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RESEARCH ARTICLE

Comparison of Six DNA Extraction Procedures and the Application of Plastid DNA Enrichment Methods in Selected Non-photosynthetic Plants

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ABSTRACT: Genomic DNA was isolated using three DNA extraction commercial kits and three CTAB-based methods for two non-photosynthetic plants, *Balanophora japonica* and *Mitrastemon kanehirai*. The quality of the isolated DNA was evaluated and subjected to following restriction enzyme digestions. All six procedures yielded DNA of sufficient quality for PCR, and the method described by Barnwell *et al*. (1998) performed well in isolating DNA from both species for restriction enzyme digestion. In addition, we succeeded to enrich plastid DNA content by using the methods depending on a high salt buffer to deplete nuclear material. The 'high salt' methods based on protocol presented by Milligan (1989) were able to increase plastid DNA effectively and significantly reduce nuclear DNA from *M. kanehirai*. The plastid DNA enrichment protocols are inexpensive and not time-consuming, and may be applicable to other non-photosynthetic plants.

KEY WORDS: CTAB, DNA isolation, heterotrophic plants, plastid DNA, polysaccharide.

INTRODUCTION

Many heterotrophic plants contain polysaccharides and other secondary metabolites that interfere with DNA isolations (Hayata, 1913; Do and Adams, 1991; Scott and Playford, 1996; Nickrent et al., 2000; Tsai et al., 2008; Yu et al., 2010; Wang et al., 2012). These compounds sometimes prevent enzymes to access DNA, and therefore inhibiting follow-up experiments such as polymerase chain reaction (PCR) or restriction enzyme digestions. Many DNA extraction methods, including most commercial kits, are generally designed for cultivated species, which contain much less interfering compounds for isolating DNAs, and therefore might be inapplicable for heterotrophic plants (Do and Adams, 1991; Scott and Playford, 1996). Previous studies (Nickrent et al., 1997a; Nickrent et al., 1997b) showed that the CTAB-based method described by Nickrent (1994) can successfully extract DNA from non-photosynthetic plants with quality good enough for PCR, but is insufficient for enzyme digestions.

We have applied the common DNA extraction methods (Doyle and Doyle, 1987) to the non-photosynthetic parasitic *Balanophora* species, and it is generally fine to obtain good quality DNA for PCR amplification (Su and Hu, 2012; Su et al., 2012). However, the *Balanophora* DNA from such methods sometimes failed to perform well in enzyme digestions in our preliminary surveys. Furthermore, ordinary DNA extraction methods cannot guarantee to have enough plastid DNA since very few plastids are present in the cells of the non-photosynthetic plants (dePamphilis and Palmer, 1990; Nickrent et al., 1997b; Nickrent et al., 2000). All available methods for plastid DNA isolation are designed for isolating ordinary non-reduced chloroplasts in green plants, for example, the gradient-based methods for plastid isolation (Kolodner and Tewari, 1975; Palmer, 1986), and others to enrich organelles (e.g. Herrmann, 1982; Palmer, 1982; Bookjans et al., 1984; Milligan, 1989; Triboush et al., 1998; Kausch et al., 1999). The isolation methods with DNAase-I treatment postulated by Herrmann (1982) require a large amount of plant samples, which is also unpractical in our study, since the plant materials are usually limited.

However, some methods (e.g. Milligan, 1989; Triboush et al., 1998) have been used without the requirement on the color of materials and just need a small amount of tissue. The Milligan's procedure (1989) depends on a high salt buffer to solubilize nuclear material in order to obtain a chloroplast fraction, and Triboush et al. (1989) isolated organelles by differential centrifugation. These methods are thus have potentials for plastid isolation in non-photosynthetic plants.

