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KARYOTYPES OF THE SNORKEL SNAIL GENERA *PTEROCYCLOS* AND *RHIOSTOMA* (PROSOBRANCHIA: CYCLOPHORIDAE)

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ABSTRACT. — Twenty-five specimens of 'snorkel snails' from the two closely related genera *Pterocyclos* (two species) and *Rhiostoma* (six species) from Thailand and Malaysia were karyotyped. The two genera showed no differences in both their haploid and diploid chromosome numbers (n = 14, 2n = 28), whilst the fundamental chromosome numbers (FN) varied in *Rhiostoma*, being 50, 54 or 56 depending upon the species. Karyotypes of the two *Pterocyclos* species from southern Thailand and northern Malaysia (with FN = 56) were distinct, being 8m + 4sm + 2st in *Pterocyclos* sp. and 7m + 5sm + 2st in *P. blandi*. The six species of *Rhiostoma* examined herein showed variations in their karyotype and chromosome morphology. The karyotypes varied from 7m + 4sm + 3st in *R. hainesi* from eastern Thailand, 6m + 5sm + 2st + 1a in *R. chupingense* from southern Thailand and northern Malaysia, 2m + 9sm + 2st + 1t in *R. jalorensis* from southern Thailand, whilst the two island species exhibited significant differences in their karyotypes, being 3m + 7sm + 1st + 3t in *R. asiphon* from the Angtong Archipelago and 5m + 5sm + 1st + 3a in *R. samuiense* from Samui Island. In *R. housei*, the karyotype varied between the central (9m + 2sm + 3st) and southern populations (9m + 2sm + 2st + 1a) of Thailand. This is the first report of the karyotypes of land operculate snails from the Thai-Malay Peninsula, and provides fundamental knowledge for the future systematic study of these groups.

KEY WORDS. — Pterocyclinae, chromosome, land snail, systematic, Thailand, biodiversity

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INTRODUCTION

The Cyclophoridae are confined to peninsular India and Sri Lanka, the east Himalayan region through Southeast Asia and the Indonesian archipelagoes, as well as to northeastern Australia and into much of Japan (Tielecke, 1940). The two genera of 'snorkel snails', *Pterocyclos* and *Rhiostoma*, are endemic operculate cyclophorid snails that occur in the limestone areas of Southeast Asia. They belong to the subfamily Pterocyclinae, which is comprised of some 20 species and are distributed in Myanmar, Vietnam, Laos, Cambodia, Malaysia, and Thailand (Möllendorff, 1894; Blanford, 1902; Tomlin, 1931; Salisbury, 1949; Solem, 1966; Ruhoff, 1980). They have unique shell shapes with the last portion of the last whorl being almost disconnected with the penultimate whorl, or having a tubular structure on the last whorl near the shell aperture. These peculiar shell characteristics have given rise to their generic name of 'snorkel snails'. Because of their remarkable characters they have attracted shell traders and large numbers of snails have been continuously hunted from their natural habitats to such an extent that their status may become endangered, and this will likely affect the limestone ecosystem.

The main problems for classification of the genera *Pterocyclos* and *Rhiostoma* are the morphological variations in the shell colour pattern, last whorl and gross shell shapes, within and between populations of the same species. Moreover, the morphology of the reproductive organs has been reported as being of limited value in species discrimination (Prasankok et al., 2011). Thus, no meaningful investigations have been carried out into the relationships between the species of these two genera based on their anatomical characters, in contrast to species in other cyclophorid genera, such as the *Cyclophorus* (see Kongim et al., 2006).

