A Karyotype Comparison of Nine Species of Aneuploid *Zamia* by Using the Conventional Orcein Staining and the Fluorochrome CMA-DAPI Differential Staining Methods

Norikazu Tagashira and Katsuhiko Kondo*

Laboratory of Plant Chromosome and Gene Stock, Faculty of Science,
Hiroshima University, Higashi-Hiroshima City 739–8526, Japan

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Summary *Zamia angustifolia*, *Z. integrifolia*, *Z. pumila* and *Z. pygmaea* had the common chromosome number of $2n=16$ and the common karyotype which consisted of 12 median-, 2 submedian- and 2 subterminal-centromeric chromosomes. The 4 species showed commonly the CMA-positive band in the centromeric region of 2 median-, 2 submedian- and 2 subterminal-centromeric chromosomes. In contrast, *Z. furfuracea*, *Z. loddigesii*, *Z. skinneri* and *Z. vazquezii* had commonly the chromosome number of $2n=18$ and 10 median- and 2 submedian-centromeric chromosomes in karyotype but exhibited 6 different chromosomes from each other. *Zamia furfuracea* and *Z. loddigesii* displayed the CMA-positive band in the centromeric region of 8 median- and 6 acro-centromeric chromosomes. *Zamia skinneri* performed the CMA-positive band in the centromeric region of 4 acro- and 2 terminal-centromeric chromosomes, while *Z. vazquezii* performed the CMA-positive band in the centromeric region of 4 median- and 6 terminal-centromeric chromosomes. *Zamia muricata* had the chromosome number of $2n=23$ differed from that of the previous count, the karyotype which consisted of 7 median- and 16 acro-centromeric chromosomes and the CMA-positive band in the centromeric region of 4 median- and 16 acro-centromeric chromosomes. Then, the 9 species of *Zamia* studied exhibited unclear DAPI-positive bands near the centromeric region of most of their chromosomes. By comparison, the closely related *Ceratozamia mexicana* had the chromosome number of $2n=16$ and the CMA-positive band at the terminal region of 6 chromosomes.

Key words Aneuploid, CMA-DAPI differential staining, Orcein karyotype analysis, *Zamia*.

Zamia is taxonomically placed in the Cycadales, the Zamiaceae, the Zamioideae (Stevenson 1990, Jones 1993), and is primarily distributed from Central to South America (Jones 1993). Classification of *Zamia* has been seen in many papers and has described 26 (e.g., Schuster 1932) to 60 species (Jones 1993, Norstog and Nicholls 1997). *Zamia* might be phylogenetically closely related with *Ceratozamia*, *Chigua* and *Microcycas* all of which are distributed in North and South America (Stevenson 1990, Jones 1993).

Zamia has been well studied in diversity of chromosome numbers from $2n=16$ to 28 due to interspecific (Marchant 1968, Vovides 1983, Moretti 1990a,b, Moretti *et al.* 1991, Caputo *et al.* 1996) and intraspecific aneuploids (Norstog 1980, Moretti and Sabato 1984, Moretti *et al.* 1991, Vovides and Olivares 1996). This aneuploid series might be promoted by the centric fusion (Marchant 1968, Norstog 1980) or centric fission (Moretti and Sabato 1984, Moretti 1990a,b, Moretti *et al.* 1991).

Chromosome marking by various banding methods has been applied to many plant species to isolate certain chromosomes which characterized specific taxonomic-groups (e.g., Moretti 1981–82, Tanaka and Hizume 1980). Fluorescence banding techniques by chromomycin A₃ (CMA) and

* Corresponding author: E-mail: kkondo@ipc.hiroshima-u.ac.jp

4',6-diaminido-2-phenyliodole (DAPI) have been applied to chromosomes of several cycads such as *Ceratozamia* (Kokubugata and Kondo 1998), *Cycas* (Hizume *et al.* 1992, Kokubugata and Kondo 1996) and *Microcycas* (Kokubugata and Kondo 1998). Kokubugata and Kondo (1998) demonstrated and stated for the first time that the CMA and DAPI staining methods promised to distinguished certain intergeneric relationships among the cycad genera.

The present study describes chromosome variation and differentiation in 9 species of the aneuploid *Zamia* detected by the conventional aceto-orcein staining and fluorescence CMA-DAPI differential staining methods.

