Universal Multiplexable matK Primers for DNA Barcoding of Angiosperms

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Primer Note

Universal multiplexable *matK* primers for DNA barcoding of angiosperms[1]

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- *Premise of the study:* PCR amplification of the *matK* barcoding region is often difficult when dealing with multiple angiosperm families. We developed a primer cocktail to amplify this region efficiently across angiosperm diversity.
- *Methods and Results:* We developed 14 *matK* primers (seven forward, seven reverse) for multiplex PCR, using sequences available in GenBank for 178 taxa belonging to 123 genera in 41 families and 18 orders. Universality of these new multiplexed primers was tested with 53 specimens from 44 representative angiosperm families in 23 different orders. Our primers showed high PCR amplification and sequencing success.
- *Conclusions:* These results show that our newly developed primers are highly effective for multiplex PCR and can be employed in future barcode projects involving taxonomically diverse samples across angiosperms. Using multiplex primers for barcoding will reduce the cost and time needed for PCR amplification.

Key words: degenerate primers; DNA barcoding; *matK*; multiplex PCR.

The rapidly evolving and highly variable gene maturase K (*matK*; Hilu and Liang, 1997) has been recommended as a locus for DNA barcoding by the Consortium for the Barcode of Life (CBOL) Plant Working Group (Hollingsworth et al., 2009). Amplification and sequencing of the *matK* barcoding region is difficult due to high sequence variability in the primer binding sites (Hollingsworth et al., 2011). Currently, there are three popular *matK* primer pairs available to amplify approximately the same region of the gene: 390F and 1326R (Sun et al., 2001; Cuénoud et al., 2002), XF and 5R (Ford et al., 2009), and 1R_KIM and 3F_KIM (Hollingsworth et al., 2009; Jeanson et al., 2011). Kress et al. (2009) used these three primer pairs to amplify DNA barcodes from 296 shrub and tree species. These primer combinations showed amplification success in 85% and sequencing success in 69% of the species, proving that reliable amplification is possible across a range of plants, using several primer combinations. However, using more than one primer pair can be time consuming as well as costly and is often complex for large-scale projects (e.g., Heckenhauer et al., unpublished data).

Here, we report a set of universal primers that can be multiplexed in one PCR to amplify *matK* successfully in angiosperms and expedite high-throughput, rapid, automated, and cost-effective species identification. We present methods that enable efficient PCR amplification and sequencing of the *matK* barcode region.

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METHODS AND RESULTS

Sequences of the *matK* gene from 178 taxa belonging to 123 genera and 41 families were obtained from GenBank (www.ncbi.nlm.nih.gov/genbank; Appendix S1) and aligned using the MAFFT plugin (Katoh and Standley, 2013) in Geneious (version 8.0.5; Kearse et al., 2012). Because primers were initially developed for a barcoding project dealing primarily with the tree flora of Southeast Asia, *matK* sequences of the most representative genera and families of dicots and monocots were used. The target DNA region was located between positions 383 and 1343 of the *matK* gene (with respect to *Arabidopsis thaliana* (L.) Heynh.) and includes the binding sites of the three commonly used *matK* primer pairs. Primers were designed at the most conserved regions, resulting in a fragment between positions 383 and 1256 (positions 414–1226, excluding the primer sequences). Forward primers are at a similar position to the 390F and XF primers, whereas the reverse primers are located downstream from the above-cited reverse primers to avoid a region of up to 11 adenine bases (e.g., *Sterculia tragacantha* Lindl. AY321178, positions 1257–1267), which could cause PCR and sequencing problems. To minimize primer degeneracy, aligned sequences were clustered into seven groups according to their genetic similarity in the MAFFT alignment, in which sequences are sorted according to their pairwise distances. Thus, for each cluster, primers with no more than five degenerate nucleotide positions were developed. Primers were developed manually considering primer properties (annealing temperature, 3′ and 5′ end stability) and primer secondary structures (cross dimers, dimers, hairpins) with the use of NetPrimer (PREMIER Biosoft International, Palo Alto, California, USA; www.premierbiosoft.com/netprimer/netprlaunch/netprlaunch.html). Primers were designed at the same positions in the *matK* gene for the forward and reverse primers so that they could be multiplexed in a single PCR for each sample. Seven forward and seven reverse primers were developed. Because using more primer combinations in a multiplex PCR reduces the probability of the most appropriate primers binding to the target region, only five forward and five reverse primers for the most frequent sequences in our alignment were multiplexed (Table 1: C_MATK_F/C_MATK_R). Primers were mixed in different ratios depending on their level of degeneration (Table 1). The remaining two forward and two reverse primers serve as spares for amplification of taxa that fail amplification using the previous five-primer combination. Primers were compared against the National Center for Biotechnology Information (NCBI) GenBank nucleotide reference database using the Mega BLAST algorithm (blast.ncbi.nlm.nih.gov/Blast.cgi). Table 2 shows BLAST results with no mismatches in forward or reverse primers at the family level. Thus, in studies where the species are identified to family level, primers can be combined accordingly in a multiplex PCR. To evaluate the universality of the primers, multiplex PCR was conducted on DNA of 54 species from 48 families, representing frequently occurring trees and palms (e.g., Arecaceae, Dipterocarpaceae, Euphorbiaceae) in