The peculiar shell characters and unresolved problems of classification make *Pterocyclos* and *Rhiostoma* attractive for systematics and biogeographical studies. There is enormous potential for genome sequencing-based studies on terrestrial snails, but to date these have been conducted almost exclusively on Stylommatophoran pulmonates (Wade et al., 2001) and involve relatively expensive consumables. In contrast, karyotyping is a low cost methodology that provides useful tools for alpha taxonomy and could be complementary to genetic sequencing for evolutionary studies. Indeed, cytogenetic studies are particularly valuable for clarifying the phylogenetic relationships and investigating mechanisms of chromosomal evolution in closely related species. Accordingly, karyological data has been used for species-level classification in several molluscan groups, such as *Atlanta*, *Bellamya*, *Goniobasis*, and *Viviparus* (Zhou et al., 1988; Dillon, 1991; Thiriot-Quièvreux & Seapy, 1997; Baršiene et al., 2000). However, karyotypes of members of *Pterocyclos* and *Rhiostoma* have not yet been examined.

In the present paper, we report on the karyotype variability in selected *Pterocyclos* and *Rhiostoma* species from Thailand and Malaysia, which bring the total number examined to date to 24 cyclophorids, and for the first time allow discussion on systematic implications.

MATERIAL AND METHODS

Localities, shell characteristics of each species, and karyotype information are given in Figs. 1, 2 and Table 2. Species identifications were made using the following literature; Reeve (1861), Möllendorff (1894), Blanford (1902), Tomlin (1931), Salisbury (1949), Solem (1966), and Ruhoff (1980). Shells were also compared with type material at the Senckenberg Museum, Frankfurt (SMF) and the Natural History Museum, London (NHM).

Chromosome preparations were made from gonadal tissue by an air-drying method, modified from Patterson & Burch (1978), Park (1994), and Kongim et al. (2006). Gonads were directly injected with 0.1 ml of 0.1% (w/v) colchicine (Sigma D-89552), and were dissected after 4–5 h and cut into small pieces in 0.07% (w/v) hypotonic KCl solution. Separated cells were collected after centrifugation at 1,000 rpm for 10 min and fixed in fresh Carnoy's fixative (3:1 (v/v) ratio of absolute methanol to glacial acetic acid). The supernatant was replaced with fresh fixative for each of the two centrifugations. Cell suspensions were dropped onto clean glass slides pre-heated to 60°C, and then air-dried and stained in 4% (w/v) Giemsa solution for 15 min. Photomicrographs of ten well-spread metaphase cells were measured for relative length and centromeric index. Mitotic karyotypes were arranged and numbered for chromosome pairs in order of decreasing mean relative length. Nomenclature of morphological chromosome types follows that of Levan et al. (1964).

RESULTS

Specimens of the two genera *Pterocyclos* (three specimens, two species) and *Rhiostoma* (22 specimens, six species) collected from Thailand and Malaysia showed no intergeneric differences in either haploid or diploid chromosome number (n = 14, 2n = 28). However, the fundamental chromosome number (FN) in *Rhiostoma* varied, being 50, 54 or 56 depending upon the species, whilst the three *Pterocyclos* specimens had a FN of 56. The chromosome pairs were categorised as metacentric (m), submetacentric (sm), subtelocentric (st), telocentric (t) and acentric (a). Karyotypes of the two *Pterocyclos* species from southern Thailand and Malaysia differed, being 8m + 4sm + 2st in *Pterocyclos* sp. from southern Thailand down to Peris, Malaysia, and 7m + 5sm + 2st in *P. blandi* from Langkawi, Malaysia. The latter species showed the distinctive chromosome markers of a short arm, whilst pair number six exhibited a wider angle arrangement, and pair number seven showed terminal