Materials and methods

Nine species of *Zamia* and *Ceratozamia mexicana* Brongn. studied are listed in Table 1. Classification of *Zamia* and *Ceratozamia* followed Jones (1993). The material plants used were male excepting *Z. angustifolia* Jacq. used was female (Table 1).

Meristematic tissues were isolated from young leaflets by the method of Kokubugata and Kondo (1994): Young leaflets were chopped into small pieces ca. 1.5 mm across and were treated with 0.002 M 8-hydroxyquinoline solution at 4°C for 13 h before they were fixed in 1:3 acetic ethanol at 4°C overnight. They were washed and stored in 70% ethanol. The materials were rinsed in distilled water and in 45% acetic acid at room temperature for 10 min each. They were macerated in 45% acetic acid at 60°C for 4 min. The preparations were placed on glass slides, were removed trichomes on their epidermis and were chopped into minute pieces about 0.5 mm across. The preparations were stained in 2% aceto-orcein in humid chamber at room temperature for 4 h. Then, they were squashed on glass slides for chromosome spreading. Chromosome identification followed Levan *et al.* (1964): Median-centromeric chromosome (=m-chromosome; arm ratio=long arm/short arm=1.0–1.7), submedian-centromeric chromosome (=sm-chromosome; arm ratio=1.8–3.0), subterminal-centromeric chromosome (=st-chromosome; arm ratio=3.1–5.0), and terminal-centromeric chromosome (=t- and T-chromosomes; arm ratio=5.1–∞). “t-chromosome” means acrocentric chromosome, and “T-chromosome” means telocentric chromosome. Additionally, microphotographs were made on Minicopy HR-II film (Fuji).

Meristematic tissues used for CMA-DAPI staining were prepared as follows: Young leaflets treated with 0.002 M 8-hydroxyquinoline and fixed in 45% acetic acid were chopped into minute pieces. They were macerated in the enzyme mixture of 3% cellulase Onozuka R-10 (Yakult) and 0.25% Pectolyase Y-23 (Seishin) in distilled water (w/v) at 37°C for 20 min. They were washed in distilled water for 10 min and were squashed in 45% acetic acid on glass slides. The slides were removed coverslip by the dry-ice method and were air-dried.

The fluorescent CMA and DAPI differential staining methods followed Kokubugata and Kondo (1996): The slides were washed in MacIlvain buffer at pH 7.0 for 30 min and were dropped 50 μ l of 0.1 g/ml Distamycin A (Sigma) dissolved in MacIlvain buffer at pH 7.0 for 10 min. Then, the slides were washed in MacIlvain buffer contained 5 mM MgSO₄ at pH 7.0 for 10 min, and then, were dropped 50 μ l of 0.1 mg/ml CMA (Sigma) dissolved in MacIlvain buffer contained 5 mM MgSO₄ at pH 7.0 for 10 min. They were washed in MacIlvain buffer contained 5 mM MgSO₄ at pH 7.0 for 10 min and then, were stained in 2 μ g/ml DAPI (Sigma) dissolved in MacIlvain buffer at pH 7.0 at room temperate for 10 min. Then, they were washed in MacIlvain buffer at pH 7.0 for 10 min and were mounted with coverslips using a drop of 50% glycerol diluted with distilled water. The slides were observed in an epifluorecence microscope with blue-violet filter (BV-2A; Nikon). Microphotographs were made on T-MAX 400 film (Kodak).

After CMA-banding observation the slides were destained in 45% acetic acid at room temperature for 30 min. They were air-dried and used for DAPI fluorescence staining as follows: The slides were washed in MacIlvain buffer at pH 7.0 at room temperature for 30 min and they were