Table 1. Primers developed for multiplex PCR used to amplify the *matK* barcoding region. The forward (C_MATK_F) and reverse (C_MATK_R) primer cocktail as well as the four additional primers are given with their proportions in the primer cocktail.

Cocktail name/Primer name (Direction)	Proportion in primer cocktail	Primer sequence (5′–3′)[a]	Primer position[b]
C_MATK_F			383–413
matK-413f-1 (Forward)	2	TAATTTAC**R**ATCAATTCATTCAATATTTCC	
matK-413f-2 (Forward)	2	TAATTTACGATC**Y**ATTCATTCAATATTTCC	
matK-413f-3 (Forward)	1	TAATTTACGATCAATTCATTCAACATTTCC	
matK-413f-4 (Forward)	2	TAATTT**M**C**R**ATCAATTCATTCCATATTTCC	
matK-413f-5 (Forward)	1	TAATTTACGATCAATTCATTCACTATTTCC	
C_MATK_R			1227–1256
matK-1227r-1 (Reverse)	3	GA**R**GA**Y**CC**R**CT**R**T**R**ATAATGAGAAAGATTT	
matK-1227r-2 (Reverse)	1	GAAGA**Y**CCGCTATGATAATGAGAAAGGTTT	
matK-1227r-3 (Reverse)	2	GA**R**GATCC**R**CT**R**T**R**ATAATGAAAAAGATTT	
matK-1227r-4 (Reverse)	2	GA**R**GATCC**R**CT**R**T**R**ATAATGAGAAAAATTT	
matK-1227r-5 (Reverse)	2	GA**R**GATCC**R**CT**R**T**R**ATAATGAGAAATATTT	
Additional primers			
matK-413f-6 (Forward)	2	TAATTTACGATC**W**ATTCATTC**M**ATTTTTCC	383–413
matK-413f-7 (Forward)	1	TAATTTACAATC**M**ATTCATTCAATATTTTC	383–413
matK-1227r-6 (Reverse)	2	GA**R**GATCCGCT**R**TAATAATGCGAAAGATTT	1227–1256
matK-1227r-7 (Reverse)	2	GA**R**GATCCGCTAT**R**ATAATGATAAATATTT	1227–1256

[a] Ambiguous bases are set in boldface.
[b] Primer position is given for *Arabidopsis thaliana* (GenBank accession no. AF144378.1).

Southeast Asia (Table 3), along with other taxa from other parts of the world to improve the coverage of angiosperms (e.g., *Leontodon* [Asteraceae], *Tillandsia* [Bromeliaceae], *Helianthemum* [Cistaceae], *Polystachya* [Orchidaceae]). Approximately 30 mg of silica gel–dried material (bark or leaves) was transferred into a 96-well plate, and genomic DNA was extracted using the DNeasy 96 Plant Kit (QIAGEN, Hilden, Germany). PCRs included 5 μL of 2× ReddyMix PCR Master Mix with 1.5 mM $MgCl_2$ (#AB-0575/DC/LD/A; Thermo Fisher Scientific, Waltham, Massachusetts, USA), 0.1 μL of forward and reverse primer cocktail each at 50 μM (final concentration 0.5 μM), 1 μL of template DNA, and H_2O up to a final volume of 10 μL. Thermocycler conditions were as follows: 95°C for 2 min; five cycles of 95°C for 25 s, 46°C for 35 s, and 70°C for 1 min; 35 cycles of 95°C for 25 s, 48°C for 35 s, and 70°C for 1 min; and a final extension at 72°C for 5 min. For samples that did not amplify using the above-mentioned protocol, the 2× Phusion Green HS II Hi-Fi PCR Master Mix with 1.5 mM $MgCl_2$ (#F-566S, Thermo Fisher Scientific) was used with the following thermocycler conditions: 98°C for 30 s; five cycles of 98°C for 10 s, 53°C for 30 s, and 72°C for 30 s; 35 cycles of 98°C for 10 s, 55°C for 30 s, and 72°C for 30 s; and a final extension at 72°C for 5 min. PCR products were visualized on a 1.5% TAE agarose gel using ethidium bromide staining. After cleaning the PCR products with 1 μL exonuclease I and FastAP thermosensitive alkaline phosphatase mixture (7 units Exo I, 0.7 units FastAP; Thermo Fisher Scientific) at 37°C for 45 min and 85°C for 15 min, barcodes were Sanger sequenced with the BigDye Terminator Kit version 3.1 (Thermo Fisher Scientific) according to the manufacturer's instructions. Sequencing was carried out using an ABI 3730xL DNA Analyzer (Applied Biosystems, Foster City, California, USA) at the Department of Botany and Biodiversity Research, University of Vienna. Bidirectional sequences were assembled in Geneious and edited.

