Assessment of three plastid DNA barcode markers for identification of *Clinacanthus nutans* (Acanthaceae)

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Abstract

This study was conducted to determine the feasibility of using three plastid DNA regions (*matK*, *trnH-psbA*, and *rbcL*) as DNA barcodes to identify the medicinal plant *Clinacanthus nutans*. In this study, *C. nutans* was collected at several different locations. Total genomic DNA was extracted, amplified by polymerase chain reaction (PCR), and sequenced using *matK*, *trnH-psbA*, and *rbcL*, primers. DNA sequences generated from PCR were submitted to the National Center for Biotechnology Information's (NCBI) GenBank. Identification of *C. nutans* was carried out using NCBI's Basic Local Alignment Search Tool (BLAST). The *rbcL* and *trnH-psbA* regions successfully identified *C. nutans* with sequencing rates of 100% through BLAST identification. Molecular Evolutionary Genetics Analysis (MEGA) 6.0 was used to analyze interspecific and intraspecific divergence of plastid DNA sequences. *rbcL* and *matK* exhibited the lowest average interspecific distance (0.0487 and 0.0963, respectively), whereas *trnH-psbA* exhibited the highest average interspecific distance (0.2029). The R package Spider revealed that *trnH-psbA* correctly identified Barcode of Life Data System (BOLD) 96%, best close match 79%, and near neighbor 100% of the species, compared to *matK* (BOLD 72%; best close match 64%; near neighbor 78%) and *rbcL* (BOLD 77%; best close match 62%; near neighbor 88%). These results indicate that *trnH-psbA* is very effective at identifying *C. nutans*, as it performed well in discriminating species in Acanthaceae.

Keywords *Clinacanthus nutans* · DNA barcodes · *matK* · *rbcL* · *trnH-psbA*

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Introduction

The Acanthaceae family of angiosperms contains 225 genera and 2894 individual species (The Plant List 2013). Many members of this family have significant medicinal uses, including antioxidant, insecticidal, antifungal, anti-inflammatory, antiviral, anti-pyretic, hepatoprotective, immunomodulatory, cytotoxic, and anti-platelet aggregation. *Clinacanthus nutans*, which belongs to the family Acanthaceae, is well known in Malaysia, Thailand, Indonesia, Vietnam, and China (Chelyn et al. 2014). It has several common names according to the region in which it is found: Sabah Snake Grass and “Belalai Gajah” (Malaysia), “Dandang Gendis” and “Ki Tajan” (Indonesia), “Phaya Yo” and “Phaya Plongtong” (Thailand), and “E Zuihua” (China) (Farsi et al. 2016). This plant can be found in most habitats, including dense or open forests, damp fields, bushes, valleys, sea shores, mangrove areas, swamps, and marine regions (Alam et al. 2016; Ismail et al. 2016). Fong et al. (2014) reported that *C. nutans* grows and primarily reproduces via

vegetative propagation, as the flowers are hard to find, which results in severely reduced sexual reproduction. *C. nutans* is well known for its antioxidant, anti-inflammatory, anticancer, antidiabetic, and antiviral properties (Sakdarat et al. 2009; Wanikiat et al. 2008; Arullappan et al. 2014; Ismail et al. 2016).

The growth habit and leaf appearance of *C. nutans* are similar to those of *Clinacanthus siamensis* (Fong et al. 2014), but these species have different medicinal properties (Fong et al. 2014; Alam et al. 2016). Kunsorn et al. (2013) conducted macroscopic and microscopic analyses of *C. nutans* and *C. siamensis* and found similar morphology and cell components, but the leaf measurement index (palisade ratio, stomata index, and stomatal number) showed different constant numbers. When using medicinal plants, accurate identification is crucial. Traditional approaches to identifying plants include organoleptic methods (identification by senses: taste, sight, smell, and touch) and use of morphological characters (identification by shape, color, and texture). At the family level, plants can be easily recognized morphologically by characteristics of their leaves (simple, opposite, decussate), flowers (zygomorph), and ovary (superior) (Alam et al. 2016). However, these methods require an expert to identify the plant species (Tehen et al. 2014).