Table 1. Nine species of *Zamia* and *Ceratozamia mexicana* used

Species	Distribution (after Jones 1993)	Cultivation source	Total number of plants (sex) used	Present count of chromosome number	Previous count of chromosome number and (reference)
Zamiaceae					
<i>Zamia</i>					
<i>Z. angustifolia</i> Jacq.	Eleuthera Island, Eastern Cuba	Satake Corporation	1 (♀)	2n=16	(Norstog 1980)
<i>Z. integrifolia</i>	America (South-Eastern Georgia and Florida), Western Cuba, South-Central Puerto Rico, Cayman Islands, Bahama	Laboratory of Plant Chromosome and Gene Stock	20 (♂), 2 (♀)	2n=16	(Johnson 1963)
L. f. in Aiton					
<i>Z. pumila</i> L.	Central Cuba	Laboratory of Plant Chromosome and Gene Stock	2 (♂)	2n=16	(Sax and Beal 1934, Moretti 1990)
<i>Z. pygmaea</i> Simis	Central Western Cuba	Satake Corporation	2 (♂), 1 (?)	2n=16	(Moretti 1990)
<i>Z. furfuracea</i>	Mexico (Veracruz)	Laboratory of Plant Chromosome and Gene Stock	1 (♂), 3 (?)	2n=18	(Norstog 1980, Moretti and Sabato 1984)
L. f. in Aiton					
<i>Z. loddigesii</i> Miq.	Mexico (Qaxaca, Chiapas, Campeche, Quintana Roo), Belize, Guatemala	Satake Corporation	1 (♂)	2n=18	(Norstog 1980, Moretti and Sabato 1984)
<i>Z. skinneri</i> Wersz. ex A. Dietrich	Southern Nicaragua, Costa Rica, Panama	Satake Corporation	2 (♂)	2n=18	(Norstog 1980)
<i>Z. vazquezii</i>	Mexico (Veracruz)	Laboratory of Plant Chromosome and Gene Stock	8 (♂), 2 (♀)	2n=22 2n=18*	(Norstog, 1980) (Moretti <i>et al.</i> 1991)
D. Stevenson, Sabato <i>et</i> Moretti					
<i>Z. muricata</i> Willd	Northern Venezuela and adjacent Colombia	Satake Corporation	1 (♂), 1 (?)	2n=23	(Norstog 1980)
<i>Ceratozamia</i>					
<i>C. mexicana</i> Brongn.	Mexico (North-Central Veracruz)	Satake Corporation	1 (♂)	2n=16	(Sax and Beal 1934, Marchant 1968)

* Reported as an aneuploid of *Z. fischeri* by Moretti *et al.* (1991).

dropped 50 μ l of 0.1 mg/ml Actinomycin D (Sigma) dissolved in MacIlvain buffer at pH 7.0 at room temperature. They were washed in MacIlvain buffer at pH 7.0 at room temperature for 10 min, and were stained in 10 mg/ml DAPI dissolved in MacIlvain buffer at pH 7.0 at room temperature for 30 min. The slides were washed in MacIlvain buffer at pH 7.0 at room temperature for 10 min and were mounted with coverslips using a drop of 50% glycerol. The slides were observed on an epifluorescence microscope using UV filter (UV-2A; Nikon). Microphotographs were made on T-MAX 400 film (Kodak).

Results and discussion

Zamia angustifolia Jacq., *Z. integrifolia* L.f., *Z. pumila* L. and *Z. pygmaea* Simis had commonly the chromosome number of $2n=16$ (Fig. 1A–D) and the karyotype consisted of 12 median- (m-chromosomes; 1st to 12th chromosomes), 2 submedian- (sm-chromosome; 13th and 14th chromosomes) and 2 subterminal-centromeric chromosomes (st-chromosomes; 15th and 16th chromosomes). The chromosome numbers of the 4 species were quite the same as those in the previous reports (Table 1). However, their karyotypes were different from those in the previous reports due to different classification of chromosome by centromeric positions; 4 submedian-centromeric chromosomes by Sax and Beal (1934) and Norstog (1980) instead of the present 2 sm- and 2 st-chromosomes. The 2 submedian-centromeric and 2 acro-centromeric chromosomes of the species have already been reported by Moretti (1990b). The secondary constriction was found only on the 1st chromosome or the first median-centromeric chromosome in *Z. pumila* in the present study (Fig. 1C), although it was found on 2 median-centromeric chromosomes by Moretti (1990b).