Using 2× ReddyMix PCR Master Mix, all samples could be amplified except for one sample with low-quality DNA (Fig. 1, slot 30). This sample was successfully amplified in a PCR with 2× Phusion Green HS II Hi-Fi PCR Master Mix (Fig. 1, slot 31). Overall, the newly designed degenerate primer cocktails were very effective (100%) in amplifying the target *matK* region, with a product of 813 bp in length in *Arabidopsis thaliana*. By multiplexing the primers in a single PCR, barcodes were recovered from all samples.

CONCLUSIONS

We developed 14 universal, partly degenerate primers suitable for DNA barcoding of angiosperms that may also be suitable for multiplexed amplicon sequencing approaches on next-generation sequencing platforms (e.g., fusion primers on the Illumina system, see Elbrecht and Leese, 2015). We confirmed the effectiveness of our multiplexed primers on 53 species from 44 different plant families. Amplification success for these multiplexed primers in the cross-transferability tests with plant families outside Southeast Asia extends their potential usefulness, especially for large-scale barcoding projects with a diverse composition of plant families. Furthermore, by improving the routine amplification of the *matK* barcode, the establishment of our multiplex PCR approach will reduce laboratory costs as well as potential laboratory errors.

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Table 2. Recommended use of primers for different families, based on BLAST matches with no mismatches.[a]

