

Supplementary Information

Selection and Validation of Reference Genes for mRNA Expression by Quantitative Real-Time PCR Analysis in *Neolamarckia cadamba*

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Supplementary Information Description

Supplementary Figures

Supplementary Fig. S1 Specificity of primer pairs for qRT-qPCR amplification. 1.0% non-denaturing agarose gel electrophoresis showed amplification of a specific product of the expected size for each reference gene. M represented DNA marker. 1-15 represented *ACT1*, *ACT7*, *ACT11*, *TUA2*, *TUA4*, *TUA5*, *EF-1- α* , *Rubisco*, *RPS25*, *RPL10A*, *MDH*, *GAPDH*, *PGK*, *SAMDC*, *F-BOX*.

Supplementary Fig. S2 Melting curve of 15 candidate reference genes. a-o referred to *ACT1*, *ACT7*, *ACT11*, *TUA2*, *TUA4*, *TUA5*, *EF-1- α* , *Rubisco*, *RPS25*, *RPL10A*, *MDH*, *GAPDH*, *PGK*, *SAMDC* and *F-BOX*, respectively.

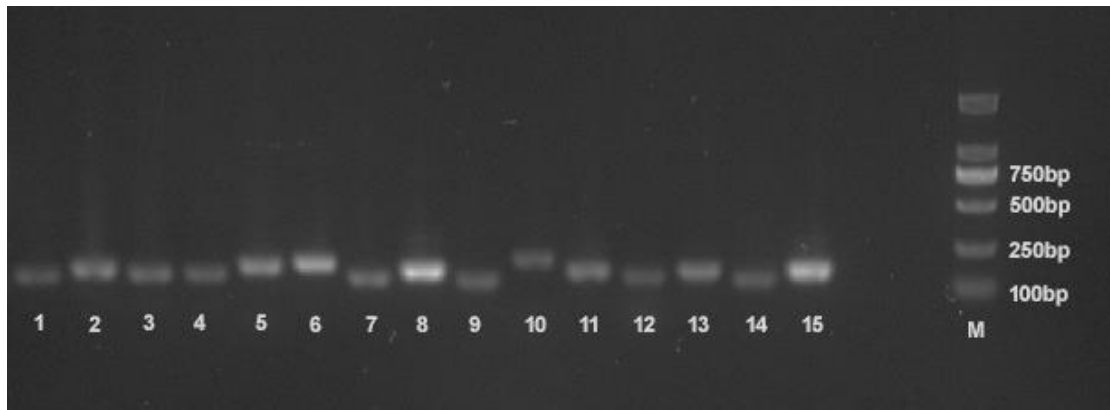
Supplementary Tables

Supplementary Table S1 Amplicon characteristics of candidate reference genes.

Supplementary Table S2 Stability ranking orders of candidates reference genes in six experimental sets by geNorm, NormFinder, BestKeeper and RefFinder.

Supplementary Table S3 Five sample sets from *Neolamarckia cadamba* used in this study.

