

# Two reported cytotypes of the emergent orchid model species *Erycina pusilla* are two different species

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**Abstract** Each species is characterized by a specific set of chromosomes, which is described as the chromosome portrait or karyotype. In general, such a karyotype is the same for all individuals in the population. An exception to that rule has recently been found in the orchid *Erycina pusilla*, which has been reported to have two cytotypes with chromosome numbers of  $2n = 10$  and  $2n = 12$ . Here, we examined the karyotypes of the two cytotypes and found differences in arm ratios and heterochromatin patterns as well as in the presence of satellite chromosomes and in the number and location of rDNA and telomeric repeat sites. These differences are extensive and would have required multiple chromosome rearrangements to generate the differences between the two karyotypes. We also found that F1 hybrids between the parents with the two different chromosome numbers resulted in sterile offspring, in accordance with our previous findings. The combination of hybrid sterility

and extensively rearranged chromosomes supports the hypothesis that these two reported cytotypes are, in fact, two different species.

**Keywords** Speciation · Karyotype · Cytotypes · Chromosome rearrangement · *Erycina pusilla*

## Introduction

The genome of any eukaryotic organism has a characteristic karyotype, with a specific chromosome number and morphology. Such a karyotype is, apart from sex chromosomes and B chromosomes, a constant feature of all individuals of a species. Cytotypes are individuals or populations with one or a few structural or numerical differences in their chromosome sets. Heterozygotes of two different cytotypes can display erratic meiotic behavior, leading to reproductive isolation under certain circumstances, followed by extinction or speciation. The most common cytotypes in a species are those with different chromosome numbers, such as polyploids that have resulted from whole-genome duplications. Polyploidy has been shown to be a common event in the evolution of most angiosperms (De Bodt et al. 2005; Soltis et al. 2008; Jiao et al. 2011; Weiss-Schneeweiss et al. 2013). Polyploids are assumed to have aided survival for land species during and/or after the Cretaceous-Tertiary

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mass extinction (Fawcett et al. 2009). Cytotypes may also differ in small chromosome number variations, which are caused by aneuploidy or dysploidy. Unlike polyploidy or aneuploidy, dysploidy always involves chromosome structural variation caused by chromosome rearrangements, such as translocations and inversions. Compared to polyploid cytotypes there are far fewer examples of cytotypes with chromosome number polymorphisms caused by dysploidy. Examples are *Graomys griseoflavus* ( $2n = 36\text{--}38$ ; Cricetidae) (Theiler et al. 1999) and *Crocus pallasii* subsp. *pallasii* ( $2n = 14$  and  $16$ ; Iridaceae) (Karamplianis et al. 2013). Comparable chromosome polymorphisms have been described in different species of the orchid family, such as *Cephalanthera* (Moscone et al. 2007) and *Paphiopedilum* (Lan and Albert 2011). This variability plays a critical role in species evolution and has been considered to be a consequence of chromosome rearrangement (Faria and Navarro 2010; Heslop-Harrison and Schwarzacher 2011). Such rearrangement may increase or decrease the chromosome number in the karyotype and is termed ascending or descending dysploidy (Schubert and Lysak 2011; Leitch et al. 2013; De Storme and Mason 2014; Yang et al. 2014).

Two cytotypes have been reported for *Erycina pusilla*, i.e., one with  $2n = 10$  (Dodson 1957a, b) and one with  $2n = 12$  (Félix and Guerra 1999, 2000). *Erycina pusilla* (synonym *Psycmorchis pusilla*) is a member of the orchid family and is known as a type of “twig epiphyte”, which refers to its characteristic of small branch habitat (less than 2.5 cm in diameter) (Kolanowska and Szlachetko 2014). Plants grow naturally under a disturbed environment with low levels of sunlight and irregular water supply, leading to the development of fast-growing and short-juvenile features (Chiu et al. 2011). Unlike most orchid species, *E. pusilla* often reaches maturity and the flowering stage within 1 year. Accompanied by a smaller genome size ( $1C = 1.5$  pg) (Chase et al. 2005) and low diploid chromosome number ( $2n = 10$  and  $12$ ), this tiny orchid (less than 10 cm in height at maturity) is a potential orchid model that has been studied intensely in recent years in such areas as in vitro fruiting and seed production (Chiu et al. 2011), categorization of miRNA (Lin et al. 2013), transcriptome analysis (Chou et al. 2013), chloroplast genome analysis (Pan et al. 2012), establishment of an agrobacterium-mediated genetic transformation