Order	Family	Appropriate forward primer	Appropriate reverse primer
Alismanthales	Alismataceae	matK-413f-2	matK-1227r-1, matK-1227r-3
	Araceae	matK-413f-2, matK-413f-5	matK-1227r-1
Apiales	Araliaceae	matK-413f-2, matK-413f-5	matK-1227r-1, matK-1227r-4
	Apiaceae	matK-413f-7	matK-1227r-1, matK-1227r-5
Aquifoliales	Aquifoliaceae	matK-413f-1	matK-1227r-1, matK-1227r-3
	Cardiopteridaceae (*Gonocaryum minus*)	matK-413f-1	matK-1227r-1, matK-1227r-3
	Stemonuraceae	matK-413f-1	matK-1227r-1, matK-1227r-3
Arecales	Arecaceae (Arecaceae sp.)	matK-413f-2	matK-1227r-1, matK-1227r-3
Asparagales	Amaryllidaceae	matK-413f-6	matK-1227r-1, matK-1227r-3
	Asparagaceae	matK-413f-6	matK-1227r-1, matK-1227r-4, matK-1227r-5
	Hyacinthaceae	matK-413f-6	matK-1227r-1, matK-1227r-3
	Iridaceae	matK-413f-6	matK-1227r-1, matK-1227r-3, matK-1227r-5
	Orchidaceae (*Polystachya humbertii*)	matK-413f-1, matK-413f-2, matK-413f-3, matK-413f-6	matK-1227r-1, matK-1227r-2, matK-1227r-3
	Tecophilaeaceae	matK-413f-6	matK-1227r-1
	Xanthorrhoeaceae	matK-413f-6	matK-1227r-1, matK-1227r-5
Asterales	Asteraceae (*Leontodon hispidus*)	matK-413f-1	matK-1227r-1, matK-1227r-2, matK-1227r-3, matK-1227r-4, matK-1227r-5
	Campanulaceae	matK-413f-2	matK-1227r-1,matK-1227r-5
	Goodeniaceae	matK-413f-4	matK-1227r-1
Austrobaileyales	Austrobaileyaceae	matK-413f-2	matK-1227r-2
	Schisandraceae	matK-413f-2	matK-1227r-2
	Trimeniaceae	matK-413f-2	matK-1227r-2
Berberidopsidales	Berberidopsidaceae	matK-413f-1	matK-1227r-1
Boraginales	Boraginaceae	matK-413f-1, matK-413f-4	matK-1227r-1, matK-1227r-3, matK-1227r-5
	Ehretiaceae	matK-413f-1	matK-1227r-1
Brassicales	Brassicaceae	matK-413f-1, matK-413f-4, matK-413f-6	matK-1227r-1, matK-1227r-5
	Capparaceae	matK-413f-1	matK-1227r-1
	Caricaeae	matK-413f-1	matK-1227r-1
	Cleomaceae	matK-413f-1, matK-413f-3, matK-413f-4, matK-413f-7	matK-1227r-1, matK-1227r-2, matK-1227r-4, matK-1227r-5
	Moringaceae	matK-413f-1	matK-1227r-1, matK-1227r-5
	Resedaceae	matK-413f-1	matK-1227r-1
Bruniales	Brunelliaceae	matK-413f-1	matK-1227r-1
Buxales	Bucaceae	matK-413f-1	matK-1227r-1
Caryophyllales	Amaranthaceae	matK-413f-1	matK-1227r-1
	Cactaceae	matK-413f-1	matK-1227r-1
	Polygonaceae	matK-413f-1	matK-1227r-1, matK-1227r-2, matK-1227r-5
	Simmondsiaceae	matK-413f-1	matK-1227r-3
	Tamaricaceae	matK-413f-1	matK-1227r-1
Celastrales	Celastraceae	matK-413f-1, matK-413f-4, matK-413f-6	matK-1227r-1, matK-1227r-2, matK-1227r-3, matK-1227r-4, matK-1227r-5
	Lepidobotryaceae	matK-413f-1	matK-1227r-5
Chloranthales	Chloranthaceae	matK-413f-2	matK-1227r-1, matK-1227r-5
Commelinales	Commelinaceae	matK-413f-2	matK-1227r-1
	Haemodoraceae	matK-413f-2	matK-1227r-1, matK-1227r-2, matK-1227r-5
Cornales	Cornaceae (*Alangium* cf. *javanicum, Mastixia* sp.)	matK-413f-1, matK-413f-3	matK-1227r-1, matK-1227r-3, matK-1227r-4, matK-1227r-5
	Grubbiaceae	matK-413f-1	matK-1227r-1
	Hydrangeaceae	matK-413f-1	matK-1227r-1, matK-1227r-4
	Loasaceae	matK-413f-1, matK-413f-7	matK-1227r-1, matK-1227r-4
Crossosomatales	Stachyuraceae	matK-413f-1	matK-1227r-1
	Staphyleaceae	matK-413f-1	matK-1227r-1, matK-1227r-5
	Strasburgeriaceae	matK-413f-1	matK-1227r-1
Cucurbitales	Anisophylleaceae (*Anisophyllea* sp.)	matK-413f-1, matK-413f-6	matK-1227r-1
	Begoniaceae	matK-413f-1, matK-413f-6	matK-1227r-1
	Coriariaceae	matK-413f-2	matK-1227r-1
	Cucurbitaceae	matK-413f-2	matK-1227r-1, matK-1227r-3, matK-1227r-4, matK-1227r-5
	Datiscaceae	matK-413f-1	matK-1227r-1
	Tetramelaceae	matK-413f-1	matK-1227r-3, matK-1227r-5
Dipsacales	Adoxaceae	matK-413f-4	matK-1227r-1
	Caprifoliaceae	matK-413f-1, matK-413f-5	matK-1227r-1

Table 2. Continued.