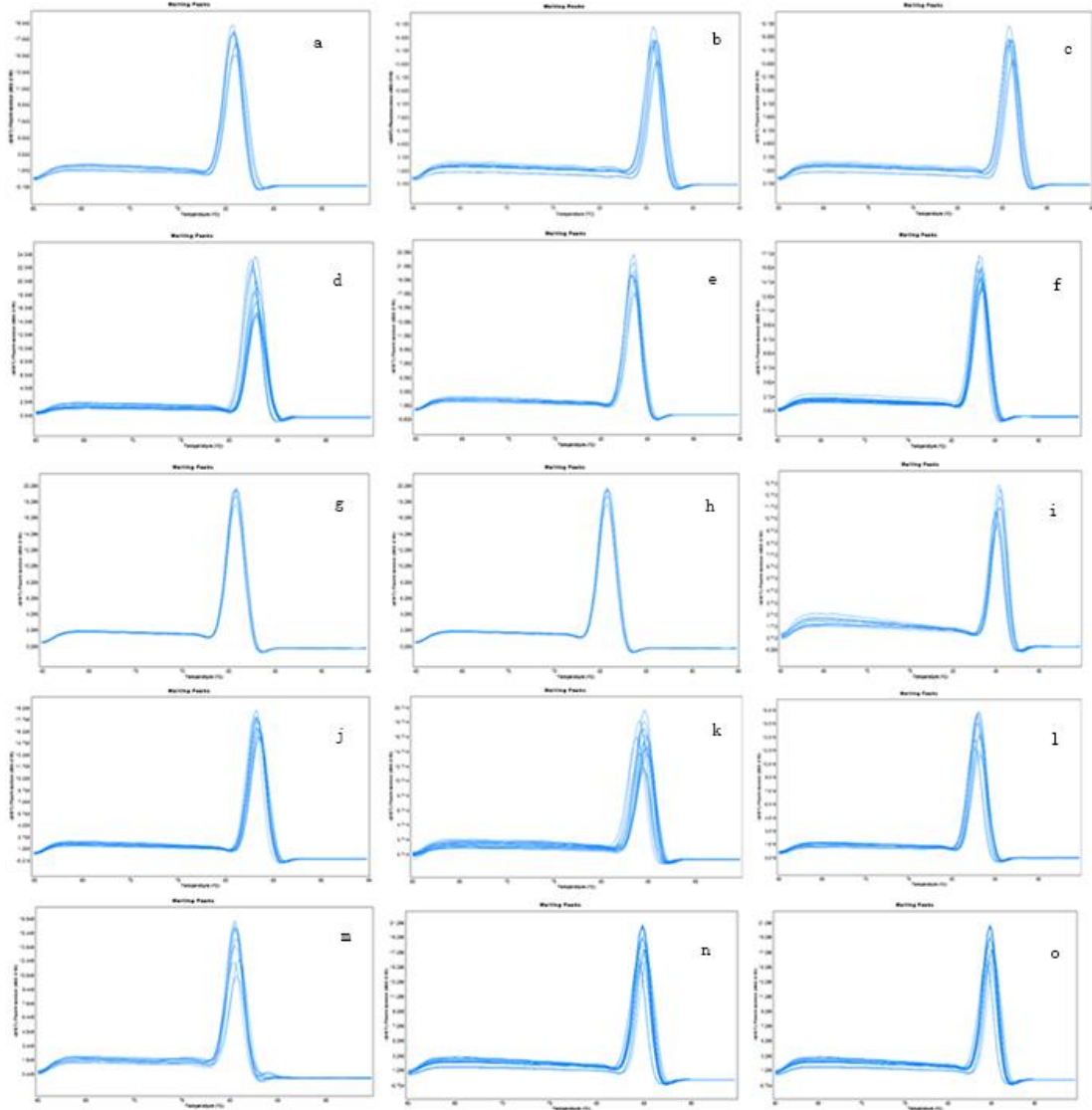
Supplementary Table S4 Characteristics of candidate reference genes and qRT-PCR primers in *Neolamarckia cadamba*. F indicates forward primer while R represents reverse primer.



Supplementary Fig. S1 Specificity of primer pairs for qRT-qPCR amplification. 1.0% non-denaturing agarose gel electrophoresis showed amplification of a specific product of the expected size for each reference gene. M represented DNA marker. 1-15 represented *ACT1*, *ACT7*, *ACT11*, *TUA2*, *TUA4*, *TUA5*, *EF-1- α* , *Rubisco*, *RPS25*, *RPL10A*, *MDH*, *GAPDH*, *PGK*, *SAMDC*, *F-BOX*.

The raw image:





Supplementary Fig. S2 Melting curve of 15 candidate reference genes. a-o referred to *ACT1*, *ACT7*, *ACT11*, *TUA2*, *TUA4*, *TUA5*, *EF-1- α* , *Rubisco*, *RPS25*, *RPL10A*, *MDH*, *GAPDH*, *PGK*, *SAMDC* and *F-BOX*, respectively.

Supplementary Table S1 Amplicon characteristics of candidate reference genes.