procedure (Lee et al. 2015) and transcriptome-wide analysis of the MADS-box gene family (Lin et al. 2016). However, little cytogenetic information has been reported: only a chromosome count and conventional karyotype analysis has been carried out in this orchid. In Yeh et al. (2015) the two  $2n = 10$  and  $2n = 12$  cytotypes, collected from two South American countries, demonstrated high similarity both in genomic composition and DNA amount based on the results of genomic in situ hybridization and flow cytometry. In addition, analyses of meiotic prophase of the  $2n = 10/12$  heterozygotes from reciprocal crosses revealed incomplete pairing, with unpaired loops between parental homoeologous chromosome segments. These results suggested that the chromosome number variation of the two cytotypes have resulted from chromosome rearrangements, rather than changes in repeat content of their genomes. However, the establishment and mechanisms of such structural rearrangements in the karyotypes between these two cytotypes remain unclear.

Identification of chromosome rearrangement and karyotype characterization is generally done by chromosome banding or painting methods. However, conventional banding techniques are limited in resolution only 4–6 Mbp chromosome segments, which covers parts of the condensed metaphase complements (de Jong et al. 1999). The Fluorescent in situ Hybridization (FISH) technique based on probes with single copy and repetitive sequences enables detection of much smaller DNA targets, and can provide unique and consistent information for karyotype comparisons for evolutionary and cytotoxic analyses. The most common repeats for such studies, for example in *Maxillaria* (Orchidaceae) (Cabral et al. 2006), Auranthioideae (Moraes et al. 2012), *Lupinus* (Hajdera et al. 2003), *Pinus* (Hizume et al. 2002) and *Nicotiana* (Chen et al. 2002), include ribosomal DNA (45S and 5S rDNA) and telomere repeat sequences (TTTAGGG). The 45S (5.8S–18S–26S) rDNA genes comprise tandemly repetitive units that are highly conserved among species, which are in most species present in the distal parts of the short arms of one or more chromosome pairs called nucleolar organizer regions (NORs). 5S rDNA genes usually localize separately from the 45S rDNA genes and do not take part in nucleolus formation. The telomere repeat sequence is a conserved marker that identifies chromosome ends; however, some studies have revealed

interstitial telomere sites (Uchida et al. 2002a, b; Tek and Jiang 2004; Shibata et al. 2005), providing strong evidence of recent chromosome fusions and Robertsonian translocations. In this study, we applied the above-mentioned molecular chromosome markers combined with FISH to determine the karyotype differences between the two cytotypes of *E. pusilla*.

## Materials and methods

### Plant material

The two cytotypes of *E. pusilla* were collected from Ecuador ( $2n = 10$ ) and from Surinam ( $2n = 12$ ) and their specimens were deposited in the herbarium of Taiwan's National Museum of Natural Science (Yeh et al. 2015). All plants were maintained and cultured on half-strength Murashige and Skoog medium (Murashige and Skoog 1962), and cultivated in growth chambers under long-day conditions (16-h photoperiod) at 22 °C.

### Chromosome slide preparation

The experimental protocol was according to Chang (Chang et al. 2007, 2008; Szinay et al. 2008) with minor modifications. Fast growing root tips were collected and pre-treated with 2 mM aqueous solution of 8-hydroxyquinoline for 3 h at 15 °C or 1:1 (v/v) 2 mM 8-hydroxyquinoline/0.1% colchicine for 2 h at 15 °C. Root tips were then fixed in freshly prepared Carnoy's solution (3:1 (v/v) 95% ethanol/glacial acetic acid) and stored at -20 °C until use. The fixed material was washed in distilled water and digested with a pectolytic enzyme mixture containing 1% (w/v) cellulose Onozuka RS (Yakult Honsha), 1% (w/v) pectolyase Y23, and 1% (w/v) pectinase solution in a humid chamber at 37 °C for 80 min. Subsequently, the fragile meristem was rinsed carefully with distilled water, squashed in a drop of 45% acetic acid, and the coverslip was removed after freezing in liquid nitrogen. The slides were dried at 42 °C and rinsed in Carnoy's solution and 95% ethanol, respectively. Finally, all slides were observed under a phase-contrast microscope to check the mitotic stage and chromosome quality.