Order	Family	Appropriate forward primer	Appropriate reverse primer
Ericales	Ebenaceae (*Diospyros* sp.)	matK-413f-1	matK-1227r-1, matK-1227r-3, matK-1227r-6
	Ericaceae	matK-413f-1, matK-413f-4	matK-1227r-1, matK-1227r-5
	Lecythidaceae (*Barringtonia curranii*)	matK-413f-5	matK-1227r-1
	Pentaphylacaceae	matK-413f-1	matK-1227r-1
	Primulaceae (*Ardisia* sp.)	matK-413f-1, matK-413f-2	matK-1227r-3, matK-1227r-1, matK-1227r-5, matK-1227r-7
	Styracaceae	matK-413f-1	matK-1227r-1
	Symplocaceae (*Symplocos crassipes*)	matK-413f-1	matK-1227r-1
	Theaceae	matK-413f-1	matK-1227r-4
Escalloniales	Escalloniaceae	matK-413f-1	matK-1227r-1
Fabales	Fabaceae (*Fordia splendidissima*)	matK-413f-1, matK-413f-2, matK-413f-4, matK-413f-6, matK-413f-7	matK-1227r-1, matK-1227r-3, matK-1227r-5
	Polygalaceae (*Xanthophyllum beccarianum*)	matK-413f-1, matK-413f-2	matK-1227r-1
Fagales	Betulaceae	matK-413f-2	matK-1227r-1
	Casuarinaceae	matK-413f-2	matK-1227r-1
	Fagaceae (*Lithocarpus* sp.)	matK-413f-2	matK-1227r-1, matK-1227r-3, matK-1227r-5
	Juglandaceae	matK-413f-1	matK-1227r-1, matK-1227r-6
Garryales	Garryaceae	matK-413f-1	matK-1227r-1, matK-1227r-4, matK-1227r-6
Gentianales	Apocynaceae (*Tabernaemontana* sp.)	matK-413f-1, matK-413f-3, matK-413f-4, matK-413f-5, matK-413f-6	matK-1227r-1, matK-1227r-2, matK-1227r-6
	Loganiaceae	matK-413f-1	matK-1227r-1, matK-1227r-5
	Rubiaceae (*Urophyllum* sp., *Psychotria* sp.)	matK-413f-1, matK-413f-5	matK-1227r-1, matK-1227r-2
Geraniales	Geraniaceae	matK-413f-1, matK-413f-6	matK-1227r-1
	Melianthaceae	matK-413f-1, matK-413f-6	matK-1227r-1
Gunnerales	Gunneraceae	matK-413f-1, matK-413f-2	matK-1227r-1
Huerteales	Dipentodontaceae	matK-413f-1	matK-1227r-1
	Gerrardinaceae	matK-413f-1	matK-1227r-1
	Tapisciaceae	matK-413f-1	matK-1227r-1, matK-1227r-5
Icacinales	Icacinaceae	matK-413f-1	matK-1227r-1, matK-1227r-3
Lamiales	Acanthaceae	matK-413f-1	matK-1227r-1, matK-1227r-2, matK-1227r-4, matK-1227r-5
	Gesneriaceae	matK-413f-1	matK-1227r-1, matK-1227r-2, matK-1227r-5
	Lamiaceae (*Teijsmanniodendron* sp.)	matK-413f-1	matK-1227r-1, matK-1227r-2, matK-1227r-5
	Lentibulariaceae	matK-413f-1	matK-1227r-1
	Myrsinaceae	matK-413f-1	matK-1227r-1
	Oleaceae	matK-413f-1	matK-1227r-1, matK-1227r-2, matK-1227r-3, matK-1227r-4
	Orobanchaceae	matK-413f-1	matK-1227r-1, matK-1227r-3, matK-1227r-4
Laurales	Hernandiaceae	matK-413f-2	matK-1227r-1
	Lauraceae (*Litsea sarawacensis*)	matK-413f-2	matK-1227r-1, matK-1227r-3
	Siparunaceae	matK-413f-2	matK-1227r-3
Liliales	Smilacaceae	matK-413f-2	matK-1227r-1, matK-1227r-5
Magnoliales	Annonaceae	matK-413f-2	matK-1227r-1, matK-1227r-4, matK-1227r-5
	Degeneriaceae	matK-413f-2	matK-1227r-1
	Eupomatiaceae	matK-413f-2	matK-1227r-1
	Himantandraceae	matK-413f-2	matK-1227r-1
	Magnoliaceae (*Magnolia* sp.)	matK-413f-2, matK-413f-6	matK-1227r-1
	Myristicaceae	matK-413f-2, matK-413f-4	matK-1227r-1, matK-1227r-5
Malpighiales	Clusiaceae (*Garcinia* sp.)	matK-413f-1	matK-1227r-1, matK-1227r-3, matK-1227r-4, matK-1227r-5
	Euphorbiaceae (*Antidesma* sp., *Drypetes* sp., *Koilodepas* sp., *Macaranga hosei*, *Mallotus* sp.)	matK-413f-1	matK-1227r-1, matK-1227r-3, matK-1227r-4, matK-1227r-5
	Linaceae	matK-413f-1	matK-1227r-1
	Passifloraceae	matK-413f-1	matK-1227r-1
	Phyllanthaceae	matK-413f-1, matK-413f-2, matK-413f-7	matK-1227r-1
	Putranjivaceae	matK-413f-1	matK-1227r-5
	Rhizophoraceae	matK-413f-5	matK-1227r-1, matK-1227r-3
	Salicaceae	matK-413f-1	matK-1227r-1, matK-1227r-5
	Violaceae (*Rinorea* sp.)	matK-413f-1	matK-1227r-1, matK-1227r-6

Table 2. Continued.