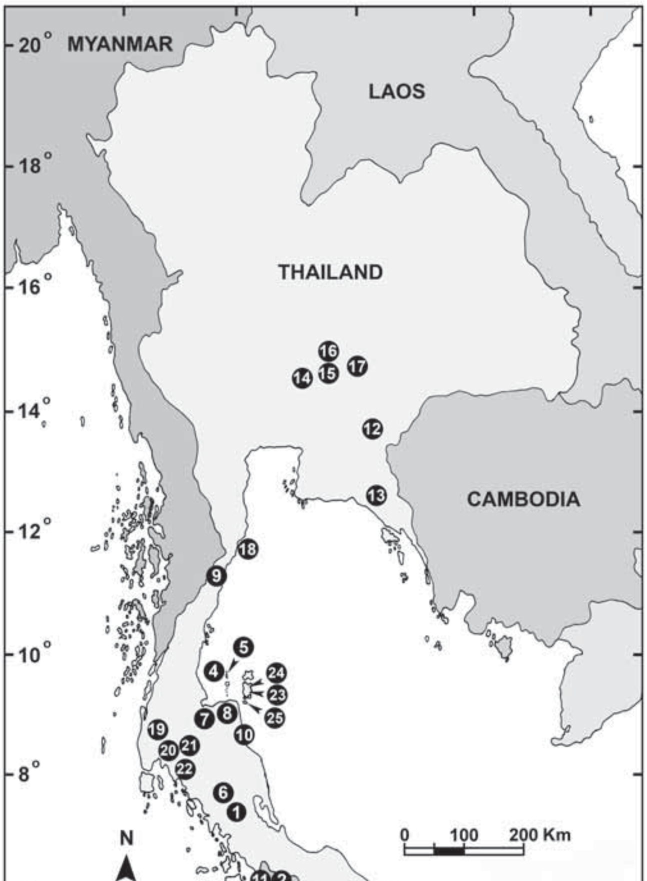

Fig. 1. A map of Thailand showing the collection localities of the *Pterocyclos* and *Rhiostoma* specimens used in this study. Locations for each indicated number are given in Table 2.

Table 1. The diploid (2n) and fundamental number (FN) of the cyclophorid species.

Species	Origin	2n	FN	Reference
P. bilabiatus (Sowerby)	India	28	—	Kasinathan & Natarajan (1975)
T. ravidus (Benson)	India	28	—	Kasinathan & Natarajan (1975)
T. shiplayi (Pfeiffer)	India	28	—	Kasinathan & Natarajan (1975)
M. scabra Theobald	India	28	—	Kasinathan & Natarajan (1975)
C. jerdoni (Benson)	India	28	—	Kasinathan & Natarajan (1975)
C. polynema (Pfeiffer)	India	28	—	Choundhury and Pandit (1997)
C. aurantiacus (Schumacher)	Thailand	28	56	Kongim et al. (2006)
C. cantori (Benson)	Thailand	28	56	Kongim et al. (2006)
C. courbeti Ancey	Thailand	28	56	Kongim et al. (2006)
C. diplochilus Möllendorff	Thailand	28	56	Kongim et al. (2006)
C. fulguratus (Pfeiffer)	Thailand	28	56	Kongim et al. (2006)
C. malayanus (Benson)	Thailand	28	56	Kongim et al. (2006)
C. saturnus Pfeiffer	Thailand	28	56	Kongim et al. (2006)
C. speciosus (Philippi)	Thailand	28	56	Kongim et al. (2006)
C. subfloridus Ancey	Thailand	28	56	Kongim et al. (2006)
C. volvulus (Müller)	Thailand	28	56	Kongim et al. (2006)
Pterocyclos sp.	Malaysia	28	56	Present study
P. blandi Benson	Malaysia	28	56	Present study
R. asiphon Möllendorft	Thailand	28	50	Present study
R. chupingense Tomlin	Thailand	28	54	Present study
R. hainesi Pfeiffer	Thailand	28	56	Present study
R. housei (Haines)	Thailand	28	56, 54	Present study
R. jalorensis Sykes	Thailand	28	54	Present study
R. samuiense Tomlin	Thailand	28	54	Present study

Genera: *Cyclophorus* (*C.*), *Pterocyclos* (*P.*), *Theobaldius* (*T.*), *Micraulax* (*M.*), and *Rhiostoma* (*R.*).