### DNA probe labeling

The 9-kb fragment of the 45S rDNA sequences were from *Triticum aestivum* in plasmid pTA71, and the 5S rDNA sequences were amplified from the genomic DNA of *E. pusilla* by PCR using the oligomer primer set: 5'-GATCCCATCAGAACTTC-3' (forward) and 5'-GGTGCTTTAGTGCTGGTAT-3' (reverse) (Kim et al. 2009). The clone pAt T4 with the *Arabidopsis*-like telomere repeat (TTTAGGG) was used to detect distal and interstitial telomere sites. All DNA probes were generated by nick translation reaction, following the manufacturer's instructions (Roche). DNA samples were randomly nicked and labeled with either biotin-16-dUTP or digoxigenin-11-dUTP. Probes were purified by ethanol precipitation and stored at -20 °C.

### Fluorescence in situ hybridization (FISH)

Selected high quality squashed root tip slides were placed in a Coplin jar and pretreated with pepsin (5 µg/mL in 0.1 M HCl) at 37 °C for 10 min, 4% formaldehyde solution for 10 min, and a graded series of ethanol (70, 90, 100%) for 3 min each. The hybridization mix (20 µL/slide), containing 50 ng of labeled probes, 50% formamide, 10% sodium dextran sulfate, 0.25% SDS (sodium dodecyl sulfate) and 2 × SSC (saline-sodium citrate) was heated in a boiling water bath for 10 min and then cooled down on ice for 10 min. For the hybridization experiments, probe mix was dropped onto each chromosome slide and covered with a coverslip. Slides were placed on a heating block at 80 °C for 2.5 min and hybridization was carried out in a humid chamber at 37 °C for 16–20 h. After hybridization, coverslips were removed and washed by 2 × SSC twice. Slides were washed three times for 5 min in 50% formamide at 42 °C. Blocking solution (5% bovine serum albumin, 0.2% Tween-20 in 4 × SSC) was added and incubated at 37 °C for 30 min. Subsequently, antibodies (labeled with streptavidin or anti-DIG conjugated fluorescent dye) were added and incubated at 37 °C for 1 h. Slides were washed with 2 × SSC, dehydrated through a graded ethanol series, and mounted with 12 µL of 5 µg/mL of 4',6-diamidino-2-phenylindole (DAPI) in Vectashield (Vector Laboratories, Burlingame, CA, USA). The chromosome structure and fluorescent signals were observed and photographed using a

fluorescence microscope (Zeiss) equipped with a digital camera. Images were pseudo-colored and adjusted using AxioVision software (Zeiss).

### Karyotype analysis

At least five metaphase cells of each cytotype, after FISH and DAPI counterstaining, were used for chromosome analysis. Every chromosome was straightened and measured using ImageJ software (<http://imagej.nih.gov/ij/>) using the plugin of Kocsis et al. (1991). Ideograms were generated according to relative chromosome length, arm ratio (length of long arm/length of short arm), heterochromatin distribution, and location of repetitive sequences (5S and 45S rDNA loci, and *Arabidopsis*-like telomere repeat sequences). Chromosomes were numbered and arranged in sequence of decreasing length.

## Results

### Karyotype comparison by DAPI staining

Figure 1 shows representative examples of mitotic metaphase complements of the  $2n = 10$  and  $2n = 12$  cytotypes of *E. pusilla*. Using the chromosome banding as produced by DAPI staining we made ideograms of the two cytotypes. Each chromosome contained a pericentromeric heterochromatin banding pattern and was asymmetrical, with arm ratios ranging from 1.62 to 1.06 (Table 1). The longest pair showed similar arm ratios and heterochromatin patterns in both cytotypes. One chromosome pair of the  $2n = 12$  cytotype displays secondary constrictions, separating

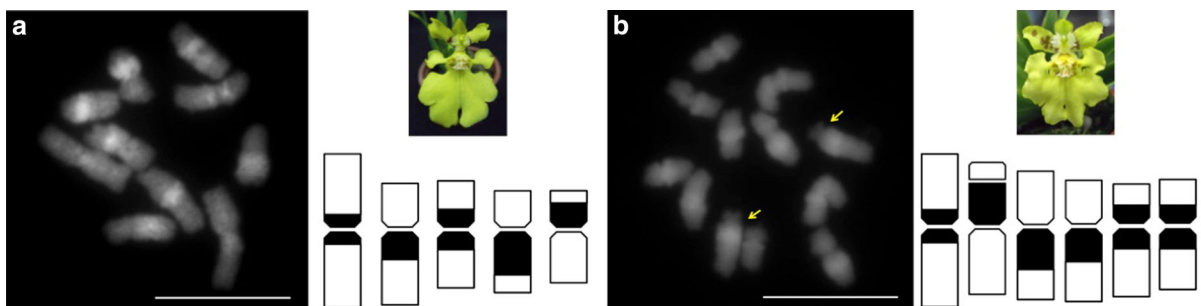
the adjacent satellite from the short arm heterochromatin.

