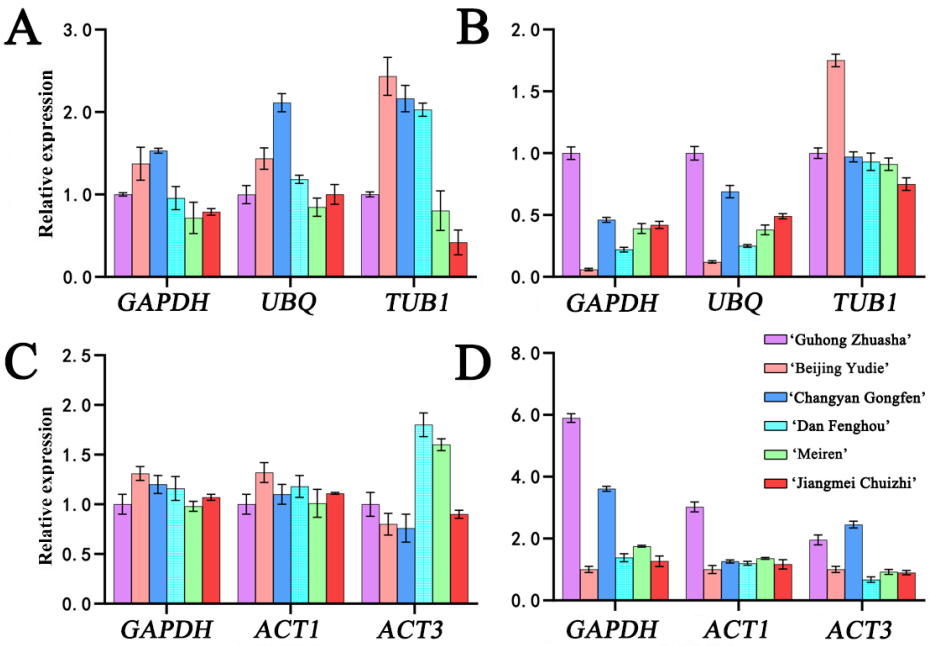


Figure 6. Relative expression of COX10 and ANR in (A, B) leaf tissue and (C, D) petal tissue of six P. mume cultivars, normalized to the most stable (GAPDH, UBQ or ACT1) versus the least stable (TUB1 or ACT3) reference genes identified for each tissue.

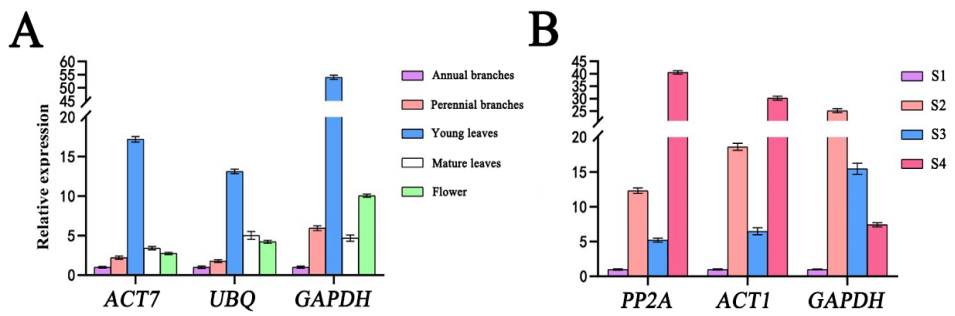


Figure 7. Relative expression of (A) CESA1 across different organs and (B) Exp-A20 across different flower-opening stages of 'Meiren', normalized to the most stable (ACT7/UBQ or PP2A/ACT1) versus the least stable (GAPDH) reference genes.

4. Discussion

The present investigation brought together a multi-attribute decision-support framework for germplasm appraisal with a methodical reference-gene validation, tackling two operational hurdles in P. mume science and breeding: the absence of standardized, end-use-oriented selection benchmarks for a large and varied germplasm pool, and the shortage of empirically validated internal calibrators for transcript-level studies that must span the species' considerable tissue and varietal breadth.

4.1. AHP as a discriminant tool for specific-use cultivar screening

The fact that every judgment matrix passed the CR < 0.1 threshold (Table 3) lends credence to the internal consistency of the three hierarchies, and the pronounced reshuffling of indicator weights across them—growth potential and hardiness carrying the load for landscape applications, decorative form and bloom dimensions dominating the potted-plant model, and fruit load and bloom period heading the dual-purpose model—makes clear that the AHP weighting framework captured genuinely separate horticultural objectives and not simply a single broad "ornamental value" dimension. This outcome echoes recent AHP-driven assessments in other woody and herbaceous ornamentals, where bloom-associated criteria likewise rose to the top for landscape and container settings ([6], in Rhododendron; [7], in Hemerocallis), and it strengthens the wider argument for AHP as a clear, statistically verifiable substitute for purely subjective variety selection within ornamental improvement programmes. That said, AHP-derived weights rest on expert pairwise judgments and are therefore sensitive both to the makeup of the judging panel and to the initial list of traits; testing the present weighting scheme against end-user or grower preference data—an approach recently employed for cut peony varieties through combined AHP and K-means clustering—would represent a worthwhile follow-up.