In contrast to traditional methods, use of DNA to identify *C. nutans* is more accurate, less time consuming, and can be done using a very small amount of material. The DNA barcoding technique is widely accepted within the scientific community (Coissac et al. 2016) and has been used commonly since the mitochondrial gene cytochrome c oxidase I (COI) was suggested as the DNA barcode for species identification in animals (Hebert et al. 2003). Subsequently, much progress has been made in identifying DNA barcodes for plants. Many barcode marker candidates have been proposed, such as *matK*, *rbcL*, *ITS*, and *ITS2*, which are short DNA sequences between 400 and 800 base pairs (bp) long. Many plastid DNA sequences have been studied and validated and are suggested as potential barcode loci. To reduce cost and time consumption, the DNA barcode method has

been used by many researchers to identify and discriminate medicinal plants without any influence of growth phase, environmental conditions, and morphological diversity within species (Chen et al. 2010; Tehen et al. 2014; Tang et al. 2016).

Kunsorn et al. (2013) and Suesatpanit et al. (2017) reported that *ITS* and *ITS2* could not discriminate *C. nutans* from *C. siamensis*. Therefore, we attempted to use other universal DNA markers (*rbcL*, *matK*, and *trnH-psbA*) from the Consortium for the Barcode of Life (CBOL) Plant Working Group to identify *C. nutans*. The objective of this study was to identify and select suitable plastid DNA barcode markers for *C. nutans*. This information will serve as a guide to provide a suitable plant identification marker for *C. nutans* and other Acanthaceae species.

Methodology

Plant sample collection

Eighty *C. nutans* samples were randomly collected from eight sites located in the northern region of Peninsular Malaysia (Table 1, Fig. 1). Four locations where *C. nutans* is cultivated in Pulau Pinang were identified: Pongsu Seribu (PBF) and Tasek Gelugor (PTG) in north Seberang Perai (mainland), Batu Maung (PBM) on the southeastern side of the island, and Batu Ferringhi (PBF) on the hilly northern side of the island. Three more locations were identified in Kedah: Jeniang (KJN), a small town situated between Gurun and Sik, Sungai Petani (KSP), and Kuala Ketil (KKK). The last cultivation site was in Perlis, located at Taman Herba Perlis at Sungai Batu Pahat (SBP). It lies on the northern part of the west coast of Peninsular Malaysia. The *C. nutans* samples were collected at elevations ranging from 8 to 44 m above sea level. Average temperature among the eight locations was 32 °C, with readings between 30 and 34 °C. Mean rainfall at all sites was calculated from data collected from

Table 1 Location of *C. nutans* sampling sites in northern regions of Peninsular Malaysia

Location code	State	Region	Coordinate		Environmental conditions			
			Latitude	Longitude	Elv (m)	T (°C)	R (mm)	LI (lux)
PBF	Penang	Batu Ferringhi	5.46913	100.24655	33	33	221	500
PTG	Penang	Tasek Gelugor	5.48783	100.50535	14	30	202	650
SBP	Perlis	Sungai Batu Pahat	6.53414	100.16906	44	30	384	500
KSP	Kedah	Sungai Petani	5.70244	100.51216	13	34	211	800
KKK	Kedah	Kuala Ketil	5.60518	100.65115	21	33	246	850
PPS	Penang	Pongsu Seribu	5.49663	100.44532	11	33	202	700
KJN	Kedah	Jeniang	5.81501	100.62629	34	33	146	500
PBM	Penang	Batu Maung	5.28496	100.28013	8	32	225	550

Elv (m) elevation, T (°C) temperature, R (mm) rainfall mean (February–April 2015), LI (lux) light intensity

Fig. 1 Location of *C. nutans* sampling sites in northern regions of Peninsular Malaysia. The code names for these locations are shown in Table 1

the nearest Malaysian Meteorological Department. Light intensity was measured using a light tester meter.

DNA extraction, amplification, purification, and sequencing

Total genomic DNA of plant leaves was extracted using NucleoSpin® Plant II DNA extraction kit (Macherey–Nagel, Bethlehem, PA, USA). The concentration and purity of DNA were quantified using a NanoDrop 2000 UV–Vis spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) by determining the absorbance at 260 and 280 nm. DNA was stored at -20°C until needed for further analysis.