In our preliminary survey on 12 DNA extraction methods for two non-photosynthetic plants, *Balanophora japonica* Makino and *Mitrastemon kanehirai* Yamamoto, six of them showed promising results, while the others performed badly, with low or no yield of DNA (see Table S1). In this report, we compared the performance of the six procedures that successfully isolated DNA from these two non-photosynthetic plants, and evaluated with the

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following PCR and restriction enzyme digestion. The procedures include three DNA extraction commercial kits, the two methods mentioned above and another CTAB-based method that Barnwell et al. (1998) developed for the highly mucilaginous succulent plants. At the same time, we attempted to enrich plastid DNA content during extraction of *M. kanehirai* DNA for studying its plastid genome by using and modifying the Milligan and Triboush methods. The results were compared with the proportion of plastid vs. nuclear DNA content among the three different plastid enrichment methods.

MATERIALS AND METHODS

Plant Materials

Balanophora japonica and *M. kanehirai* are both non-photosynthetic plants native to Taiwan. The materials (*B. japonica*: Mt. Datong, Taipei County, Sep. 29, 2005, *Hu1567*; *M. kanehirai*: Lienhuachih, Nantou County, Oct. 12, 2010, *Hu1810*) were freshly collected and stored in -20°C. Frozen tissues were used for each DNA extraction method.

DNA extraction methods

The methods used for comparison are listed in Table 1, including three commercial kits (*Method 1–3*) and three CTAB-based methods (*Method 4–6*).

Total genomic DNAs were extracted by *Method 1–3* according to the corresponding manufacturer's protocols. *Method 1*, the DNeasy Plant Mini Kit (QIAGEN), uses the QIAshredder spin column to remove initial precipitates and cell debris, and a DNeasy column to capture DNA. *Method 2*, the Tri-Plant Genomic DNA Reagent Kit (Geneaid), and *Method 3*, the TRI Reagent (Molecular Research Center), both use their own particular reagents to lyse plant samples and then follow by an isopropanol or ethanol precipitation.

Method 4 denoted for the standard CTAB method described by Doyle and Doyle (1987). Plant materials were ground in liquid nitrogen and then incubated in 10 volumes of preheated 2× CTAB buffer (100 mM Tris-HCl, 1.4 M NaCl, 20 mM EDTA, 2% w/v CTAB, 2% w/v PVP40 and added 0.2% β-mercaptoethanol just before use.) at 65°C for 1 h with occasional swirling. The solution was mixed with 10 mL of Chloroform:isoamyl alcohol (24:1, v/v) and was blended thoroughly. This was followed by a centrifugation at 9,000 g for 10 min, and the aqueous phase was transferred to a new centrifuge tube. The DNA was precipitated by adding 0.7 volume of isopropanol and incubated at -20°C for 30 min. The DNA pellet was collected by a centrifugation for 10 min at 10,000 g and washed with cold 75% ethanol. The pellet was resuspended in 2 mL of TE buffer, and then RNase digestion was performed.

Method 5 denoted for the "delayed hot CTAB" method described by Nickrent (1994). The sample was cut into small pieces and homogenized with hot 95°C CTAB buffer (about 25 mL for every 2–3 g of plant tissue). The modified 2× CTAB buffer is composed of 100 mM Tris-HCl, 1.4 M NaCl, 30 mM EDTA, 2% w/v CTAB, 5 mM ascorbic acid, 4 mM diethyldithiocarbamic acid and 2% w/v PVP40, the latter two ingredients were added just before use. The extract was strained through cheesecloth into 50-mL centrifuge tube and then incubated at 70–80°C for 30 min with occasional swirling. The sample was briefly centrifuged without pausing, and the supernatant was transferred to a new tube. Chloroform:isoamyl alcohol (24:1) (0.7 volume) was added and the solution was mixed for 5 min. This was then centrifuged at 9,000 g for 15 min, and the aqueous phase was transferred to a new centrifuge tube. The DNA was precipitated by adding 0.7 volume of ice-cold isopropanol and incubated at -20°C for at least 1 h. The DNA pellet was collected by a centrifugation for 20 min at 10,000 g. Then the pellet was resuspended in 3 mL of TE and 2 mL of 4 M NH_4OAc. This was followed by an extraction with an equal volume of phenol:chloroform (1:1), and 2 volumes of ethanol were added to the aqueous phase. The content was incubated at -20°C for at least 30 min, and the DNA pellet was collected by a centrifugation, then proceeded an RNase treatment.