aligned discontinuities. The six species of *Rhiostoma* examined herein also showed karyotype variations and differentiation in chromosome morphology. The karyotype of *R. hainesi* from eastern Thailand was 7m + 4sm + 3st, whilst the two species from southern Thailand differed again being 6m + 5sm + 2st + 1a in *R. chupingense*, with chromosome number four exhibiting telomere end union on the long arm, and 2m + 9sm + 2st + 1t in *R. jalorensis*. The two island species differed again, being 3m + 7sm + 1st + 3t in *R. asiphon* from Angtong Archipelagoes and 5m + 5sm + 1st + 3a in *R. samuiense* from Samui Island, Thailand. In *R. housei*, the karyotype differed between populations in the central (9m + 2sm + 3st) and southern regions (9m + 2sm + 2st + 1a) of Thailand (Figs. 3, 4 and Table 2).

DISCUSSION

In the Pterocyclinae (Cyclophoridae), the chromosome number is highly conserved at $n = 14$, $2n = 28$. Several characteristics are common to all analysed species so far, including the invariable diploid number (28), which is most similar to the chromosome number of the genus *Cyclophorus* in the Cyclophorinae (Kongim et al., 2006; Lee et al., 2008). Ten tiny cyclophoroideans belonging to the genus *Diplommatina* also show a similar conserved chromosome number ($2n = 26$) and karyotype characters (metacentric and submetacentric; Ieyama & Tada, 1991; Ogaito & Ieyama, 1997; Ieyama & Ogaito, 1998, 2000), as did the land operculate snail *Pollicaria* (Pupinidae) from Southeast Asia (Kongim et al., 2009, 2010). This conservation of haploid number is distinctly different from the subclass Streptoneura (Caenogastropoda), which vary rather extensively between taxa, ranging from 7 to 60 (Burch, 1967), with each taxon exhibiting its own chromosome number and appearing not to vary by more than ± 2 bivalents.

The Pterocyclinae show unbalanced p and q chromosome arm lengths with subtelocentric and telocentric chromosomes in their karyotype formulae, and so differ from the karyotype of the globosed-shelled Cyclophoridae, whilst the genus *Cyclophorus* differ in the number of submetacentric chromosomes and in the absence of subtelocentric and telocentric types. Moreover, from a cytogenetics point of view, these groups are quite distinguishable, with *Pterocyclos* being characterised by the number of asymmetric chromosomes, and a lower number of subtelocentric chromosomes than in *Rhiostoma*. Some differences could also be seen in the chromosome size. The diverse karyotypes of the genus *Rhiostoma* exhibit subtelocentric, telocentric and acentric chromosome types. Remarkably, the three *Rhiostoma* species from southern Thailand, *R. chupingense*, *R. housesi* and *R. samuiense*, show the presence of one, one and three pairs of acentric chromosomes, respectively.

Their acentrics are paired chromatids that lie parallel to one another but in which there is no centromere, as reported before (Griffiths et al., 2008). Moreover, the chromosomes have terminal aligned discontinuities and telomere end union. These data indicated that several chromosomal rearrangements seem to have taken place through the evolutionary history of *Rhiostoma* species, mainly driven by reciprocal translocation (Halnan, 1989; Rooney & Czepulkowski, 1992; Clark & Wall, 1996; Rickart et al., 1999). Furthermore, within *Rhiostoma*, a single nominal species *R. housei*, displayed variable karyotypes, with 9m + 2sm + 3st and 9m + 2sm + 2st + 1a from central and southern Thailand, respectively.

Variations in karyotypes may represent a primary species-isolating mechanism, as discussed by Halnan (1989) and King (1993). The distribution of *R. housei* is currently recognised to range from northern Thailand (type locality) to southern Thailand and the species is notable for exhibiting considerable variation in shell morphology. With these clear variations in the karyotype in Thailand it now remains to be determined whether they are truly genetically isolated or reproductively incompatible and whether they actually comprise two or more distinct species. Our results suggest that eight *Rhiostoma* species and some subspecies occur in Thailand. Further chromosomal surveys, combined with comparative analyses and geographic variation of morphological characters, including allozyme or DNA sequence analysis are required to determine the extent of gene flow and species limitation in the *R. housei* species complex (Prasankok et al., 2011). The karyotypes of the two endemic species, *R. chupingense* and *R. housei*, are different in fundamental number (FN) and karyotype formula. Moreover, *P. blandi*, *R. chupingense*, *R. housei*, and *R. jalorensis* exhibit unique chromosomal characters, especially in the first six pairs.