The plastid DNA regions (*rbcL*, *trnH-psbA*, and *matK*) were amplified using universal DNA barcoding primers from the CBOL Plant Working Group (2009). For the *rbcL* gene, primers were *rbcLa-F*: 5'-ATGTCACCACAAACAGAG ACTAAAGC-3' and *rbcLa-R*: 5'-GTAAAATCAAGTCCA CCRG-3'; for the *matK* gene they were *matK-1RKIM-f*: 5'-CCAGTCCATCTGGAAATCTTGGTTC-3' and *matK-3FKIM-r*: 5'-CGTACAGTACTTTTGTGTTTACGAG-3'; and for the *trnH-psbA* gene they were *psbA3_f*: 5'-GTT ATGCATGAACGTAATGCTC-3' and *trnHf_05*: 5'-CGC GCATGGTGGATTCAATCC-3'. The polymerase chain reaction (PCR) was performed in a final volume of 25 μL containing 2.50 μL of 10 $\times$ Taq buffer, 0.75 μL of 50 mM MgCl_2 , 0.50 μL of 10 mM dNTP mix, 1.00 μL of 0.4 μM primers, 0.13 μL of 5 U of Taq DNA polymerase, 1.00 μL

of genomic DNA, and 19.13 μL of sterile deionized water. PCR amplification was performed in a My Cycle™ Thermal Cycle (Bio-Rad, Hercules, CA, USA) programmed for an initial 1 min denaturation at 94°C , 45 cycles of 1 min denaturation at 94°C , 1 min annealing at 53°C for *matK*, *rbcL*, and *trnH-psbA* primers, and 2 min extension at 72°C followed by a final extension for 5 min at 72°C and a hold at 4°C . Amplified PCR products were analyzed using 1.5% agarose gel at 90 V for 30 min. The bright bands produced from the gel were purified using the Wizard® SV Gel and PCR Clean-Up System (Promega, Madison, WI, USA). The purified PCR products were sent to 1st BASE DNA Sequencing Services (Selangor, Malaysia) for bidirectional sequencing with appropriate amplification primers.

Data sequencing and alignment

Trimming the low-quality sequence and primer areas was performed by CodonCode Aligner 3.7.1 (CodonCode Co., Centerville, MA, USA). Forward and reverse sequences were aligned using the Basic Local Alignment Search Tool (BLAST) from the National Center for Biotechnology Information (NCBI). We followed Maloukh et al. (2017) when identifying the sequence in BLAST; they considered a score in a sequence comparison of species with *E* value of a $< 1 \times 10^{-5}$ and maximum hits (99 or 100%) in the reference database. All *matK*, *rbcL*, and *trnH-psbA* DNA sequences

from different species were obtained from BLAST. The DNA sequences were aligned using ClustalX version 2.1.

Statistical analysis

The parameter used to analyze DNA barcodes included interspecific and intraspecific divergence by the Kimura 2-Parameter (K2P) model using Molecular Evolutionary Genetics Analysis 6.0 (MEGA 6.0) software. Three parameters were used to describe intraspecific divergence: (1) intraspecific divergence theta, where theta is the mean pairwise distance within each species with at least two representatives; theta eliminates biases associated with unequal sampling among a species, (2) coalescent depth, which is the maximum intraspecific distance within each species with at least two individuals, and (3) all intraspecific distances, which showed the average of all samples collected within each species with more than one individual.

Three other parameters were used to characterize interspecific divergence: (1) theta prime, which is the mean pairwise distance within each genus with more than one species, thus eliminating biases associated with different numbers of species among genera, (2) smallest interspecific distance (i.e., the minimum interspecific distance within each genus with at least two species), and (3) all interspecific distances, which showed the average of all species in each genus with at least two species.

Interspecific and intraspecific genetic distances from the DNA sequences determined using MEGA 6.0 were used to calculate the DNA barcode gap. The barcoding gap is the difference between interspecific and intraspecific genetic distances within a group of organisms. The Wilcoxon signed-rank test was conducted using IBM SPSS Statistics v19 to analyze the distribution divergence of the genetic distance between different sequences to assess the barcoding gap for different candidate loci. The test statistics $W+$ and $W-$ were calculated for the two-sided test (Chen et al. 2010; Tang et al. 2016). The following three methods were used to test the ability of the barcode to accurately identify the species: best close match (Meier et al. 2006), nearest neighbor, and BOLD identification criteria; they were implemented in the R package Spider (Brown et al. 2012). Neighbor-joining (NJ) phylogenetic trees generated by the software MEGA 6.0 with the K2P distance were assessed using a bootstrap

test with 1000 replicates of NJ run options for all *matK*, *rbcL*, and *trnH-psbA* DNA sequences.