Gene symbol	Linear regression equation	Correlation (R²)	Amplification efficiency (%)	Slope
<i>ACT1</i>	$y = -3.4600x + 31.185$	0.9917	0.945	-3.4600
<i>ACT7</i>	$y = -3.2903x + 33.513$	0.9907	1.013	-3.2903
<i>ACT11</i>	$y = -3.3053x + 32.799$	0.9918	1.007	-3.3053
<i>TUA2</i>	$y = -3.2273x + 32.080$	0.9986	1.041	-3.2273
<i>TUA4</i>	$y = -3.2273x + 32.080$	0.9986	1.041	-3.2273
<i>TUA5</i>	$y = -3.3399x + 32.758$	0.9975	0.993	-3.3399
<i>EF-1-α</i>	$y = -3.3950x + 31.979$	0.9955	0.970	-3.3950
<i>Rubisco</i>	$y = -3.2863x + 33.577$	0.9902	1.015	-3.2863
<i>RPS25</i>	$y = -2.9840x + 32.645$	0.9991	1.163	-2.9840
<i>RPL10A</i>	$y = -3.4037x + 33.979$	0.9954	0.967	-3.4037
<i>MDH</i>	$y = -3.1153x + 32.282$	0.9944	1.094	-3.1153
<i>GAPDH</i>	$y = -3.3027x + 34.511$	0.9984	1.008	-3.3027
<i>PGK</i>	$y = -3.2188x + 31.943$	0.9953	1.045	-3.2188
<i>SAMDC</i>	$y = -3.4283x + 31.231$	0.9946	0.957	-3.4283
<i>F-BOX</i>	$y = -3.1690x + 32.654$	0.9936	1.068	-3.1690

Supplementary Table S2 Stability ranking orders of candidates reference genes in six experimental sets by geNorm, NormFinder, BestKeeper and RefFinder.