The fifteen standout varieties pinpointed here (Table 9) span a practically useful range of bloom windows (mid-January through March), petal colours, and growth architectures. Such breadth should aid nursery operators in aligning germplasm with site conditions and market needs, and in broadening the relatively narrow cultivar palette that currently holds sway in commercial nurseries [4]. Because the foundational genome assembly [2,8] and associated QTL/candidate-gene inventories for decorative traits in P. mume continue to grow rapidly, marrying AHP-based phenotypic evaluation with marker-assisted selection at the loci currently being resolved for bloom colour, scent, and plant form [8] would be a logical next step toward expediting the breeding of purpose-built mei cultivars.

4.2. Tissue- and stage-specific reference genes in Prunus mume

The disappointing performance of GAPDH as a normalizer in the organ-type and flowering-stage comparisons of 'Meiren'—set against its solid results in the varietal leaf and petal comparisons—serves as a pointed reminder of a recurrent theme in the reference-gene literature: abundant, steady transcript levels in one comparison (say, across genotypes at a fixed developmental point) by no means guarantee similar stability along a different axis of biological variability (e.g., across organs or developmental time) within the same organism [14]. It is precisely this context-dependent behaviour that prompted the MIQE guidelines for qRT-PCR [13] to insist that reference genes be validated for the actual tissues, growth stages, and genotypes under examination, rather than settling for a single putatively "universal"

housekeeping gene borrowed from earlier publications. The present data, along with the divergent target-gene patterns that emerged when GAPDH, ACT3, or TUB1 served as normalizers in lieu of the top-ranked choices (Figures 6–7), drive this caution home for P. mume specifically.

The imperfect agreement among the four base algorithms—most conspicuous in petal tissue, where BestKeeper leaned toward TUB2/PHK while NormFinder and ΔCt backed ACT1/GAPDH—parallels patterns documented in other Prunus and horticultural species ([24], in peach; [14]) and stems from each method's differential emphasis on Cq dispersion versus pairwise or within-/between-group variance components. This divergence is exactly the reason we leaned on the RefFinder synthesis, rather than any single algorithm, for arriving at our final recommendations, in keeping with what is now regarded as best practice [14].

4.3. Limitations and future directions

Several limitations should be acknowledged. The AHP assessment drew on a single germplasm repository and, for fruit-composition traits, a lone fruiting season; conducting trials across multiple years and locations would reinforce confidence in the absolute rankings, although the weighting scheme itself—being derived from expert pairwise comparisons rather than from the phenotypic measurements—should be less vulnerable to year-to-year environmental fluctuation. The reference-gene evaluation concentrated on vegetative and floral material grown under standard conditions; widening the candidate panel to encompass additional abiotic and biotic stress scenarios, along the lines of earlier cold-stress work in P. mume and P. sibirica [17], would extend the panel's applicability to stress-responsive gene-expression experiments. Finally, although RefFinder pools the output of four well-established algorithms, newer machine-learning-based stability estimators may yield added refinement as the volume of P. mume transcriptomic data continues to grow [8].

5. Conclusions

A three-tier, AHP-grounded evaluation framework was constructed and empirically tested for the purpose of screening Prunus mume germplasm toward landscape, container, and combined flower-and-fruit applications; all constituent judgment matrices satisfied conventional consistency benchmarks, and deploying the framework across an 80-accession collection brought to light five elite varieties for each usage category. In a parallel effort, a methodical stability assessment of 14 candidate reference genes spanning leaf and petal samples from six varieties, plus organ types and bloom phases of 'Meiren', pinpointed GAPDH/UBQ, GAPDH/ACT1, ACT7/UBQ, and PP2A/ACT1 as the optimal normalizers for the respective comparisons, and showed that substituting a poorly performing calibrator can tangibly skew the apparent expression profiles of target genes. Taken together, the

tools developed here offer a practical foundation for the horticultural advancement of elite P. mume germplasm and for the rigorous molecular dissection of the genes that will inform its future improvement.

Author Contributions

Conceptualization, B.D.; methodology, H.L.I. and B.D.; investigation, H.L.I.; formal analysis, H.L.I.; writing—original draft preparation, H.L.I.; writing—review and editing, H.L.I. and B.D.; supervision, B.D.; funding acquisition, B.D. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest

The authors declare no conflict of interest.

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Supplementary Materials

Table S1: Investigation methods and replicate numbers for all 16 evaluation indicators. Table S2: Full comprehensive-evaluation ranking of all 50 accessions assessed for greening use. Table S3: Full ranking of all 50 accessions assessed for potted-plant use. Table S4: Full ranking of all 46 accessions assessed for dual flower-and-fruit use. Tables S5–S7: Complete pairwise AHP judgment matrices for the greening, potted-plant, and dual-use hierarchies, respectively.