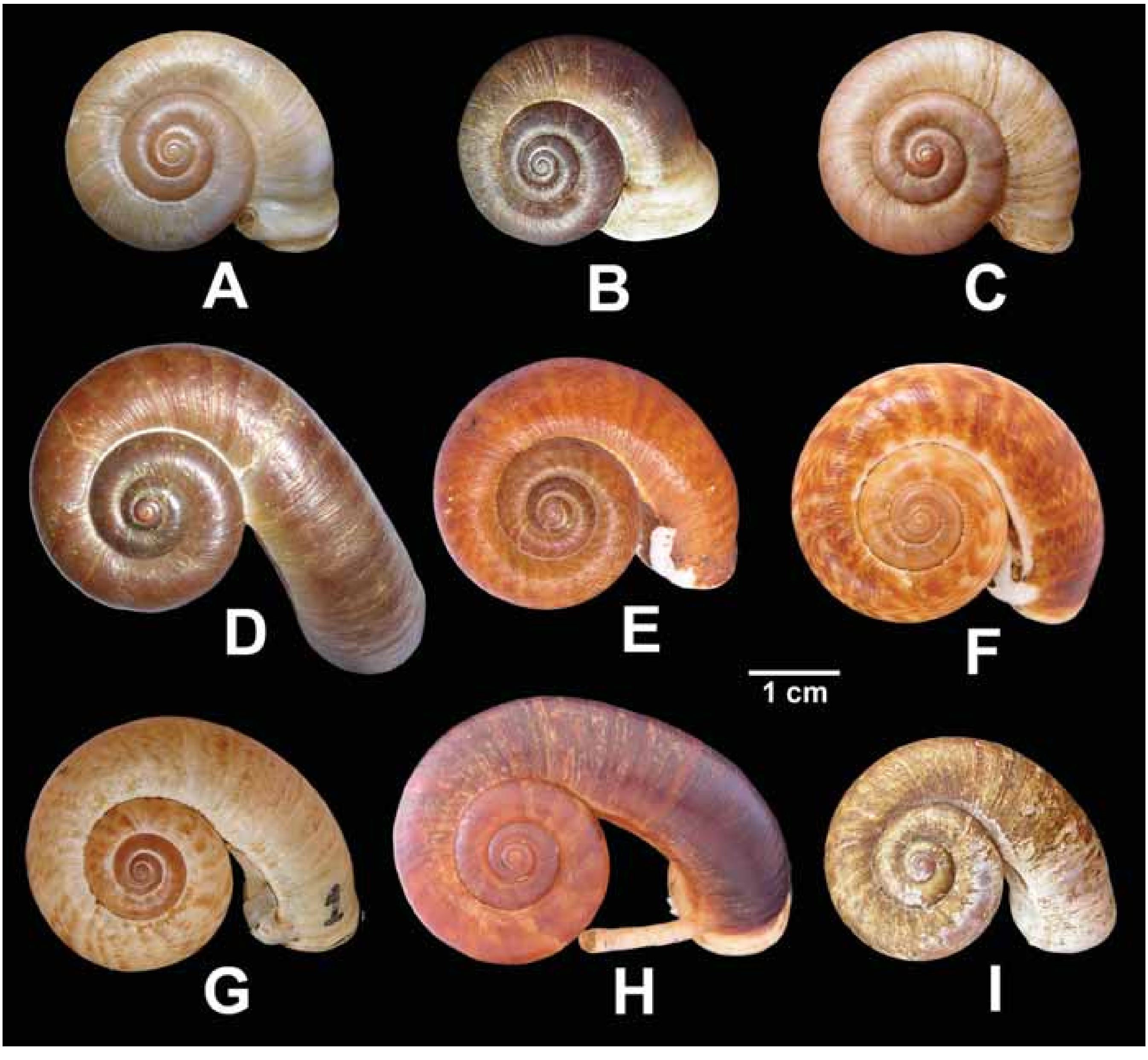

Fig. 2. Shell characteristics of: A, *Pterocyclos* sp.; B, *P. blandi*; C, *Rhiostoma asiphon*; D, *R. chupingense*; E, *R. hainesi*; F, *R. housei* (from central Thailand, localities 14–17); G, *R. housei* (from southern Thailand, locality 18); H, *R. jalorensis*; and I, *R. samuiense*.

Table 2. The diploid number (2n), fundamental number (FN), and karyotype pattern of the *Pterocyclos* (*P.*) and *Rhiostoma* (*R.*) species in the present study.

Species	**Locality (number and site)**	**2n, FN**	**Karyotype**
Pterocyclos sp.	1. Tam Sumano, Patthalung 2. Gua Cenderawasih, Peris, Malaysia	28, 56	8m + 4sm + 2t
P. blandi	3. Kedah Cement, Langkawi, Malaysia	28, 56	7m + 5sm + 2t
R. asiphon	4. Koh Wua Talub, Mu Koh Angtong N.P., Suratthani 5. Koh Samsao, Suratthani	28, 50	3m + 7sm + 1st + 3t