Method	Ranking order (The 1st is the most stable, and the 15th is the least stable)														
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
(A) Ranking order in different tissues															
Genorm	<i>RPL10A / SAMDC</i>		<i>ACT11</i>	<i>RPS25</i>	<i>ACT7</i>	<i>PGK</i>	<i>EF-1-α</i>	<i>ACT1</i>	<i>TUA5</i>	<i>F-BOX</i>	<i>TUA4</i>	<i>GAPDH</i>	<i>MDH</i>	<i>TUA2</i>	<i>Rubisco</i>
Normfinder	<i>RPL10A</i>	<i>ACT7</i>	<i>SAMDC</i>	<i>ACT11</i>	<i>PGK</i>	<i>RPS25</i>	<i>ACT1</i>	<i>EF-1-α</i>	<i>F-BOX</i>	<i>TUA4</i>	<i>TUA5</i>	<i>GAPDH</i>	<i>TUA2</i>	<i>MDH</i>	<i>Rubisco</i>
BestKeeper	<i>ACT7</i>	<i>SAMDC</i>	<i>RPL10A</i>	<i>PGK</i>	<i>ACT11</i>	<i>EF-1-α</i>	<i>TUA5</i>	<i>RPS25</i>	<i>F-BOX</i>	<i>ACT1</i>	<i>TUA4</i>	<i>TUA2</i>	<i>GAPDH</i>	<i>Rubisco</i>	<i>MDH</i>
RefFinder	<i>RPL10A</i>	<i>SAMDC</i>	<i>ACT7</i>	<i>ACT11</i>	<i>PGK</i>	<i>RPS25</i>	<i>EF-1-α</i>	<i>ACT1</i>	<i>F-BOX</i>	<i>TUA5</i>	<i>TUA4</i>	<i>GAPDH</i>	<i>TUA2</i>	<i>MDH</i>	<i>Rubisco</i>
(B) Ranking order under different developmental stages															
Genorm	<i>PGK / SAMDC</i>		<i>ACT7</i>	<i>RPL10A</i>	<i>F-BOX</i>	<i>TUA2</i>	<i>Rubisco</i>	<i>TUA4</i>	<i>TUA5</i>	<i>MDH</i>	<i>ACT1</i>	<i>RPS25</i>	<i>GAPDH</i>	<i>EF-1-α</i>	<i>ACT11</i>
Normfinder	<i>F-BOX</i>	<i>PGK</i>	<i>SAMDC</i>	<i>RPL10A</i>	<i>ACT7</i>	<i>MDH</i>	<i>TUA4</i>	<i>TUA2</i>	<i>Rubisco</i>	<i>TUA5</i>	<i>GAPDH</i>	<i>ACT1</i>	<i>RPS25</i>	<i>EF-1-α</i>	<i>ACT11</i>
BestKeeper	<i>PGK</i>	<i>SAMDC</i>	<i>RPL10A</i>	<i>ACT7</i>	<i>TUA5</i>	<i>TUA2</i>	<i>RPS25</i>	<i>F-BOX</i>	<i>MDH</i>	<i>TUA4</i>	<i>EF-1-α</i>	<i>Rubisco</i>	<i>ACT11</i>	<i>GAPDH</i>	<i>ACT1</i>
RefFinder	<i>PGK</i>	<i>SAMDC</i>	<i>F-BOX</i>	<i>RPL10A</i>	<i>ACT7</i>	<i>TUA2</i>	<i>MDH</i>	<i>TUA4</i>	<i>TUA5</i>	<i>Rubisco</i>	<i>RPS25</i>	<i>GAPDH</i>	<i>ACT1</i>	<i>EF-1-α</i>	<i>ACT11</i>
(C) Ranking order under different photosynthetic cycles															
Genorm	<i>ACT7 / SAMDC</i>		<i>ACT1</i>	<i>TUA5</i>	<i>TUA2</i>	<i>EF-1-α</i>	<i>RPL10A</i>	<i>PGK</i>	<i>RPS25</i>	<i>GAPDH</i>	<i>ACT11</i>	<i>MDH</i>	<i>F-BOX</i>	<i>TUA4</i>	<i>Rubisco</i>
Normfinder	<i>ACT7</i>	<i>SAMDC</i>	<i>PGK</i>	<i>RPS25</i>	<i>ACT1</i>	<i>TUA5</i>	<i>RPL10A</i>	<i>TUA2</i>	<i>EF-1-α</i>	<i>GAPDH</i>	<i>ACT11</i>	<i>MDH</i>	<i>F-BOX</i>	<i>TUA4</i>	<i>Rubisco</i>
BestKeeper	<i>SAMDC</i>	<i>ACT7</i>	<i>PGK</i>	<i>RPS25</i>	<i>ACT1</i>	<i>TUA5</i>	<i>TUA2</i>	<i>RPL10A</i>	<i>EF-1-α</i>	<i>ACT11</i>	<i>GAPDH</i>	<i>MDH</i>	<i>F-BOX</i>	<i>TUA4</i>	<i>Rubisco</i>
RefFinder	<i>SAMDC</i>	<i>ACT7</i>	<i>ACT1</i>	<i>PGK</i>	<i>TUA5</i>	<i>RPS25</i>	<i>TUA2</i>	<i>RPL10A</i>	<i>EF-1-α</i>	<i>GAPDH</i>	<i>ACT11</i>	<i>MDH</i>	<i>F-BOX</i>	<i>TUA4</i>	<i>Rubisco</i>
(D) Ranking order under drought stress															
Genorm	<i>RPS25 / SAMDC</i>		<i>TUA4</i>	<i>GAPDH</i>	<i>F-BOX</i>	<i>ACT11</i>	<i>RPL10A</i>	<i>ACT1</i>	<i>PGK</i>	<i>TUA5</i>	<i>Rubisco</i>	<i>TUA2</i>	<i>MDH</i>	<i>EF-1-α</i>	<i>ACT7</i>
Normfinder	<i>SAMDC</i>	<i>RPL10A</i>	<i>RPS25</i>	<i>F-BOX</i>	<i>ACT11</i>	<i>ACT1</i>	<i>TUA4</i>	<i>GAPDH</i>	<i>PGK</i>	<i>TUA5</i>	<i>Rubisco</i>	<i>TUA2</i>	<i>EF-1-α</i>	<i>MDH</i>	<i>ACT7</i>
BestKeeper	<i>RPL10A</i>	<i>SAMDC</i>	<i>RPS25</i>	<i>ACT1</i>	<i>ACT11</i>	<i>F-BOX</i>	<i>TUA5</i>	<i>TUA4</i>	<i>PGK</i>	<i>MDH</i>	<i>Rubisco</i>	<i>TUA2</i>	<i>ACT7</i>	<i>EF-1-α</i>	<i>GAPDH</i>
RefFinder	<i>SAMDC</i>	<i>RPS25</i>	<i>RPL10A</i>	<i>F-BOX</i>	<i>ACT11</i>	<i>ACT1</i>	<i>TUA4</i>	<i>GAPDH</i>	<i>TUA5</i>	<i>PGK</i>	<i>Rubisco</i>	<i>TUA2</i>	<i>MDH</i>	<i>EF-1-α</i>	<i>ACT7</i>
(E) Ranking order under cold stress															
Genorm	<i>RPS25 / SAMDC</i>		<i>Rubisco</i>	<i>TUA5</i>	<i>F-BOX</i>	<i>ACT1</i>	<i>PGK</i>	<i>GAPDH</i>	<i>ACT11</i>	<i>TUA4</i>	<i>RPL10A</i>	<i>ACT7</i>	<i>MDH</i>	<i>TUA2</i>	<i>EF-1-α</i>
Normfinder	<i>Rubisco</i>	<i>RPS25</i>	<i>ACT11</i>	<i>SAMDC</i>	<i>GAPDH</i>	<i>TUA5</i>	<i>ACT1</i>	<i>RPL10A</i>	<i>PGK</i>	<i>F-BOX</i>	<i>ACT7</i>	<i>TUA2</i>	<i>MDH</i>	<i>EF-1-α</i>	<i>TUA4</i>