Zamia furfuracea L.f., *Z. loddigesii* Miq., *Z. skinneri* Wersz. ex A. Dietrich and *Z. vazquezii* D. Stevenson, Sabato *et* Moretti had commonly the chromosome number of $2n=18$ (Fig. 1E–H) and 10 m- (1st to 10th chromosomes) and 2 sm-chromosomes (11th and 12th chromosomes) and uncommonly 6 chromosomes (13th to 18th chromosomes) which were different in karyomorphology from each other and specified respective species: For instance, the 6 chromosomes of *Z. furfuracea* and *Z. loddigesii* were t-chromosomes and were characterized by their heterochromatic short arm. The 6 chromosomes of *Z. skinneri* consisted of 4 t-chromosomes and 2 T-chromosomes which were characterized by their quite small short arm. The 6 chromosomes of *Z. vazquezii* were T-chromosomes. Even on same slide of each of the 4 species, sizes of the heterochromatic short arms of the t-chromosomes varied among the nucleus plates studied. These heterochromatic short arms had been observed on t-chromosome by Marchant (1968) and Moretti (1990b). Then, these arms had not been well-studied and concerned for karyotype analysis. The 6 variable chromosomes of each of the 4 species were carefully concluded here to consist of 2 A-chromosomes referred to st-chromosomes and 4 T-chromosomes referred to t-chromosomes.

Zamia muricata Willd had the chromosome number of $2n=23$ and the karyotype consisted of 7 m-chromosomes (1st to 7th chromosomes) and 16 t-chromosomes (8th to 23rd chromosomes) (Fig. 1I). Sizes of the short arm of the t-chromosomes in the species varied among the nucleus plates studied as well as those in the 4 species with $2n=18$ (Fig. 1E–H).

Ceratozamia mexicana had the chromosome number of $2n=16$ and the karyotype consisted of 12 m-chromosomes (1st to 12th chromosomes), 2 st-chromosomes (13th and 14th chromosomes) and 2 T-chromosomes (15th and 16th chromosomes) (Fig. 1J). *Ceratozamia mexicana* had small satellites on the terminal region of the short arm of the 11th and 12th chromosomes and of the long arm of the 13th and 14th chromosomes. These satellites were not observed in the *Zamia* species studied.

Speciation of the 9 species of the aneuploid *Zamia* studied might be promoted by the phenomenon of the Robertsonian change (Robertson 1916), since total 30 chromosome-arms were presented without counting the short arms of the st-, t- and T-chromosomes (Fig. 1).

Fig. 1. Karyotypes of 10 species of *Zamia* and *Ceratozamia mexicana*. A) *Z. angustifolia*. B) *Z. integrifolia*. C) *Z. pumila*. D) *Z. pygmaea*. E) *Z. furfuracea*. F) *Z. loddigesii*. G) *Z. skinneri*. H) *Z. vazquezii*. I) *Z. muricata*. J) *Ceratozamia mexicana*. Bar=10 μ m.

The 4 species of *Zamia* with the chromosome number of $2n=16$ showed CMA-positive bands on the proximal region of both short and long arms of the 11th and 12th chromosomes, the proximal region of the short arm of the 13th and 14th chromosomes, and the proximal region of both the short and long arms of the 15th and 16th chromosomes (Figs. 2A, C, E, G, 4A–D). Exceptionally, *Z. pumila* showed a CMA-positive band on the secondary constriction of the 1st chromosome (Figs. 2E, 4D). On the other hand, the 4 species showed DAPI-positive band, which was weak in brightness and quickly disappeared, on the centromeric region of 12 chromosomes (Figs. 2B, D, F, H,

Fig. 2. CMA-DAPI-stained chromosomes of 10 species of *Zamia* and *Ceratozamia mexicana*. A, C, E, G, I, K, M and O CMA-stained chromosomes. B, D, F, H, J, L, N, P DAPI-stained chromosomes. A, B) *Z. angustifolia*. C, D) *Z. integrifolia*. E, F) *Z. pumila*. G, H) *Z. pygmaea*. I, J) *Z. furfuracea*. K, L) *Z. loddigesii*. M, N) *Z. skinneri*. O, P) *Z. vazquezii*. CMA-positive bands were corresponded to DAPI-negative bands. Bar = 10 μ m.

4A–D). The majority of these chromosomes contained no CMA-positive band. CMA specifically stained guanine (G)- and cytosine (C)-rich regions on chromosome (Schweizer and Nagl 1976), while DAPI specifically stained adenine (A)- and thimine (T)-rich regions (Timmis *et al.* 1975). The present study in the chromosomes of all of the 9 species of *Zamia* studied indicated that the CMA-positive regions were corresponded to the strong DAPI-negative region and were considered as high GC-rich regions.