R. chupingense	6. Khao Poo Khao Ya N.P., Phatthalung 7. Khao Panomwang, Suratthani 8. Kao Chang, Donsak, Suratthani 9. Wat Khao Thammarong, Prachuap khirikhan 10. Khao Wangtong, Nakhon Srithammarat 11. Sugai Jenis Peris, Malaysia	28, 54	6m + 5sm + 2st + 1a
R. hainesi	12. Khao Chakan, Sra Kaeo 13. Namtok Plio N.P., Chanthaburi	28, 56	7m + 4sm + 3st
R. housei	14. Muaglek, Saraburi 15. Khao Lukchang, Pakchong, Nakhon Ratchsrima 16. Wat Theppithakpunnaram, Kang Koi, Saraburi 17. Tam Dao, Saraburi	28, 56	9m + 2sm + 3st
	18. Khao Lommuak, Muang, Prachuap Khirikhan	28, 54	9m + 2sm + 2st + 1a
R. jalorensis	19. Wat Tam Suwannakuha, Muang, Pang-nga 20. Wat Tam Sua, Muang, Krabi 21. Namtok Taotong, Pang-nga 22. Tam Nampud, Pang-nga	28, 54	2m + 9sm + 2st + 1t
R. samuiense	23. Namtok Namuang, Koh Samui, Suratthani 24. Namtok Hinlad, Koh Samui, Suratthani 25. Koh Tan, Suratthani	28, 50	5m + 5sm + 1st + 3a

Abbreviation: m, metacentric; sm, submetacentric; st, subtelocentric; t, telocentric; a, acentric chromosome.
Remark: Locality numbers refer to the locality shown in Fig. 1.