### Cytological mapping of 45S and 5S rDNA genes

A pair of 45S and three pairs of 5S rDNA loci were located on the  $2n = 10$  cytotype (Fig. 2a-c). The 45S rDNA signals were on the terminal sites of a homologous chromosome. One pair of 5S signals was present interstitial on one chromosome pair. The other two 5S signals were situated on both ends of another homologous pair, where the 45S rDNA genes were colocalized with 5S rDNA genes at the terminal ends of the long arms. In the  $2n = 12$  cytotype, one pair of 45S and 5S rDNA loci, were mapped on the distal and interstitial regions of two different homologous chromosomes (Fig. 2d-f), respectively. Thus, the number and location of the 5S rDNA loci differed between these two cytotypes.

### Chromosome positions of the telomere sequences

Telomere foci were observed on both ends of each chromosome (Fig. 3a, b). In addition, in the  $2n = 12$  cytotype, there were four obvious signals located in the centromere regions (Fig. 3b, arrows). Combining conventional (DAPI counterstaining) and molecular cytogenetic data, we constructed ideograms of the two cytotypes (Fig. 4) according to relative chromosome length, heterochromatin banding, satellite structure, arm ratio, and location of fluorescent markers (rDNA and telomere repeat sequence). In addition, Table 2 summarizes the number of rDNA loci, telomere repeat signals, and satellite (SAT) chromosomes between the two cytotypes.



**Fig. 1** Somatic metaphase chromosomes stained with DAPI and ideograms showing comparative chromosome lengths (arm ratio) with distributions of euchromatic (white) heterochromatic

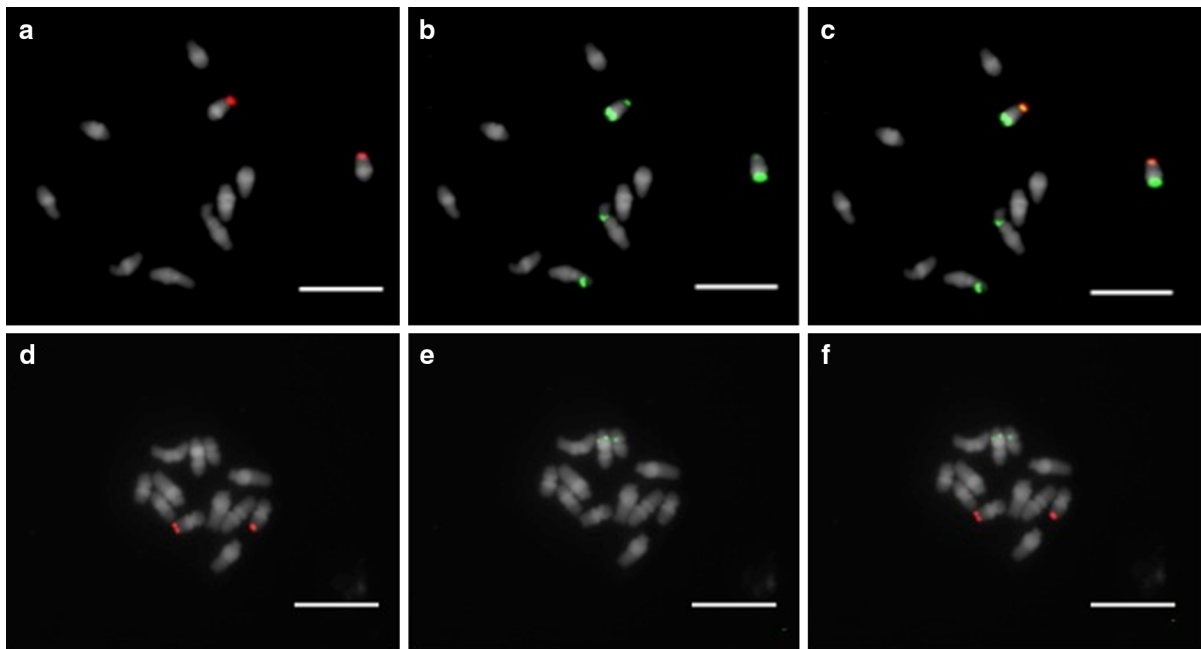
(black) DNA. **a** *Erycina pusilla*,  $2n = 10$ . **b** *Erycina pusilla*,  $2n = 12$ . Arrows indicate satellite chromosomes (SAT chromosomes). The bar represents 10  $\mu$ m