Order	Family	Appropriate forward primer	Appropriate reverse primer
Malvales	Elaeocarpaceae	matK-413f-1	matK-1227r-1
	Malvaceae (*Durio griffithii*, *Leptonychia* sp., *Sterculia* sp.)	matK-413f-1	matK-1227r-1
Myrtales	Lythraceae	matK-413f-1, matK-413f-5	matK-1227r-1, matK-1227r-3
	Melastomataceae	matK-413f-7	matK-1227r-1, matK-1227r-4
	Myrtaceae (*Syzygium* sp.)	matK-413f-1, matL-413f-4, matK-413f-6	matK-1227r-1, matK-1227r-3, matK-1227r-4, matK-1227r-5
	Onagraceae	matK-413f-3	matK-1227r-1
Oxalidales	Brunelliaceae	matK-413f-1	
	Cunoniaceae	matK-413f-1	matK-1227r-1
	Huaceae	matK-413f-6	matK-1227r-1
Pandanales	Cyclanthaceae	matK-413f-2	matK-1227r-1
	Pandanaceae	matK-413f-2	matK-1227r-1
Paracryphiales	Paracryphiaceae	matK-413f-1	matK-1227r-1
Piperales	Aristolochiaceae	matK-413f-2	matK-1227r-1, matK-1227r-5
	Piperaceae	matK-413f-2	matK-1227r-3
	Saururaceae	matK-413f-2	matK-1227r-1
Poales	Bromeliaceae (*Tillandsia* cf. *caloura*)	matK-413f-2, matK-413f-6	matK-1227r-1, matK-1227r-3
	Typhaceae	matK-413f-2	matK-1227r-1, matK-1227r-3
Proteales	Nelumbonaceae	matK-413f-1	matK-1227r-1
	Platanaceae	matK-413f-1	matK-1227r-1
	Proteaceae	matK-413f-1, matK-413f-2, matK-413f-3	matK-1227r-1, matK-1227r-3, matK-1227r-4, matK-1227r-5
Ranunculales	Berberidaceae	matK-413f-3	matK-1227r-1
	Eupteleaceae	matK-413f-1, matK-413f-2	matK-1227r-1
	Lardizabalaceae	matK-413f-1	matK-1227r-1, matK-1227r-5
	Papaveraceae	matK-413f-1, matK-413f-2, matK-413f-3, matK-413f-5	matK-1227r-1, matK-1227r-3, matK-1227r-5
	Ranunculaceae	matK-413f-4	matK-1227r-1, matK-1227r-6, matK-1227r-4, matK-1227r-5
Rosales	Cannabaceae (*Gironniera nervosa*)	matK-413f-1, matK-413f-3	matK-1227r-1, matK-1227r-3
	Moraceae (*Artocarpus elasticus*)	matK-413f-1	matK-1227r-3
	Rhamnaceae (*Ziziphus angustifolius*)	matK-413f-1, matK-413f-7	matK-1227r-1, matK-1227r-3
	Rosaceae	matK-413f-1, matK-413f-2, matK-413f-6	matK-1227r-1, matK-1227r-3, matK-1227r-4, matK-1227r-5
	Ulmaceae	matK-413f-1	matK-1227r-3
	Urticaceae	matK-413f-1	matK-1227r-3
Sabilales	Sabiaceae (*Meliosma sumatrana*)	matK-413f-1, matK-413f-2	matK-1227r-1, matK-1227r-4
Santalales	Loranthaceae	matK-413f-4	matK-1227r-1, matK-1227r-4
	Opiliaceae	matK-413f-1, matK-413f-2	matK-1227r-1
	Santalaceae	matK-413f-1, matK-413f-2	matK-1227r-1, matK-1227r-5
	Schoepfiaceae	matK-413f-1	matK-1227r-1, matK-1227r-4
Sapindales	Meliaceae (*Aglaia* sp.)	matK-413f-1, matK-413f-7	matK-1227r-1, matK-1227r-5
	Rutaceae (*Glycosmis macrantha*)	matK-413f-1	matK-1227r-1, matK-1227r-6, matK-1227r-5
	Sapindaceae (*Lepisanthes* sp.)	matK-413f-4	matK-1227r-1, matK-1227r-3, matK-1227r-5
Saxifragales	Cercidiphyllaceae	matK-413f-1, matK-413f-7	matK-1227r-1
	Haloragaceae	matK-413f-1	matK-1227r-1
	Hamamelidaceae	matK-413f-1, matK-413f-5	matK-1227r-1
	Paeoniaceae	matK-413f-1	matK-1227r-1
	Saxifragaceae	matK-413f-1, matK-413f-4, matK-413f-5	matK-1227r-1
Solanales	Montiniaceae	matK-413f-1	matK-1227r-1
	Solanaceae	matK-413f-1, matK-413f-3	matK-1227r-3
Trochodendrales	Trochodendraceae	matK-413f-1, matK-413f-6	matK-1227r-1
Vitales	Vitaceae	matK-413f-1	matK-1227r-1, matk-1227r-2, matK-1227r-5

[a] Species/genera in parentheses were successfully amplified in the family using the primer cocktail C_MATK_F/C_MATK_R.