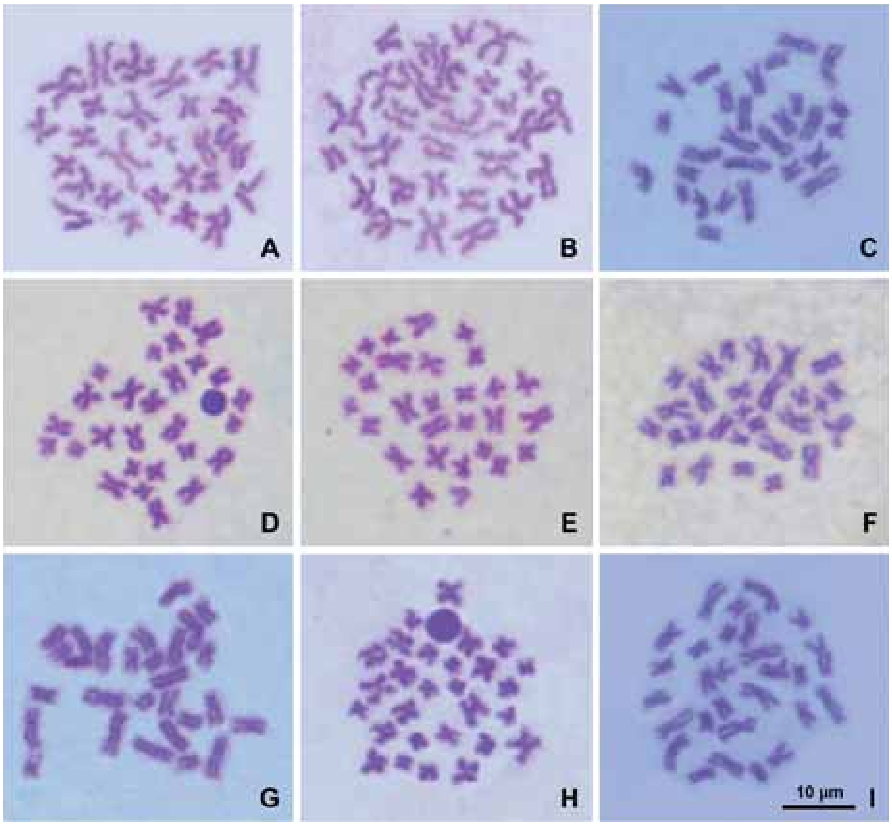

Fig. 3. Mitotic chromosomes of: A, *Pterocyclos* sp.; B, *P. blandi*; C, *Rhiostoma asiphon*; D, *R. chupingense*; E, *R. hainesi*; F, *R. housei* (from central Thailand, localities 14–17); G, *R. housei* (from southern Thailand, locality 18); H, *R. jalorensis*; and I, *R. samuiense*.

Cytotaxonomy is an efficient tool for systematics in many animal groups, by discriminating between morphologically similar species (cryptic species), since the karyotype itself probably represents a trait resistant to environmental, behavioral or physiological influences (White, 1978; Sumner, 2003). Therefore, chromosomal alterations are usually significant events in the evolutionary processes of a species. Chromosome variations, in terms of both the number and karyotype pattern, have been implicated as a primary isolating mechanism for speciation. However, karyotype differentiation appears to result most frequently from population fragmentation via founder events (Dillion, 1991; King, 1993). Thus, interpretation of chromosome numbers as an indicator of evolutionary relationship must be viewed with caution, especially from outside of closely related individuals. For example, molluscan chromosome numbers appear to have a tendency to increase from the traditional Archaeogastropoda to the Neogastropoda, whereas a tendency to decrease is noticeable in the Opisthobranchia from the Cephalaspidea to the Nudibranchia. In contrast, pulmonate molluscs are generally stable in chromosome number within taxonomic groups (Patterson & Burch, 1978).

The present results are in accordance with the minimum interaction theory proposed by Kolnicki (2000), which has been suggested as a common model for karyotype evolution in eukaryotes. According to this theory the increase in chromosome number during evolution acts as an adaptive mechanism to reduce the risk of deleterious chromosomal recombination within the genome. This process results in a higher chromosome number coupled with a reduction in chromosome size.

Regardless, the chromosomal structures reported in the subfamily Pterocyclinae support the taxonomic status of *Pterocyclos* and *Rhiostoma* as distinct genera, since members from both genera show clear chromosomal differences.