Zamia furfuracea and *Z. loddigesii* showed CMA-positive bands on the proximal region of both the short and long arms of the 3rd to 10th chromosomes, the whole region of the short arm and the proximal region of the long arm of the 13th to 18th chromosomes (Figs. 2I, K, 4E, F).

Zamia skinneri had CMA-positive bands on the whole region of the short arm and the proximal region of the long arm of the 13th to 16th chromosomes, while were similar to the t-chromosomes of *Z. furfuracea* and *Z. loddigesii*. The species also had CMA-positive dots on the consider-

Fig. 3. CMA-DAPI-stained chromosomes of *Zamia muricata* (A, B) and *Ceratozamia mexicana* (C, D). A, C) CMA-stained chromosomes. B, D) DAPI-stained chromosomes. Bar=10 μ m.

able short arm of the 17th and 18th chromosomes (Figs. 2M, 4G).

Zamia vazquezii displayed CMA-positive bands on the proximal region of both the short and long arms of the 3rd to 6th chromosomes and CMA-positive dots on the considerable short arm of the 13th to 18th chromosomes.

The CMA-positive region appeared on the whole short arm of all of the t- and T-chromosomes in the 4 species with the chromosome number of $2n=18$. Those regions allowed t- and T-chromosomes to be separated by the characteristics of band-size if the short arm was too small due to the constriction of heterochromatic region to distinguish either t- or T-chromosome.

The chromosomes of the 4 species with the chromosome number of $2n=18$ indicated DAPI-positive band on the centromeric region of the chromosomes which had no CMA-positive band (Fig. 2J, L, N, P). These DAPI-positive bands were weak in brightness and quickly disappeared as seen in the chromosomes of $2n=16$ species.

Zamia muricata exhibited CMA-positive bands on the proximal region of both short and long arms of the 2nd to 4th chromosomes and the whole region of the short arm and the proximal region of the long arm of the 8th to 23rd chromosomes (Figs. 3A, 4H). The bands in the 8th to 23th chromosomes were the brightest among the bands of the 9 species of the genus studied. *Zamia muricata* also showed DAPI-positive dots on the centromeric region of three m-chromosomes and 4 t-chromosomes (Fig. 3B) and faint DAPI-positive dots indistinct as seen in the other species of *Zamia* studied.

Ceratozamia mexicana had CMA-positive bands on the terminal region of the long arm of the 9th and 10th chromosomes, the terminal region of both the short and long arms, the satellite region of the 11th and 12th chromosomes, and the terminal region of either the long arm or the satellite region of the 13th and 14th chromosomes (Fig. 3C). Sizes of CMA-positive bands in the 9th and 10th chromosomes of the species were clearly different from each other (Fig. 3C). On the other hand, the species has DAPI-positive bands on the centromeric region of all the chromosomes, which were weak in brightness and quickly disappeared (Fig. 3D).

The numbers of CMA-positive regions in the 5 chromosome types found in the species studied were shown in Fig. 4 and Table 2. They suggested that high similarities in number and position of CMA- and DAPI-positive regions were seen among the $2n=16$ species studied and high variabilities in number and position of CMA- and DAPI-positive regions were seen among the $2n=18$ species studied. Differences of those numbers and positions were not correlated with differences of

Fig. 4. Ideograms of CMA-DAPI-stained chromosomes in 10 species of *Zamia* and *Ceratozamia mexicana*. A) *Z. angustifolia*. B) *Z. integrifolia*. C) *Z. pumila*. D) *Z. pygmaea*. E) *Z. furfuracea*. F) *Z. loddigesii*. G) *Z. skinneri*. H) *Z. vazquezii*. I) *Z. muricata*. J) *Ceratozamia mexicana*. Black solid areas represent CMA-positive regions and small-dotted areas represent DAPI-positive regions. The *Zamia* species show the CMA-positive band always on the proximal region of chromosomes, while *Ceratozamia mexicana* shows CMA-positive bands on the terminal regions of chromosomes. Bar=10 μ m.

chromosome numbers caused by the simple Robertsonian change an suggested occurrence of the specific structural changes of chromosomes.