Results and discussion

PCR amplification and sequence analysis

According to the CBOL Plant Working Group (2009), several DNA barcode loci have been proposed and are highly recommended for barcoding applications. Kress and Erickson (2008) described the criteria for the best DNA barcodes. First, DNA barcodes should be composed of significant species-level genetic variability and divergence. Furthermore, they must contain conserved flanking sites for the development of universal PCR primers for wide taxonomic application and a short sequence length to enable application of recently developed DNA extraction and amplification methods. In the current study, the following three DNA markers were able to successfully identify *C. nutans* with sequencing success rates of 94.3% (*matK*), 100% (*rbcL*), and 100% (*trnH-psbA*) through BLAST identification (Table 2). Although both *rbcL* and *trnH-psbA* had a 100% success rate, *trnH-psbA* was a more suitable DNA marker for identification of *C. nutans*. The CBOL Plant Working Group rejected *trnH-psbA* due to difficulties in bidirectional sequencing and because it was long (> 1000 bp) in monocots and conifers (Chase et al. 2007; Hollingsworth et al. 2009; Tripathi et al. 2013). However, we obtained high quality bidirectional sequences for *trnH-psbA* with a higher identification success rate than *matK* and *rbcL*. Tripathi et al. (2013) also reported a high identification success rate for tropical tree species using the *trnH-psbA* marker.

In this study, *matK* exhibited the poorest identification rate for *C. nutans*, likely due to its large amplification length (average of 796 bp). In contrast, the DNA sequence from *trnH-psbA* ranged from 374 and 387 bp (mean 384 bp). Degtjareva et al. (2012) reported that *trnH-psbA* had shorter sequences because of variability in insertions and deletions in the intergenic spacer, which makes sequence alignment difficult. However, deletions and insertions in intergenic spacer regions make the *trnH-psbA* gene one of the fastest evolving chloroplast gene markers because of its high level

Table 2 Analysis of the *matK*, *rbcL* and *trnH-psbA* PCR products from NCBI's BLAST

Items	<i>matK</i>	<i>rbcL</i>	<i>trnH-psbA</i>
Length range (average) (bp)	788–812 (796)	577–580 (579)	374–387 (384)
Number of species	42	60	63
Number of accessions	86	75	94
BLAST method identification efficiency (%)	94.3	100	100

of species discrimination (Fazekas et al. 2008; Lahaye et al. 2008; Newmaster et al. 2008; Liu et al. 2011).

Identification efficiency of *matK*, *rbcL*, and *trnH-psbA*

Many molecular markers for identifying plant species have been described in the literature. Nonetheless, one marker is not sufficient to compare the identification results of the species (Dong et al. 2012; Tallei and Kolondam 2015). Our findings show that the identification of plant species sequences obtained from NCBI's BLAST were from the Acanthaceae family, with 86 accessions of *matK* DNA sequences from 42 species, 75 accessions of *rbcL* DNA sequences from 60 species, and 93 accessions of *trnH-psbA* DNA sequences from 63 species (Table 2, Supplementary Table S1). Analysis using BOLD, best close match, and nearest neighbor showed that *trnH-psbA* had the highest success rate for the correct identification of species (BOLD 96%; best close match 79%; nearest neighbor 100%) compared to *matK* (BOLD 72%; best close match 64%; nearest neighbor 78%), and *rbcL* (BOLD 77%; best close match 62%; nearest neighbor 88%) (Table 3). The presence of the barcoding gap between genetic distances assures that there is a cut-off value for species delimitation (Sundberg et al. 2016). If a barcoding gap is present, the identification of plant species is correctly identified. We examined the distributions of interspecific and intraspecific divergence for the three markers at a scale of

0.001 distance units. The results demonstrated that distinct gaps were observed in the distribution of interspecific variability of *trnH-psbA* followed by *matK* and *rbcL* (Fig. 2). The most obvious gap was found in *trnH-psbA*.

Based on these results, we propose *trnH-psbA* as the universal barcode for the identification of *C. nutans*. This locus has also been recommended as a DNA barcode for other studies in other plants because it performs best as a single locus due to high amounts of interspecific variation compared to other DNA barcode loci (Sun et al. 2013; Aziz et al. 2015; Zheng et al. 2015). Additionally, Gere et al. (2013) and Tang et al. (2016) reported that *trnH-psbA* is a good candidate for authentication of their plants of interest. Although the CBOL Plant Working Group proposed using *rbcL* and *matK* for land plant identification, we found that species discrimination of *C. nutans* by *rbcL* and *matK* was lower than that of *trnH-psbA* (Table 3). Thus, *matK* and *rbcL* are not the best choices for certain studies (Starr et al. 2009; Steven and Subramanyam 2009; Pettengill and Neel 2010; Roy et al. 2010; Clement and Donoghue 2012).