Table 3. Taxa used for primer testing.

No.[a]	Order: Family	Species	GenBank accession no.
1	Laurales: Lauraceae	*Litsea sarawacensis* Gamble	KU519656
2	Malphigiales: Euphorbiaceae	*Antidesma* L.	KU519677
3	Magnoliales: Myristicaceae	*Knema* Lour.	KU519655
4	Asparagales: Orchidaceae	*Polystachya humbertii* H. Perrier*	KU519659
5	Arecales: Arecaceae	Arecaceae Bercht. & J. Presl	KU519652
6	Poales: Bromeliaceae	*Tillandsia* cf. *caloura* Harms*	KU519653
7	Dilleniales: Dilleniaceae	*Dillenia suffruticosa* Martelli	KU519692
8	Malpighiales: Achariaceae	*Hydnocarpus borneensis* Sleumer	KU519671
9	Malpighiales: Calophyllaceae	*Kayea oblongifolia* Ridl.	KU519679
10	Malpighiales: Euphorbiaceae	*Macaranga hosei* King ex Hook. f.	KU519674
11	Malpighiales: Euphorbiaceae	*Koilodepas* Hassk.	KU519675
12	Malpighiales: Pandaceae	*Galearia fulva* Miq.	KU519670
13	Gentianales: Apocynaceae	*Tabernaemontana* L.	KU519697
14	Malpighiales: Violaceae	*Rinorea* Aubl.	KU519676
15	Malpighiales: Clusiaceae	*Garcinia* L.	KU519698
16	Malpighiales: Euphorbiaceae	*Drypetes* Vahl	KU519669
17	Malpighiales: Ctenolophonaceae	*Ctenolophon parvifolius* Oliv.	KU519672
18	Fabales: Fabaceae	*Fordia splendidissima* (Blume ex Miq.) Buijsen	KU519701
19	Fabales: Polygalaceae	*Xanthophyllum beccarianum* Chodat	KU519700
20	Rosales: Cannabaceae	*Gironniera nervosa* Planch.	KU519681
21	Rosales: Moraceae	*Artocarpus elasticus* Reinw.	KU519682
22	Rosales: Chrysobalanaceae	*Atuna racemosa* Raf.	KU519699
23	Rosales: Rhamnaceae	*Ziziphus angustifolia* (Miq.) Hatus. ex Steenis	KU519680
24	Curcurbitales: Anisophyllaceae	*Anisophyllea* R. Br. ex Sabine	KU519651
25	Fagales: Fagaceae	*Lithocarpus* Blume	KU519693
26	Sapindales: Anacardiaceae	*Gluta laxiflora* Ridl.	KU519684
27	Sapindales: Meliaceae	*Aglaia* F. Allam.	KU519686
28	Sapindales: Sapindaceae	*Lepisanthes* Blume	KU519685
29	Sapindales: Rutaceae	*Glycosmis* Corrêa	KU519687
30, 31	Malvales: Dipterocarpaceae	*Dipterocarpus palembanicus* Slooten	KU519691
32	Malvales: Cistaceae	*Helianthemum obscurum* Pers.*	KU519702
33	Malvales: Malvaceae	*Leptonychia* Turcz.	KU519688
34	Malvales: Malvaceae	*Durio griffithii* Bakh.	KU519689
35	Malvales: Malvaceae	*Sterculia* L.	KU519690
36	Cornales: Cornaceae	*Alangium* cf. *javanicum* (Blume) Wangerin	KU519664
37	Cornales: Cornaceae	*Mastixia* Blume	KU519663
38	Sapindales: Anacardiaceae	*Saurauia* Willd.	KU519661
39	Ericales: Ebenaceae	*Diospyros* L.	KU519660
40	Ericales: Lecythidaceae	*Barringtonia curranii* Merr.	KU519662
41	Ericales: Primulaceae	*Ardisia* Sw.	KU519667
42	Ericales: Symplocaceae	*Symplocos crassipes* C. B. Clarke	KU519658
43	Gentianales: Rubiaceae	*Urophyllum* Jack ex Wall.	KU519696
44	Solanales: Convolvulaceae	*Erycibe* cf. *glomerata* Blume	KU519694
45	Gentianales: Rubiaceae	*Psychotria* L.	KU519695
46	Magnoliales: Magnoliaceae	*Magnolia* L.	KU519654
47	Myrtales: Myrtaceae	*Syzygium* P. Browne ex Gaertn.	KU519678
48	Sabiales: Sabiaceae	*Meliosma sumatrana* (Jack) Walp.	KU519657
49	Malpighiales: Euphorbiaceae	*Mallotus* Lour.	KU519673
50	Lamiales: Lamiaceae	*Teijsmanniodendron* Koord.	KU519668
51	Santalales: Olacaceae	*Strombosia ceylanica* Gardner	KU519665
52	Aquifoliales: Cardiopteridaceae	*Gonocaryum minus* Sleumer	KU519666
53	Sapindales: Burseraceae	*Dacryodes excelsa* Vahl	KU519683
54	Asterales: Asteraceae	*Leontodon hispidus* L.*	KU519703