**Table 1** The arm ratios and relative lengths of metaphase chromosomes of two cytotypes (or different species)

2n = 10	Chr. 1	Chr. 2	Chr. 3	Chr. 4	Chr. 5	
Arm ratio	1.06	1.50	1.33	1.35	1.26	
Relative length (%)	25.38	21.15	18.85	18.08	16.54	
2n = 12	Chr. 1	Chr. 2	Chr. 3	Chr. 4	Chr. 5	Chr. 6
Arm ratio	1.06	1.11	1.28	1.62	1.65	1.27
Relative length (%)	19.41	17.35	16.76	16.18	15.59	14.71

Arm ratio = long arm/short arm

Relative length = one chromosome/total chromosomes



**Fig. 2** Cytological mapping of 45S (red) and 5S (green) rDNA loci on the two cytotypes (or different species), 2n = 10 (**a**, **b**, **c**) and 2n = 12 (**d**, **e**, **f**) using bicolor FISH with DAPI

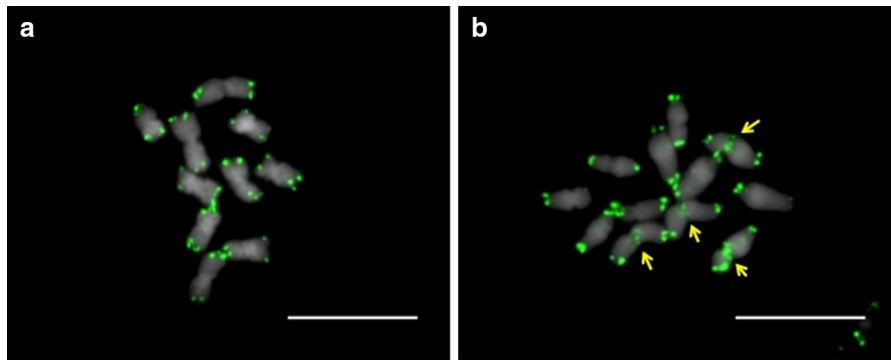
counterstaining. **c** Merged image of (**a**) and (**b**), **f** is the merged image of (**d**) and (**e**). The bar represents 10  $\mu$ m

## Discussion

We performed FISH and found strikingly different karyotypes between the two cytotypes, based on the different distributions of rDNA, telomere repeat sequences, arm ratio, heterochromatin banding, and satellite location. Furthermore, the karyotypes of each cytotype could provide a valuable reference for chromosome evolution and karyotype analysis in orchids.

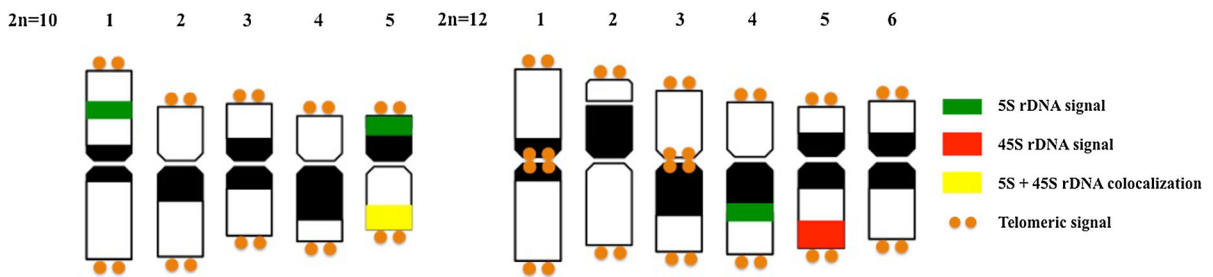
## Karyotype characterization after DAPI staining