Phylogenetic analysis

Using the NJ method, plants can be easily categorized into different species. The partial amplification sequences of *trnH-psbA* were used to understand the phylogenetic link between *C. nutans* and other Acanthaceae species. Use of

Table 3 Discriminatory power of DNA regions based on near neighbor, BOLD, and best close match (Meier et al. 2006) methods

DNA region	Nearest neighbor		BOLD (1% threshold)				Best close match		
	False (%)	True (%)	Ambiguous (%)	Correct (%)	Incorrect (%)	No ID (%)	Ambiguous (%)	Correct (%)	Incorrect (%)
<i>rbcL</i>	12	88	8	77	2	13	38	62	0
<i>matK</i>	22	78	8	72	4	16	21	64	15
<i>trnH-psbA</i>	0	100	4	96	0	0	18	79	3

Fig. 2 Relative distribution of interspecific divergence and intraspecific divergence of *rbcL*, *trnH-psbA*, and *matK*. Data for each genetic region (i.e., barcode) are shown in two layers, with the top layer representing interspecific distance and the bottom layer showing intraspecific distance

trnH-psbA as the DNA barcode showed that all species from NCBI's BLAST database are from the same Acanthaceae family, consisting of 48 species belonging to subtribe Justiciinae, four species belonging to subtribe Acanthoideae incertae sedis, two species belonging to Barleriinae, two species belonging to Andrographinae, and 38 species belonging to subtribe Ruelliinae (Fig. 3). The phylogenetic tree of *trnH-psbA* sequences indicated that nearly all species

are from distinct clades. The genetic distance within and between species clearly illustrates the difference between individuals within and between species in the Acanthaceae family. Table 4 shows the performance of genetic distance of each candidate DNA barcode marker locus. The genetic distance of *C. nutans* from closely related species was higher in interspecific divergence compared to intraspecific divergence. Based on genetic divergence, *rbcL* exhibits the lowest

Fig. 3 The NJ tree of *trnH-psbA* was constructed using Mega 6.0 based on the K2P distance model. The clades are color coded

Table 4 Interspecific and intraspecific divergences for DNA barcode marker

Divergence	<i>trnH-psbA</i>	<i>matK</i>	<i>rbcL</i>
Interspecific			
Average interspecific distance	0.2029 ± 0.0253	0.0963 ± 0.0110	0.0487 ± 0.0097
Average theta prime	0.2044 ± 0.0000	0.0949 ± 0.0049	0.0329 ± 0.0040
Smallest interspecific distance	0.0054 ± 0.0038	0.0247 ± 0.0061	0.0036 ± 0.0027
Intraspecific			
Average intraspecific distance	0.0255 ± 0.0037	0.0225 ± 0.0033	0.0450 ± 0.0063
Theta	0.1850 ± 0.0165	0.0691 ± 0.0054	0.0074 ± 0.0021
Average coalescent depth	0.0000 ± 0.0000	0.0000 ± 0.0000	0.0052 ± 0.0030

average interspecific distance (0.0487), whereas *trnH-psbA* exhibits the highest average interspecific distance (0.2029), with *matK* in the middle (0.0963). For DNA barcoding analyses, interspecific divergence must be higher than intraspecific divergence for species delimitation (Gao et al. 2010). The genetic variation test based on K2P distance and the Wilcoxon sum rank test for *trnH-psbA* showed that interspecific distance was significantly higher than intraspecific distance ($p < 0.00$) (Supplementary Table S2), indicating that a barcode gap exists for *trnH-psbA*.

Suesatpanit et al. (2017) reported that two species, *C. nutans* and *C. siamensis*, are synonymous according to The Plant List (2013). Furthermore, the combination of DNA barcoding and the High Resolution Melting (Bar-HRM) technique based on the *ITS2* marker showed that the melting curves of *C. nutans* and *C. siamensis* were the only samples in the species-level test that could not be discriminated from each other (Suesatpanit et al. 2017). Nevertheless, Queen Sirikit Botanic Garden recognizes them as different species based on their Thai common name, “Pha-Ya-Yo” or “Sa-Led-Porn-Tua-Mea” for *C. nutans*, which means female mongoose’s saliva, and “Lin-Ngu-Hao” or the less common “Sa-Led-PangPorn-Tua-Poo” for *C. siamensis*, which means male mongoose’s saliva. Our findings are similar to those of Alam et al. (2016), who reported that the two species have different pharmacological characteristics, molecular aspects, and anti-herpes simplex virus (HSV) type 1 and type 2 activities (Kunsorn et al. 2013). In summary, our data show that molecular identification using *trnH-psbA* can distinguish *C. nutans* from *C. siamensis*.