Continued

BestKeeper	<i>RPS25</i>	<i>SAMDC</i>	<i>Rubisco</i>	<i>TUA5</i>	<i>ACT11</i>	<i>TUA4</i>	<i>RPL10A</i>	<i>F-BOX</i>	<i>ACT1</i>	<i>ACT7</i>	<i>PGK</i>	<i>GAPDH</i>	<i>EF-1-α</i>	<i>TUA2</i>	<i>MDH</i>
RefFinder	<i>RPS25</i>	<i>Rubisco</i>	<i>SAMDC</i>	<i>TUA5</i>	<i>ACT11</i>	<i>F-BOX</i>	<i>GAPDH</i>	<i>ACT1</i>	<i>RPL10A</i>	<i>PGK</i>	<i>TUA4</i>	<i>ACT7</i>	<i>TUA2</i>	<i>MDH</i>	<i>EF-1-α</i>

(F) Ranking order under all samples

Genorm	<i>RPS25 / SAMDC</i>	<i>PGK</i>	<i>F-BOX</i>	<i>TUA5</i>	<i>ACT1</i>	<i>RPL10A</i>	<i>Rubisco</i>	<i>TUA4</i>	<i>MDH</i>	<i>ACT11</i>	<i>ACT7</i>	<i>GAPDH</i>	<i>TUA2</i>	<i>EF-1-α</i>	
Normfinder	<i>SAMDC</i>	<i>F-BOX</i>	<i>RPL10A</i>	<i>RPS25</i>	<i>PGK</i>	<i>ACT1</i>	<i>TUA5</i>	<i>TUA4</i>	<i>ACT11</i>	<i>ACT7</i>	<i>Rubisco</i>	<i>MDH</i>	<i>GAPDH</i>	<i>TUA2</i>	<i>EF-1-α</i>
BestKeeper	<i>SAMDC</i>	<i>RPS25</i>	<i>PGK</i>	<i>F-BOX</i>	<i>TUA5</i>	<i>ACT11</i>	<i>RPL10A</i>	<i>ACT1</i>	<i>TUA4</i>	<i>Rubisco</i>	<i>MDH</i>	<i>ACT7</i>	<i>GAPDH</i>	<i>TUA2</i>	<i>EF-1-α</i>
RefFinder	<i>SAMDC</i>	<i>RPS25</i>	<i>F-BOX</i>	<i>PGK</i>	<i>RPL10A</i>	<i>TUA5</i>	<i>ACT1</i>	<i>TUA4</i>	<i>ACT11</i>	<i>Rubisco</i>	<i>MDH</i>	<i>ACT7</i>	<i>GAPDH</i>	<i>TUA2</i>	<i>EF-1-α</i>

Supplementary Table S3 Five sample sets from *Neolamarckia cadamba* used in this study.