Method 6 denoted for the extraction procedure developed by Barnwell et al. (1998) for the highly mucilaginous succulent plants. The frozen plant tissue was ground to powder, 5 volumes of extraction buffer (100 mM Tris-HCl, 1.4 M NaCl, 20 mM Na_2 EDTA, 2% w/v CTAB, 1% w/v PVP40) were added and mixed. The homogenate was then incubated at 65°C for 30 min with occasional shaking followed by a centrifugation at 3,000 g for 5 min. The supernatant was mixed with 1.25 volumes of 10% CTAB (w/v, in 0.7 M NaCl), and the mixture was vortexed for 10 s and centrifuged at 3,000 g for 5 min. The supernatant was thoroughly mixed with 3 volumes of precipitation buffer (50 mM Tris-HCl, 10 mM Na_2 EDTA, 1% w/v CTAB). The mixture was incubated at room temperature for 30 min and then centrifuged at 5,000 g for 15 min. The pellet was dissolved in high salt TE buffer (10 mM Tris-HCl, 1.0 M NaCl, 1 mM Na_2 EDTA), and 2 volumes of ice-cold ethanol were added followed by incubation at -20°C for 1 h. The DNA was pelleted by a centrifugation and washed twice with 70% ethanol.

Plastid DNA enrichment methods

Three plastid enrichment methods were analyzed in this study. *Method PE1* denoted for the procedure

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Table 1. The methods used in this study.

Method		Reference
DNA isolation		
Method 1	Column-based commercial kit	DNeasy Plant Mini Kit, QIAGEN, Manchester, UK
Method 2	Particular reagent- based commercial kit	Tri-Plant Genomic DNA Reagent Kit, Geneaid, New Taipei City, Taiwan
Method 3	Particular reagent- based commercial kit	TRI Reagent – RNA, DNA, protein isolation reagent, Molecular Research Center, Cincinnati, OH, USA
Method 4	The standard CTAB method	Doyle and Doyle, 1987
Method 5	The delayed hot CTAB method	Nickrent, 1994
Method 6	The increased CTAB method	Barnwell et al., 1998
Plastid DNA enrichment		
PE1	Depleted nuclear material by using a high salt buffer	Milligan, 1989
PE2	Combined *PE1* with *Method 2*	Milligan, 1989
PE3	Isolated organelles by differential centrifugation	Milligan, 1989; Triboush et al., 1998

developed by Milligan (1989) that incorporates several other methods of extracting chloroplast DNA. The procedure is summarized below. The tissue was ground with 6 volumes of ice-cold isolation buffer (50 mM Tris-HCl, 1.25 M NaCl, 5 mM EDTA, 0.1% w/v BSA, 0.1% β-mercaptoethanol), and the homogenate was filtered through 4 layers of cheesecloth. The plastids were pelleted by a centrifugation at 3,000 g for 10 min and then resuspended in 10 mL of cold isolation buffer. The centrifugation and resuspension were repeated once, and 0.1 volume of 10% CTAB was added to lyse the plastids. The extract was then incubated at 60°C for 1 h, and followed by extraction with Chloroform:iso-amyl alcohol (24:1). The DNA was precipitated by adding 0.7 volume of cold isopropanol to the aqueous phase and incubated at -20°C for at least 30 min. During the isolation of plastids, the materials should be kept at 4°C.

Method PE2 denoted for a method combined with Milligan's protocol (1989) and the Tri-Plant Genomic DNA Reagent Kit. Plastid pellet was isolated by centrifugation at 6,000 g for 20 min instead of 3,000 g for 10 min following Milligan's protocol. The DNA was then extracted from pellet by using Tri-Plant Genomic DNA Reagent Kit.

Method PE3 denoted for the method mainly based on Triboush's DNA extraction method (Triboush et al., 1998), combined with Milligan's protocol (1989), as described below. All the operations of isolating plastids were conducted in ice. The sample was homogenized with 6 volumes of STE buffer (50 mM Tris-HCl, 400 mM sucrose, 20 mM Na_2EDTA, 0.2% w/v BSA, 0.2% β-mercaptoethanol). The homogenate was filtered through 4 layers of cheesecloth, and centrifuged at 200 g for 20 min. The supernatant was centrifuged at 3,700 g for 20 min, and the pellet was resuspened in 20 mL of isolation buffer (based on Milligan's protocol). It was then repeated the centrifugation and resuspension once, and then the DNA was obtained by following Milligan's protocol.