Conclusions

Results of this study showed that *trnH-psbA* is a suitable plastid DNA marker for identification of *C. nutans*. This result illustrates that a single plastid DNA marker can have strong discriminatory value when comparing closely related species. Thus, use of that *trnH-psbA* will be helpful in the study of taxonomy, ecology, phylogeny, and morphology of *C. nutans* and other species in the Acanthaceae family. This inexpensive and simple method likely will prove to be significant in the medicinal plant industry to avoid misidentification of medicinal plants.

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Author contributions NZI carried out the experiment and wrote a manuscript draft with support from the HA. MRS and MRH supervised the project, and the ASO conceived the original idea.

Compliance with ethical standards

Conflict of interest On behalf of all authors, the corresponding author states that there is no conflict of interest.

References

- Alam A, Ferdosh S, Ghafoor KA, Hakim A, Juraimi AS, Khatib A, Sarker ZI (2016) *Clinacanthus nutans*: a review of the medicinal uses, pharmacology and phytochemistry. Asian Pac J Trop Med 9(4):402–409. <https://doi.org/10.1016/j.apjtm.2016.03.011>
- Arullappan S, Rajamanickam P, Thevar N, Kodimani CC (2014) In vitro screening of cytotoxic, antimicrobial and antioxidant activities of *Clinacanthus nutans* (Acanthaceae) leaf extracts. Trop J Pharm Res 13(9):1455–1461. <https://doi.org/10.4314/tjpr.v13i9.11>
- Aziz NA, Ahmad MI, Naim DM (2015) Molecular DNA identification of medicinal plants used by traditional healers in Malaysia. Genet Mol Biol 14(4):5937–15947. <https://doi.org/10.4238/2015.december.7.5>
- Brown SD, Collins RA, Boyer S, Lefort MC, Malumbres-Olarte JAGODA, Vink CJ, Cruickshank RH (2012) Spider: an R package for the analysis of species identity and evolution, with particular reference to DNA barcoding. Mol Ecol Resour 12(3):562–565. <https://doi.org/10.1111/j.1755-0998.2011.03108.x>
- CBOL Plant Working Group (2009) A DNA barcode for land plants. PNAS 106:12794–12797. <https://doi.org/10.1073/pnas.0905845106>
- Chase MW, Cowan RS, Hollingsworth PM, Van den Berg C, Madrinan S et al (2007) A proposal for a standardised protocol to barcode all land plants. Taxon 56:295–299
- Chelyn JL, Omar MH, Mohd Yousof NSA, Rangasamy R, Wasiman MI, Ismail Z (2014) Analysis of flavone C-glycosides in the leaves of *Clinacanthus nutans* (Burm. f.) Lindau by HPTLC and HPLC- UV/DAD. Sci World J 2014:1–6. <https://doi.org/10.1155/2014/724267>
- Chen S, Yao H, Han J, Liu C, Song J, Shi L, Zhu Y, Ma X, Gao T, Pang X (2010) Validation of the *ITS2* region as a novel DNA barcode for identifying medicinal plant species. PLoS One 5(1):e8613. <https://doi.org/10.1371/journal.pone.0008613>
- Clement WL, Donoghue MJ (2012) Barcoding success as a function of phylogenetic relatedness in *Viburnum*, a clade of woody angiosperms. BMC Evol Biol 12(1):1–13. <https://doi.org/10.1186/1471-2148-12-73>
- Coissac E, Hollingsworth PM, Laverne S, Taberlet P (2016) From barcodes to genomes: extending the concept of DNA barcoding. Mol Ecol 25:1423–1428. <https://doi.org/10.1111/mec.13549>
- Degtjareva G, Logacheva M, Samigullin T, Terentieva E, Valiejo-Roman C (2012) Organization of chloroplast *psbA-trnH* intergenic spacer in dicotyledonous angiosperms of the family Umbelliferae. Biochem (Mosc) 77(9):1056–1064. <https://doi.org/10.1134/S0006297912090131>
- Dong W, Liu J, Yu J, Wang L, Zhou S (2012) Highly variable chloroplast markers for evaluating plant phylogeny at low taxonomic levels and for DNA barcoding. PLoS One 7(4):e35071. <https://doi.org/10.1371/journal.pone.0035071>
- Farsi E, Majid AMSA (2016) *Clinacanthus nutans*, yesterday’s practice, and future’s drug: a comprehensive review. Am J Phytomed Clin Ther 4(4):113–126
- Fazekas AJ, Burgess KS, Kesanakurti PR, Graham SW, Newmaster SG, Husband BC, Percy DM, Hajibabaei M, Barrett SC (2008) Multiple multilocus DNA barcodes from the plastid genome