*Species not found in Southeast Asia.
[a] Number according to Fig. 1.

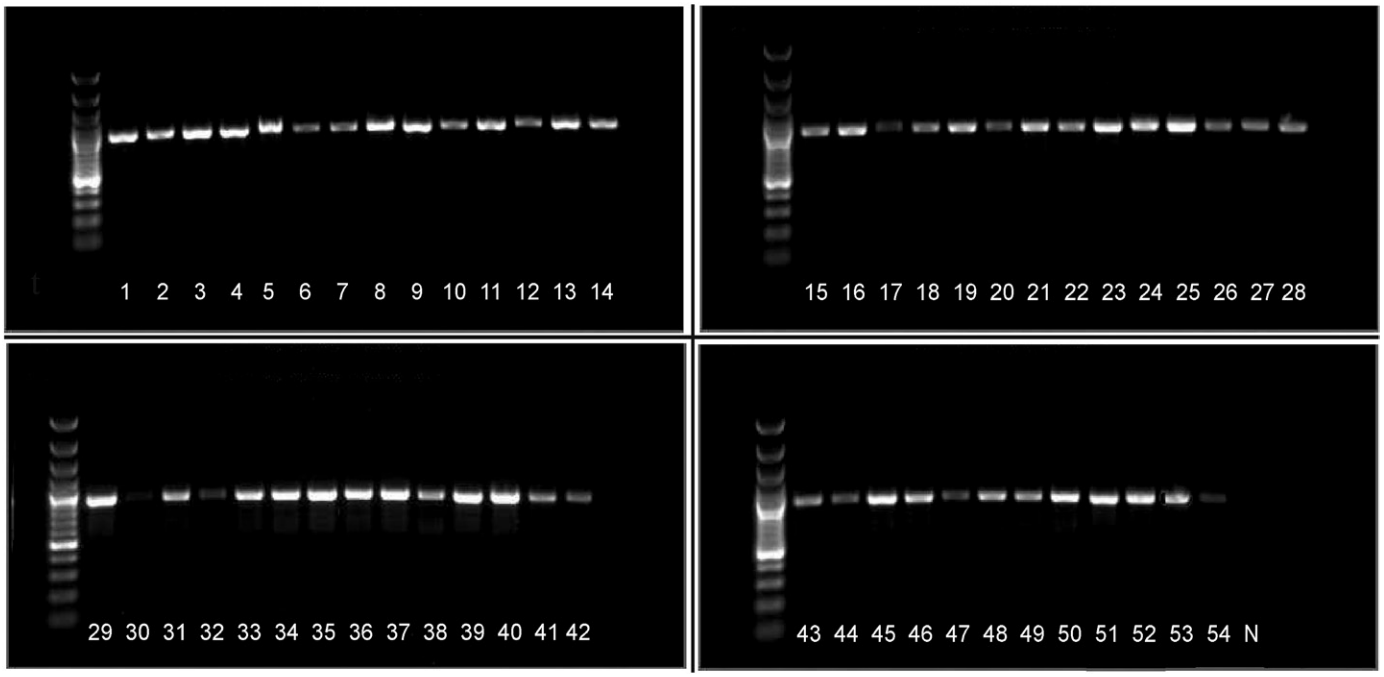

Fig. 1. Images of PCR amplicons for representatives of 53 angiosperm families using multiplex PCR with the newly developed degenerate primers (matK-413f-1 to matK-413f-5, matK-1227r-1 to matK-1227r-5). Bands are approximately 900 bp. Most of the samples were amplified using 2× ReddyMix. Low-quality DNA samples (slot 30) that failed PCR could be amplified using 2× Phusion Green HS II Hi-Fi PCR Master Mix (slot 31). For detailed sample description, see Table 3. Ladder: GeneRuler 100 bp Plus DNA Ladder (#SM0321; Thermo Fisher Scientific, Waltham, Massachusetts, USA). N = negative control.