In both cytotypes, the heterochromatic bands were distributed adjacent to primary constrictions with varying quantities of fluorescence. Integrating the banding patterns with chromosome length and arm ratio allowed us to generate ideograms. There is only one chromosome (the longest) that seems to be morphologically identical in both cytotypes. All of the other chromosomes differ in arm ratio, fraction and/or location of heterochromatin, or presence of a satellite. There was one chromosome pair (No. 2) of



**Fig. 3** FISH mapping of *Arabidopsis*-like telomeric repeat sequences (green) on the mitotic chromosomes of *E. pusilla*. **a** The cytotype/species with  $2n = 10$ . **b** The cytotype/species

with  $2n = 12$ . Arrows indicate telomeric signals on the centromeric regions. The bar represents  $10 \mu\text{m}$



**Fig. 4** Ideograms of the two cytotypes (different species) showing relative chromosome length, arm ratio, and physical mapping of three molecular makers using FISH techniques: 5S rDNA loci (green), 45S rDNA loci (red), *Arabidopsis*-like telomeric repeat sequences (orange), and rDNA co-localization

(5S + 45S rDNA, yellow). The chromosome pairs are arranged in line with by decreasing length. The numbering of each chromosome is presented according to the decreasing length of each homologous pair of each cytotype/species, respectively

**Table 2** Number of rDNA sites, telomeric repeat signals, and SAT chromosomes of the two cytotypes (or different species) (ND not detectable)

	$2n = 10$	$2n = 12$
Satellite chromosome numbers	ND	2
Number of 5S rDNA sites	6	2
Number of 45S rDNA sites	2	2
Number of 5S + 45S rDNA co-localization	2	ND
Number of telomeric repeat signals	20 (terminal ends)	24 (terminal ends) + 4 (centromeric regions)

cytotype  $2n = 12$  with a secondary constriction. Such constrictions are usually sites of the Nucleolar Organizer Regions (NOR) that provide an essential function in nucleolus formation and function after cell division (Heslop-Harrison and Schwarzacher 2011; Falistocco and Marconi 2013). The NOR is typically the major site of the 45S rDNA gene, but physical mapping of the 45S rDNA loci showed them to be

present on chromosome pair No. 5, rather than No. 2. This might have been caused by translocation between these two chromosomes, leading to the relocation of the 45S rDNA genes. Alternatively, the secondary constrictions could be merely a structural feature, not the location of the NOR (Sato et al. 1980). A previous study on *Allium sativum* indicated that not all NORs are associated with the morphological appearance of

secondary constrictions. Another cytological study implied that both the translocation and deletion could be concerned in numerical variation and the polymorphism of the satellite chromosomes of *Allium cepa* (Sato 1981).

#### Cytogenetic characterization of the rDNA genes and telomeric repeat sequences

Only one pair of terminal 45S rDNA loci was observed in both the  $2n = 10$  and  $2n = 12$  cytotypes. However, the 5S rDNA patterns differed in the two cytotypes. In Fig. 2b, the  $2n = 10$  cytotype showed three pairs of 5S rDNA signals. One pair of 5S rDNA signals was located interstitially in both cytotypes, similar to the pattern observed in *Rhynchospora* (Sousa et al. 2011). The other two pairs were located on the same homologous chromosome at the terminus of each arm. This type of arrangement is not observed frequently, but has been found in *Cephalanthera longifolia* (Moscone et al. 2007), suggesting it could be related to chromosome inversion, with breakages of the 5S rDNA cluster in subtelomere regions. The divergent number and physical location of the 5S rDNA genes could be caused not only by chromosome rearrangement, including inversions, duplications, deletions and translocations (Hanson et al. 1996; Thomas et al. 1996; Raskina et al. 2008; Wang et al. 2013), but also by transposon-mediated transposition events (Raskina et al. 2004; Altinkut et al. 2006; Datson and Murray 2006). Several cases of 5S rDNA-derived sequences being found within transposable elements have been reported (Kapitonov and Jurka 2003; Kalendar et al. 2008). By contrast, loss of rDNA loci during evolution has occurred in Brassicaceae and Rosaceae (Mishima et al. 2002; Hasterok et al. 2006; Liu and Davis 2011). However, the exact mechanism and biological consequence of the variable number of 5S rDNA loci between these two cytotypes remain unknown.