- discriminate plant species equally well. PLoS One 3(7):e2802. <https://doi.org/10.1371/journal.pone.0002802>
- Fong SY, Piva T, Urban S, Huynh T (2014) Genetic homogeneity of vegetatively propagated *Clinacanthus nutans* (Acanthaceae). J Med Plants Res 8(25):903–914. <https://doi.org/10.5897/JMPR2013.5328>
- Gao T, Yao H, Song J, Zhu Y, Liu C, Chen S (2010) Evaluating the feasibility of using candidate DNA barcodes in discriminating species of the large Asteraceae family. BMC Evol Biol 10(1):1–7. <https://doi.org/10.1186/1471-2148-10-324>
- Gere J, Yessoufou K, Daru BH, Mankga LT, Maurin O, van der Bank M (2013) Incorporating trnH-psbA to the core DNA barcodes improves significantly species discrimination within southern African Combretaceae. DNA barcoding: a practical tool for fundamental and applied biodiversity research. ZooKeys 365:127–147. <https://doi.org/10.3897/zookeys.365.5728>
- Hebert PD, Cywinska A, Ball SL (2003) Biological identifications through DNA barcodes. Proc R Soc Lond B Biol Sci 270(1512):313–321. <https://doi.org/10.1098/rspb.2002.2218>
- Hollingsworth PM, Andra Clark A, Forrest LL, Richardson J, Pennington RT et al (2009) Selecting barcoding loci for plants: evaluation of seven candidate loci with species-level sampling in three divergent groups of land plants. Mol Ecol Resour 9:439–457. <http://doi.org/10.1111/j.1755-0998.2008.02439.x>
- Ismail NZ, Arsad H, Samian MR, Ab Majid AH, Hamdan MR (2016) Evaluation of genetic diversity of *Clinacanthus nutans* (Acanthaceae) using RAPD, ISSR and RAMP markers. Physiol Mol Biol Plants 22(4):523–534. <https://doi.org/10.1007/s12298-016-0391-x>
- Kress WJ, Erickson DL (2008) DNA barcodes: genes, genomics, and bioinformatics. Proc Natl Acad Sci USA 105(8):2761–2762. <http://doi.org/10.1073/pnas.0800476105>
- Kunsorn P, Ruangrunsi N, Lipipun V, Khanboon A, Rungsirunrat K (2013) The identities and anti-herpes simplex virus activity of *Clinacanthus nutans* and *Clinacanthus siamensis*. Asian Pac J Trop Med 3(4):284–290. [https://doi.org/10.1016/S2221-1691\(13\)60064-7](https://doi.org/10.1016/S2221-1691(13)60064-7)
- Lahaye R, van der Bank M, Bogarin D, Warner J, Pupulin F, Gigot G, Maurin O, Duthoit S, Barraclough TG, Savolainen V (2008) DNA barcoding the floras of biodiversity hotspots. Proc Natl Acad Sci USA 105(8):2923–2928. <https://doi.org/10.1073/pnas.0709936105>
- Liu J, Moeller M, Gao LM, Zhang DQ, Li DZ (2011) DNA barcoding for the discrimination of *Eurasian yews* (Taxus L., Taxaceae) and the discovery of cryptic species. Mol Ecol Resour 11(1):89–100. <https://doi.org/10.1111/j.1755-0998.2010.02907.x>
- Maloukh L, Kumarappan A, Jarrar M, Salehi J, El-wakil H, Lakshmi TR (2017) Discriminatory power of rbcL barcode locus for authentication of some of United Arab Emirates (UAE) native plants. 3Biotech 7(2):1–7. <https://doi.org/10.1007/s13205-017-0746-1>
- Meier R, Shiyang K, Vaidya G, Ng PK (2006) DNA barcoding and taxonomy in Diptera: a tale of high intraspecific variability and low identification success. Syst Biol 55(5):715–728. <https://doi.org/10.1080/10635150600969864>
- Newmaster S, Fazekas A, Steeves R, Janovec J (2008) Testing candidate plant barcode regions in the Myristicaceae. Mol Ecol Resour 8(3):480–490. <https://doi.org/10.1111/j.1471-8286.2007.02002.x>