Real-time PCR

Real-time PCR and data analysis were performed in the CFX96™ Real-Time PCR Detection Systems (Bio-Rad Laboratories). Sequences of nuclear and plastid SSU fragments were amplified from *M. kanehirai* DNA extracted by different methods. The primers SSU1594F: 5'-CTACGTCCCTGCCCTTTGTA-3' and SSU1703R: 5'-GGACTTCTCGCGGCATCACGAG-3' were used to amplify a nuclear 18S rDNA fragment; the primers 16S298F: 5'-GGAAACAGCCCAGATCATCA-3' and 16S436R: 5'-GCCGACATTCTCACTTCTGC-3' were used to amplify the plastid 16S rDNA. The primers were designed based on

Mitrastemon kanehirai sequences in our preliminary survey. The PCR mixture (20 μL) contained 10 μL KAPA SYBR FAST qPCR Master Mix (Kapa Biosystems), 50 nM (nr18S rDNA) or 100 nM (pt16S rDNA) of each primer and 20 ng of extracted DNA was used as template. The amplification program initiated at 95°C for 3 min, followed by 40 cycles at 95°C for 10 s and 64°C for 30 s, and finally 95°C for 10 s. Melting curve analysis was carried out after amplification. All experiments were performed in triplicate.

RESULTS AND DISCUSSIONS

Performance of different isolation procedures

Balanophora japonica and *M. kanehirai* both lost their photosynthetic ability completely and plants are very rich in polysaccharides and other secondary metabolites (Hayata, 1913; Wang et al., 2012). Many of the commercial kits and methods failed to extract high quality DNA from heterotrophic plants (listed in supplementary data Table S1). Here we show the six methods that can successfully extract the DNAs from

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these two non-photosynthetic plants.

The ratios of the absorbance at 260 and 280 nm ($A_{260/280}$) of DNA isolated from two species with different procedures are in the range of 1.28–2.11 (Table 2). Two of the three CATB-based methods (*Method 4* and *6*) yielded better results than all the others, including the commercial kits, for *B. japonica*. In comparison, *Method 2* and *6* worked best for *M. kanehirai*.

Only *Method 6* (Barnwell et al., 1998) performed well on both *B. japonica* and *M. kanehirai*, with acceptable $A_{260/280}$ ratio. However, *Methods 2* and *4* showed inconsistent results between *Balanophora* and *Mitrastemon*. Nonetheless, the quality of all DNA isolated by different procedures were good enough for the following PCR (data not shown).

To further examine the DNA quality, we proceeded with restriction enzyme digestion on the obtained DNA extracts. The result shows that the *Method 6* (Barnwell protocol) performed better than the other procedures in extracting DNA from *B. japonica* (Fig. 1A), and *Method 2* (the Tri-Plant Genomic DNA Reagent Kit) performed best in *M. kanehirai* (Fig. 1B). In general, all procedures performed better in *M. kanehirai* which is likely because it contains less polysaccharides than *B. japonica*. However, *Method 3* (the TRI Reagent) and *Method 4* (the Doyle and Doyle protocol) could not produce DNA bands for *M. kanehirai* (M3 and M4 in Fig. 1), compared to *B. japonica*. From all of the commercial kits tested, we found that most kits extracted DNA by using columns did not perform well. It is probably because these kits were unable to eradicate polysaccharides that prevent the elution of DNA from columns and result in the low yields (Fleischmann and Heubl, 2009). Among the three CTAB-based DNA isolation methods, it seems that shortening the initial incubation time, and increasing the incubation temperature could improve DNA purity, since the *Method 5* (Nickrent protocol) performed better than *Method 4*. However, *Method 6* that Barnwell *et al*. (1998) developed increases CTAB concentration in a step-wise manner in order to avoid co-precipitation of polysaccharides with DNA, which was the most effective procedure to obtain DNA with high quality. As for yields, all the commercial kits produced more amount of DNA than the CTAB-based methods. The *Method 6* produced least but the purest DNA among all procedures, which might result from the protocol's additional purification steps and less efficient precipitation buffer.