Experimental sample sets	Tissue type	Number of treatments	Biological replicates	Sampling dates	Total number of samples (treatments * replicates * dates)
Different tissues	Roots, young leaves, Mature leaves, xylem, phloem	1	3	1	15
Different developmental stages	Mature leaves	1	3	4	12
Different photosynthetic cycle	Mature leaves	3	3	1	9
Drought treatment	Mature leaves	5	3	1	15
Cold treatment	Mature leaves	5	3	1	15

Supplementary Table S4 Characteristics of candidate reference genes and qRT-PCR primers in *Neolamarckia cadamba*. F represents forward primer while R represents reverse primer.

Gene symbol	GeneBank accession number (CDS)	GeneBank accession number (gDNA)	Annotation	Primer sequence (5'-3')	Amplicon length (bp)
<i>ACT1</i>	KY922888	KY922871	Actin 1	F:GTCAAGGCTGGGTTTGCTGGGG R:CCCAACATAAGCATCTTTTGC	117
<i>ACT7</i>	KY922889	KY922872	Actin 7	F:AACTGGTATTGTCCTGGATTCTGG R:CGGTGAGGATCTTCATGAGATG	104
<i>ACT11</i>	KY922890	KY922873	Actin 11	F:TGCTAGTGGTCGTACAACGG R:ATCTGTAAGATCGCGCCCAG	133
<i>TUA2</i>	KY922891	KY922874	Tubulin alpha 2	F:ATACCATTTGGCAAAGAGATCGT R:GGACCCAAGACCAGATCCAGTG	131
<i>TUA4</i>	KY922892	KY922875	Tubulin alpha 4	F:CCCGTGGCCACTACAC R:CAACAGGGATCCCAACC	149
<i>TUA5</i>	KY922893	KY922876	Tubulin alpha 5	F:CGGATGTTGTAGTGCTTTTG R:TGGTTTGGAACCGGTGA '	180
<i>EF-1-α</i>	KY922894	KY922877	Elongation factor 1-alpha	F:GCAACAAGATGGATGCCACCAC R:GAACCCAGAGATGGGAACAAAG	134
<i>Rubisco</i>	KY922902	KY922879	Ribulose bisphosphate carboxylase	F:GATTAGTTTTGCGGGTTCT R:TTCCACCGAGACGACCAG	143
<i>RPS25</i>	KY922900	KY922883	Ribosomal protein S25	F:CCCTCTGTTCTTTCCGACCG R:GGCTTGCATGAGATGAGACC	109
<i>RPL10A</i>	KY922901	KY922884	Ribosomal protein L10A	F:TGGCGTTGCCTGTCTATCAG R:CCGAACCTCGTGAATCCTCC	176
<i>MDH</i>	KY922902	KY922885	Malate dehydrogenase	F:TATTCTTCACGGTTTTCTCTAC R:GCTTCTCTTGGTATCCTCA	157
<i>GAPDH</i>	KY922898	KY922881	Glyceraldehyde-3-phosphate dehydrogenase	F:TTGTCTCAAACCGCAACCTT R:TTAGCTCCGGCTTGGATGTG	124
<i>PGK</i>	KY922899	KY922882	Phosphoglycerate kinase	F:GGTGTCAACCCGAAGTACAG R:ATTCTCCAGGAGCAGCACAC	153
<i>SAMDC</i>	KY922897	KY922880	S-adenosylmethionine decarboxylase	F:GGGACCAACTGGATGAAA R:TGTAAGGGAAGACAAAGAG	117
<i>F-BOX</i>	KY922895	KY922878	F-Box protein	F:CTTCCACTCCTCCCATCA R:CAGACTTCATCAGTAGCAAAAAC	149