Notably, the 5S rDNA genes were co-localized with the 45S rDNA genes at the long terminal regions of chromosome 5 in cytotype  $2n = 10$ . Such co-localization (adjacent sites) has been reported in studies of the subfamily Aurantioideae (e Silva et al. 2013) and the orchid genus *Paphiopedilum* (Lan and Albert 2011). In Aurantioideae, the closely linked rDNAs were regarded as an ancestral circumstance and the positional changes of the rDNA linkage might have

been caused by chromosome rearrangements or transposition. Furthermore, the adjacent rDNA sites at the terminal ends might be associated with the stabilization of centromere fission events (Dobigny et al. 2003; Chang et al. 2009).

Telomere repeat sequences were located on the ends of every chromosome. Surprisingly, there were two pairs of telomere signals at the centromere regions in the  $2n = 12$  cytotype. We expected that telomere signals should be found at centromeres or interstitial regions of chromosomes in the  $2n = 10$  cytotype, because a reduction in chromosome number results commonly from chromosome fusion. However,  $2n = 10$  possesses a lower chromosome number but has no telomere signals within centromere or interstitial regions. A similar event was documented in the genus *Cephalanthera* (Moscone et al. 2007), where the species *C. damasonium* ( $2n = 36$ ) contained centromeric telomeric signals whereas *C. longifolia* ( $2n = 32$ ) did not. The authors suggested the centromere telomere repeats could have been eliminated after Robertsonian fusion in the lower chromosome number species or that telomere signals in the higher chromosome species were not generated by chromosome fusion, and probably occurred through double-stranded DNA breaks associated with telomerase repair or integration of mobile elements. The rDNA loci and telomere patterns between these two cytotypes indicated that *E. pusilla* has been subjected to complicated chromosome rearrangements during evolution.

#### Two cytotypes or different species?

Conventional staining techniques combined with molecular markers revealed different karyotype characters in two reported cytotypes of *E. pusilla*. The variability of chromosome number and genome organization (heterochromatin and rDNA localization) can provide reliable information to analyze the taxonomic relationship (Félix and Guerra 2000, 2010; Koehler et al. 2008). The elevation of chromosome number has been discovered in many orchid genera, generated from the basic chromosome number by hybridization, polyploidization or dysploid formation (Cox et al. 1998; Félix and Guerra 2005). For example, the basic chromosome number of *Paphiopedilum* was proposed as  $2n = 26$ , and the chromosome number variation under the genus ranged from  $2n = 26$ –42 caused by a

Robertsonian centric fission mechanism (Jones 1998; Lee et al. 2011). It has been proposed that the change in chromosome number is from 12 to 10, even initially from 14, in this species (Yeh et al. 2015) because *E. pusilla* is in the Cymbidioid group, in which the most probable basic chromosome number is  $x = 7$  (Félix and Guerra 2000, 2005, 2010). In this study, we have demonstrated divergent chromosome profiles between two karyotypes that are mostly likely due to multiple steps in chromosome rearrangements. The cytotype/species with  $2n = 12$  showed comparatively dramatic chromosome differences compared with the cytotype/species with  $2n = 10$ , such as multiple steps with a reduction or duplication in 5S rDNA loci, integration of telomeric repeat sequences in centromere regions, and the formation of a secondary constriction structure (Table 2). In addition, we found a chromosome rearrangement that resulted in co-localization of 5S rDNA and 45S rDNA in the  $2n = 12$  cytotype. Furthermore, the arm ratios and heterochromatin patterns are also different in some chromosomes except for chromosome 1s from both cytotypes/species and between chromosome 6 from  $2n = 12$  and chromosome 3 from  $2n = 10$ , which are more stable with least degree of rearrangement. The different arm ratios and heterochromatin patterns are combinations of chromosome segment translocations and the heterochromatin expansion or deletion events. There is a series of multiple chromosome rearrangements required to generate the differences between the two karyotypes. Our previous result from Yeh et al. (2015) as well as our subsequent efforts also showed no evidence so far for fertile F1 hybrids between the parents with the two different karyotypes. The sterile F1 hybrids and our findings in the study support the hypothesis that these are two separate species, not just cytotypes.

To understand the different karyotypes in details and evolutionary relationship between these two species, more chromosome markers and comparative genetics maps should be generated. This study has demonstrated that cytogenetic work is essential to identify species and the karyotype information also provided a preliminary framework for genome sequencing, genetic map construction, and determining the chromosome evolutionary machinery in these potential orchid model plants.

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#### Compliance with ethical standards

**Conflict of interest** The authors declare no conflict of interest.

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