- Pettengill JB, Neel MC (2010) An evaluation of candidate plant DNA barcodes and assignment methods in diagnosing 29 species in the genus *Agalinis* (Orobanchaceae). Am J Bot 97(8):1391–1406. <http://doi.org/10.3732/ajb.0900176>
- Roy S, Tyagi A, Shukla V, Kumar A, Singh UM, Chaudhary LB, Datt B, Bag SK, Singh PK, Nair NK (2010) Universal plant DNA barcode loci may not work in complex groups: a case study with Indian *Berberis* species. PLoS One 5(10):e13674. <https://doi.org/10.1371/journal.pone.0013674>
- Sakdarat S, Shuyprom A, Pientong C, Ekalaksananan T, Thongchai S (2009) Bioactive constituents from the leaves of *Clinacanthus nutans* Lindau. Bioorg Med Chem 17(15):1857–1860. <https://doi.org/10.1016/j.bmc.2009.01.059>
- Starr JR, Naczi RF, Chouinard BN (2009) Plant DNA barcodes and species resolution in sedges (*Carex*, Cyperaceae). Mol Ecol Resour 9(s1):151–163. <https://doi.org/10.1111/j.1755-0998.2009.02640.x>
- Steven GN, Subramanyam R (2009) Testing plant barcoding in a sister species complex of pantropical *Acacia* (Mimosoideae, Fabaceae). Mol Ecol Resour 9(s1):172–180. <https://doi.org/10.1111/j.1755-0998.2009.02642.x>
- Suesatpanit T, Osathanunkul K, Madesis P, Osathanunkul M (2017) Should DNA sequence be incorporated with other taxonomical data for routine identifying of plant species? BMC Complement Altern Med 17(437):1–10. <https://doi.org/10.1186/s12906-017-1937-3>
- Sun X, Bai M, Yao H, Guo J, Li M, Hang Y (2013) DNA barcoding of populations of *Fallopia multiflora*, an indigenous herb in China. Genet Mol Res 12(3):4078–4089. <https://doi.org/10.4238/2013.September.27.9.4>
- Sundberg P, Kvist S, Strand M (2016) Evaluating the utility of single-locus DNA barcoding for the identification of ribbon worms (phylum Nemertea). PLoS One 11(5):e0155541. <https://doi.org/10.1371/journal.pone.0155541>
- Tallei TE, Kolondam BJ (2015) DNA barcoding of Sangihe Nutmeg (*Myristica fragrans*) using matK gene. HAYATI J Biosci 22(1):41–47. <https://doi.org/10.4308/hjb.22.1.41>
- Tang YL, Wu YS, Huang RS, Chao NX, Liu Y, Xu P, Li KZ, Cai DZ, Luo Y (2016) Molecular identification of *Uncaria* (Gouteng) through DNA barcoding. Chin Med J 11(1):1–14. <https://doi.org/10.1186/s13020-015-0072-7>
- Techen N, Parveen I, Pan Z, Khan IA (2014) DNA barcoding of medicinal plant material for identification. Curr Opin Biotechnol 25(2014):103–110. <https://doi.org/10.1016/j.copbio.2013.09.010>
- The Plant List (2013). *Clinacanthus nutans* (Burm.f.) Lindau. <http://www.theplantlist.org/tpl1.1/record/kew-2727901>. Accessed 22 Mar 2015
- Tripathi AM, Tyagi A, Kumar A, Singh A, Singh S, Chaudhary LB, Roy S (2013) The internal transcribed spacer (ITS) region and trnH-psbA are suitable candidate loci for DNA barcoding of tropical tree species of India. PLoS One 8(2):e57934. <https://doi.org/10.1371/journal.pone.0057934>
- Wanikiat P, Panthong A, Sujayanon P, Yoosook C, Rossi AG, Reutrakul V (2008) The anti-inflammatory effects and the inhibition of neutrophil responsiveness by *Barleria lupulina* and *Clinacanthus nutans* extracts. J Ethnopharmacol 116(2):234–244. <https://doi.org/10.1016/j.jep.2007.11.035>
- Zheng SH, Ren WG, Wang ZH, Huang LF (2015) Use of chloroplast DNA barcodes to identify *Osmunda japonica* and its adulterants. Plant Syst Evol 301(7):1843–1850. <https://doi.org/10.1007/s00606-015-1197-y>