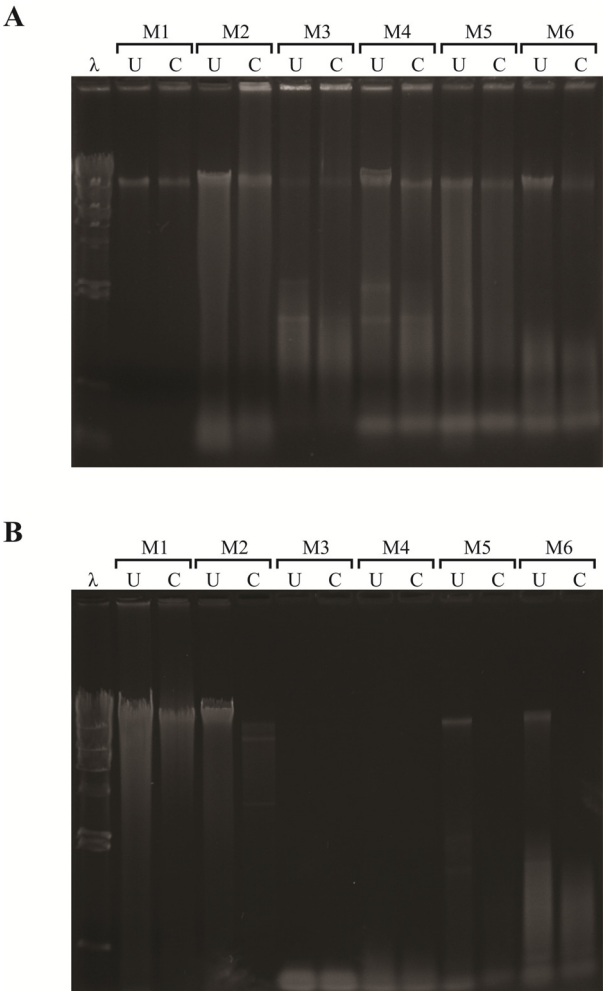


Fig. 1. The results of restriction enzyme digestion on the isolated DNAs. A: *Balanophora japonica*. B: *Mitrastemon kanehirai*. 4 μg DNA was digested with 4 U *Eco*RI/μg DNA for 1 h at 37°C, and then were separated on a 0.8% TAE agarose gel. U, uncut DNA; C, cut DNA; λ, lambda DNA/*Hin*dIII marker; M1, QIAGEN DNeasy Plant Mini Kit; M2, Geneaid Tri-Plant Genomic DNA Reagent Kit; M3, Molecular Research Center TRI Reagent; M4, the Doyle and Doyle protocol; M5, the Nickrent protocol; M6, the Barnwell protocol.

Plastid DNA enrichment

The Milligan's 'high salt buffer' method (1989) and the Triboush's differential centrifugation method (1998) were used and modified to enrich plastid DNA content for extracting *M. kanehirai* DNA. The performance of these procedures was evaluated by relative quantification of nr18S and pt16S rDNA in the DNAs extracted by different methods with Barnwell protocol as the reference. DNA isolated by all methods (included the Barnwell protocol) could amplify nr18S and pt16S rDNA fragments by real-time PCR (Fig. 2). However, *Method PE3* (Triboush-based method) not only failed to enrich plastid DNA in our tests but also yielded the lowest quality DNA that the *Cq* (quantification cycle) values of *PE3* were the largest among all methods in

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Table 2. $A_{260/280}$ ratios of DNA extracted by commercial kits and CTAB-based methods.