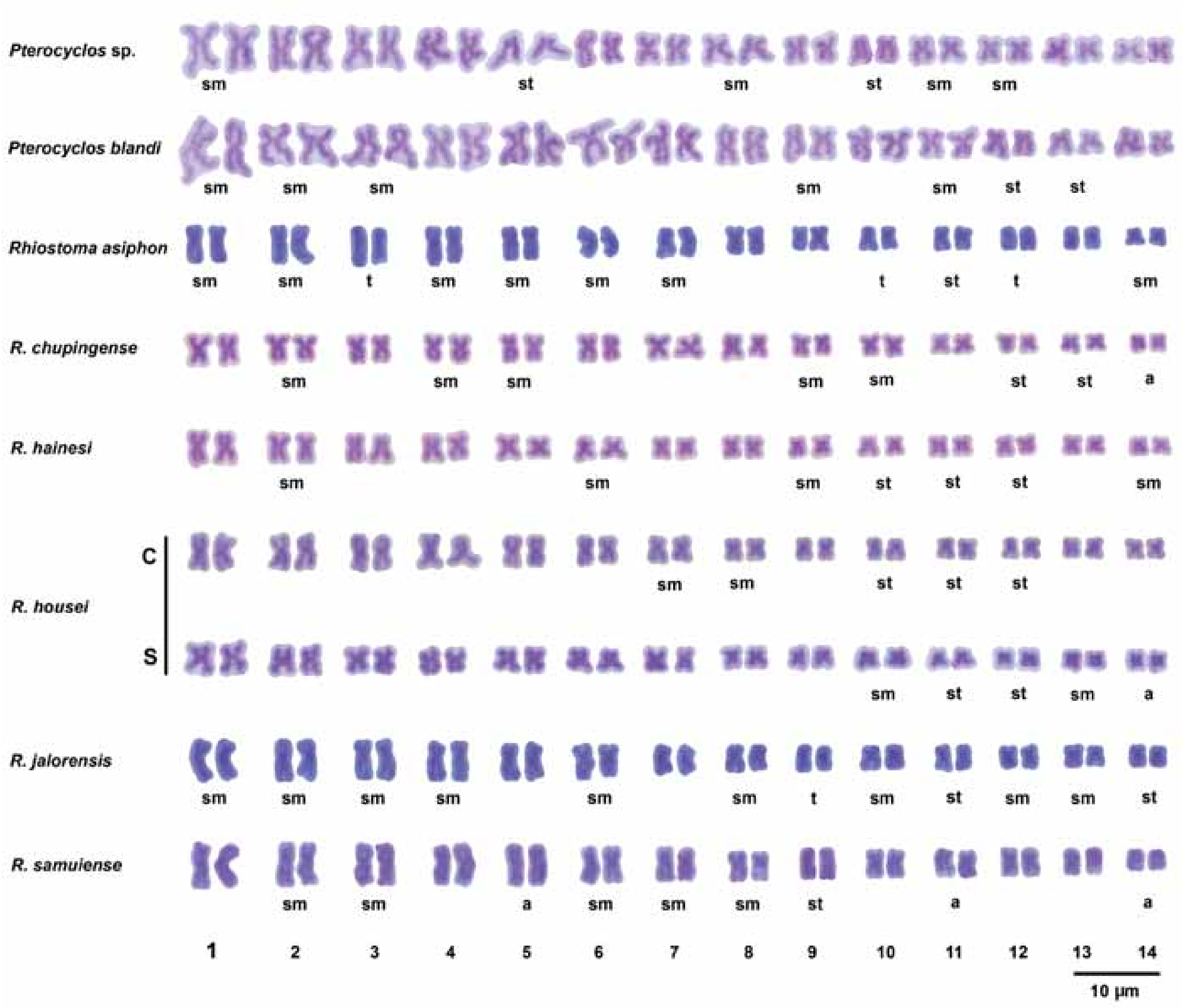

Fig. 4. Karyotype of *Pterocyclos* sp., *P. blandi*, *Rhiostoma asiphon*, *R. chupingense*, *R. hainesi*, *R. housei* (from central Thailand, localities 14 to 17), *R. housei* (from southern Thailand, locality 18), *R. jalorensis*, and *R. samuiense* (as indicated in Fig. 3), showing the metacentric (not labelled), submetacentric (sm), subtelocentric (st), telocentric (t), and acentric (a) chromosomes.

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