The position of CMA-positive regions in *Z. muricata* with the chromosome number of $2n=23$ was similar to that in the species with the chromosome number of $2n=18$, with comparatively various patterns of karyotype components, and position of CMA-positive region: 4 m-chromosomes of *Z. muricata* as well as the 4 species with $2n=18$ had the CMA-positive region while only 2 m-chromosomes in the 4 species with $2n=16$ species had CMA-positive region (Fig. 4). Additionally, the chromosomes of *Z. muricata* showed large CMA-positive regions on the whole short arm of all of the t-chromosomes (Figs. 2I, K, M, 3A).

The karyotypes and the CMA-positive regions in the 9 species of *Zamia* studied could be correlated to their geographical distribution: The 4 species, characterized with the chromosome number of $2n=16$ and high similarity in CMA- and DAPI-positive regions of chromosome were mainly distributed in islands of the Caribbean Sea, while the 4 species with the chromosome number of $2n=18$ and *Z. muricata* with $2n=23$, all varied in karyotype and position and size of CMA- and DAPI-positive regions, were distributed in the continent of Central and South America. Norstog and

Table 2. Numbers of chromosomes which have CMA-positive region in 9 species of *Zamia* and *Ceratozamia mexicana* studied

Species	Numbers of chromosomes with CMA-positive region/total chromosomes					
	m	sm	st	t	T	sum
<i>Zamia angustifolia</i>	2/12	2/2	2/2	—	—	6/16
<i>Z. integrifolia</i>	2/12	2/2	2/2	—	—	6/16
<i>Z. pumila</i>	3/12	2/2	2/2	—	—	7/16
<i>Z. pygmaea</i>	2/12	2/2	2/2	—	—	6/16
<i>Z. furfuracea</i>	8/10	0/2	—	6/6	—	14/18
<i>Z. loddigesii</i>	8/10	0/2	—	6/6	—	14/18
<i>Z. skinneri</i>	0/10	0/2	—	4/4	2/2	6/18
<i>Z. vazquezii</i>	4/10	0/2	—	—	6/6	10/18
<i>Z. muricata</i>	4/7	—	—	16/16	—	20/23
<i>Ceratozamia mexicana</i>	4/12	2/2	—	—	2/2	8/16

m=median-centromeric chromosome, sm=submedian-centromeric chromosome, st=subterminal-centromeric chromosome, t=acrocentric chromosome, T=telocentric chromosome.

Nicholls (1997) morphologically and phylogeographically divided the species of *Zamia* into 4 groups. They assumed that *Z. muricata* was a member of the Caribbean Basin group since it is distributed in islands of the Caribbean Sea as well as in Eastern South America. *Zamia muricata* has been reported to have the chromosome number of $2n=24$ (Norstog 1980) which has been different from $2n=16$ of the other species of the Caribbean Basin group. *Zamia muricata* used in this study had the chromosome number of $2n=23$, differed from $2n=24$ documented by Norstog (1980), and the position of CMA bands similar to the bands seen in the $2n=18$. If *Z. muricata* might be a member of the continent of Central and South America instead of the Caribbean Basin, it would be more simple and easy to explain taxonomic, phylogeographic, and phylogenetic position of the species.

The CMA and DAPI staining methods demonstrated that all of the species of *Zamia* studied here had CMA-positive regions on the proximal region of certain chromosomes while *Ceratozamia mexicana* had CMA-positive regions on the terminal region of certain chromosomes. *Microcycas calocoma* reported by Kokubugata and Kondo (1998) showed the CMA-positive regions positioned in its chromosomes similar to *Zamia* studied here. Thus, it suggested that *Zamia* was closely related to *Microcycas* rather than *Ceratozamia* with respect to chromosome characteristics. The majority of the species of *Zamia* studied had symmetrical karyotypes and *Microcycas calocoma* had an asymmetrical karyotype (Kokubugata and Kondo 1998). This cytological observation supported the morphological and systematical studies of Stevenson (1990).

The 1st chromosome of *Z. muricata* did not have a homologous pair and had no CMA-positive region near the centromere. In contrast, the t-chromosomes, 2 of which might be a homologous match with the first chromosome, contained large CMA-positive region near the centromere. These phenomena may be clarified and justified if those chromosomes were explained in convenience to be involved and caused with the Robertsonian centric fusion/fission at the CMA-positive region near the centromere.

Further systematic and structural analyses in *Zamia* as well as the other members of the Zamiaceae should be made to clarify our concepts of species relationships.

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