Procedure	$A_{260/280}$	
	B. japonica	***M. kanehirai***
Kit		
Method 1	1.75	1.76
Method 2	1.28	2.08
Method 3	1.91	1.48
CTAB-based Method		
Method 4	2.06	1.73
Method 5	1.83	1.81
Method 6	2.10	2.11

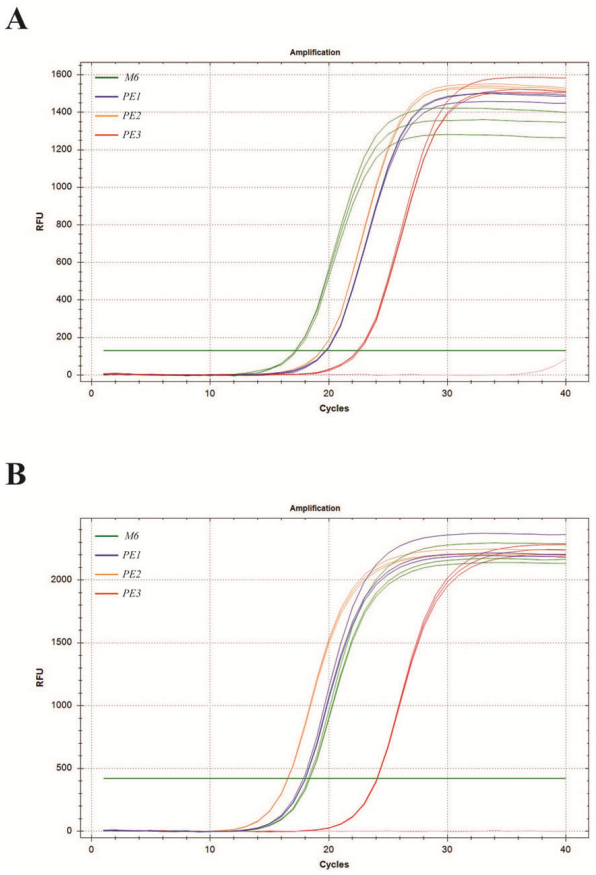


Fig. 2. Real-time PCR amplification plot of *Mitrastemon kanehirai* DNA. A: nr18S rDNA. B: pt16S rDNA. M6, the Barnwell protocol; PE1, the Milligan protocol; PE2, the Milligan protocol combined with Geneaid Tri-Plant Genomic DNA Reagent Kit; PE3, the Triboush method combined with Milligan protocol.

both nr18S and pt16S rDNA. The two Milligan-based protocols (*Method PE1* and *PE2*) were capable of increasing plastid DNA content more than 1.5 times and meanwhile reduced the proportion of nuclear DNA effectively (Fig. 3). The pt16S/nr18S rDNA ratios of extracted DNA by using these two procedures are significantly higher than *Method 6* (Fig. 4). The best performed method was *Method PE2*, the Milligan protocol combined with the Tri-Plant Genomic DNA Reagent Kit, which yielded pt16S/nr18S rDNA ratio almost 15 times higher than *Method 6* with good quality of DNA from *M. kanehirai*. It suggests that increasing centrifugal speed in the beginning step of collecting plastids could enrich plastid DNA further since *Method PE3* performed better than *Method PE2* (Fig. 3 and Fig. 4).

The result shows that the procedure based on differential centrifugation failed to apply to our studying materials. Although the high salt buffer-based protocols could not eliminate nuclear DNA completely, they still could enrich plastid DNA content significantly (*Method PE2 and PE3*). These protocols are inexpensive and not time-consuming, and may be applicable to other non-photosynthetic plants, which will be useful in studying the plastid genome of heterotrophic plants.

Overall, the Barnwell protocol performed best among all examined methods, but it is inapplicable for small amount of plant samples. However, our results suggest that there is no DNA isolation protocol can be applied to all plants because of the presence of various compounds in the plant tissue and it cannot have DNA with both the highest quality and quantity from the same protocols. Additional effort to find out or modify isolation procedures is necessary in order to obtain high quality DNA for non-photosynthetic plants.

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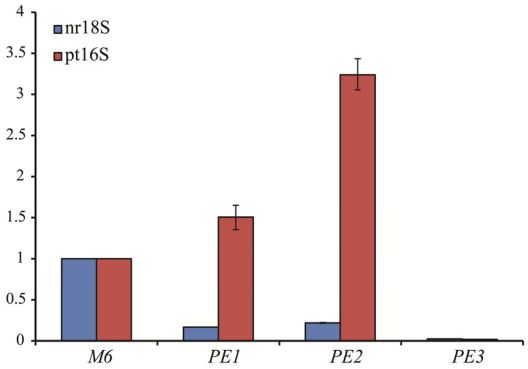


Fig. 3. Relative quantification of nr18S and pt16S rDNA. *Mitrastemon kanehirai* DNAs extracted by three methods were compared with Barnwell protocol as the reference. M6, the Barnwell protocol; PE1, the Milligan protocol; PE2, the Milligan protocol combined with Geneaid Tri-Plant Genomic DNA Reagent Kit; PE3, the Triboush method combined with Milligan protocol.

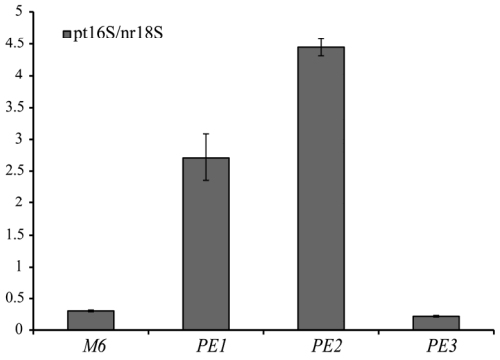


Fig 4. The ratio of pt16S/nr18S rDNA in extracted *Mitrastemon kanehirai* DNAs. The pt16S rDNA content was compared with nr18S rDNA in the same DNA samples extracted by four different procedures. M6, the Barnwell protocol; PE1, the Milligan protocol; PE2, the Milligan protocol combined with Geneaid Tri-Plant Genomic DNA Reagent Kit; PE3, the Triboush method combined with Milligan protocol.

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Supplementary data

Table S1. Other methods have been tested in this study.

Method	Reference
DNA isolation	
Column-based commercial kit	Fast ID Genomic DNA Extraction Kit, Genetic ID, Fairfield, IA, USA[a]
Column-based commercial kit	Plant Genomic DNA Mini Kit, BIOMAN, New Taipei City, Taiwan[a]
Column-based commercial kit	Plant Genomic DNA Purification Kit, GeneMark, Taichung City, Taiwan[a]
Particular reagent- based commercial kit	TRIzol® Reagent, Invitrogen, Carlsbad, CA, USA[b]
The rainforest method	Scott and Playford, 1996[c]
Modified CTAB methods	Doyle and Doyle, 1987; Croy et al., 1993; Sytsma, 1994[c]
Plastid DNA enrichment	
The sunflower method	Triboush et al., 1998[b]

[a] The yields of DNA from these methods were very low, and $A_{260/280}$ ratios of DNAs were below 1.2.
[b] The methods failed to extract DNA from *B. japonica* and *M. kanehirai*.
[c] The methods failed to improve DNA quality compared with *Method 4*.

DNA 萃取方式以及分離葉綠體 DNA 方法在非光合作用植物中的比較與應用

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摘要：利用三種植物DNA抽取套組及三種以CTAB為基礎的萃取方式，萃取兩種非光合作用植物日本蛇菰（*Balanophora japonica*）和菱形奴草（*Mitrastemon kanehirai*）的DNA，隨後測試並比較各種不同方式抽取所得DNA的品質。所有方法抽取出的DNA品質均足以進行PCR；而利用Barnwell等人（1998）針對富含黏液的多肉植物發展出的方法所抽出的蛇菰及奴草DNA，其純度皆能夠進行限制酶切割反應。此外我們採取了Milligan的方法成功地抽取出富含質體DNA（plastid DNA）的菱形奴草DNA。這些基於Milligan在1989年所敘述利用高鹽溶液以去除細胞核DNA的方法能夠有效的增加質體DNA，並且明顯減少了細胞核DNA的含量。這種增加質體DNA的萃取方式不但花費低廉且不需耗費大量時間，並有相當大的可能性能夠成功的運用在其他非光合作用植物上。

關鍵詞：CTAB、DNA萃取、異營性植物、質體DNA、多醣體。

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