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Amplified fragment length polymorphism for identification of *Habenaria* and *Pecteilis*

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Abstract

Habenaria and *Pecteilis* are terrestrial orchids that belong to the same subtribe. These two genera exhibit some similar features and are sometimes mistaken for the same genera. Nowadays, the molecular marker method can be used to elucidate the relationship between plants which are different in terms of morphology, but molecular markers can also show similarities between and within genera. There are several molecular markers which can be employed. One of the popular methods is the amplified fragment length polymorphism (AFLP) technique. This technique was used to classify 30 samples of *Habenaria* and *Pecteilis.* Among 64 combinations of 8 *Eco*RI primers and 8 *Tru*9I primers, four primer combinations (E-ATT/M-CAC), (E-AGT/M-CTG), (E-ACA/M-CAC) and (E-ACC/M-CAC) produced clear and polymorphic bands. Similarity coefficients between species indicated that *H. erichmichaelii* and *H. carnea* had the closest genetic relationship of 0.60. All samples could be divided into 2 groups. Group 1 consisted of 27 samples of *Habenaria* with small to large colorful flowers. These samples could be further separated into 3 subgroups, with subgroup 1.1 consisting of *Habenaria*, which have big and colorful flowers, subgroup 1.2 consisted of *Habenaria,* which has white, light green or green flowers and subgroup 1.3 consisted of Habenaria, which had small yellow and pink flowers. Group 2 consisted of 3 samples of *Pecteilis* with large white and yellow flowers. The results indicated that this technique could be used to distinguish *Habenaria* and alliances.

Keywords: Genetic diversity, Plant identification, Similarity, Terrestrial orchids, Orchidaceae

1. Introduction

Orchids are monocotyledonous flowering plants that belong to the Orchidaceae [1], the largest family of plant species, with 796 genera worldwide and more than 17,500 species. In Thailand, Orchidaceae comprises 168 genera, with over 1,170 species found in diverse habitats [2], including 60 terrestrial orchid genera, with more than 200 species. Some orchid genera such as *Habenaria* and *Pecteilis* show potential for use as pot plants or cut flowers. Both *Habenaria* and *Pecteilis* genera belong to the subtribe *Habenariinae*. The 600 *Habenaria*, species are distributed throughout tropical and temperate grasslands and most are deciduous. In Thailand, there are 46 *Habenaria* and *Pecteilis* species. *Habenaria* flowers consist of sepals, petals, lips and protruded columns with long spurs [3]. The lips tend to be deeply lobed with a slightly-rough border. The *Pecteilis* genus is found in Myanmar and Thailand. *Pecteilis* species have white or creamy-white flowers, short spur and column, and does not protrude as found in *Habenaria* species. Species of these two genera have underground tubers with a single pseudostem.

Nowadays, genetic relationship analysis of orchids can be carried out by molecular techniques, e.g., random amplified polymorphic deoxy ribonucleic acid (DNA) (RAPD) such as *Dendrobium* [4] and *Doritis* [5], amplified fragment length polymorphism (AFLP) such as *Paphiopedilum* [6], simple sequence repeats (SSR) such as *Aeridinae* [7], and internal transcribed spacer (ITS) such as *Vanda* [8]. In the past, colorful *Habenaria* were placed in species *H. rhodocheila*. Later on, this species was separated into several species based on flower color and other plant structures. The relationship between this species and other *Habenaria* species was tested in order to identify whether they could be separated into different species or if it was only a variation. Therefore, the AFLP

technique was employed in order to analyze the genetic relationship between some *Habenaria* and *Pecteilis* found in Thailand.

2. Materials and methods

2.1 Plant materials

Habenaria and *Pecteilis* plants were obtained from Kamthieng Flower Market in Chiang Mai and the orchid nursery of the Horticulture Division, Department of Plant and Soil Sciences, Faculty of Agriculture, Chiang Mai University. Thirty samples of *Habenaria* and *Pecteilis* were applied for AFLP analysis (Table 1, Figure 1).

2.2 DNA extraction

Young leaves were used for DNA extraction following the cetyl trimethyl ammonium bromide (CTAB) method [9]. Leaf tissue was ground in a mortar to a fine powder form mixed with 1 mL 2x CTAB buffer and transferred into a 1.5 mL centrifuge tube. Ten µL of 1 mg/µL proteinase K and 1 µL of 0.2% (v/v) 2-mercaptoethanol were added into the mixture and incubated at 60°C for 30 min in a water bath and mixed every 10 min. Then, 500 µL 25 phenol: 24 chloroforms: 1 isoamyl alcohol was added to the mixture and centrifuged at 10,000 rpm for 10 min. The supernatant was transferred into a new tube and added with an equal amount of isopropanol, mixed and then incubated at 4°C overnight. The mixture was then centrifuged at 10,000 rpm for 5 min and the supernatant was discarded. The precipitate was washed with 500 µL of wash buffer as 10 mM ammonium acetate and 75% ethanol and centrifuged at 10,000 rpm for 5 min. The liquid was then carefully poured out. The pellet was air-dried before dissolution by 40 µL of TE buffer containing 10 mM Tris-HCl and 0.5 mM Ethylenediaminetetraacetic acid (EDTA). Ten units of RNase A were then added to the mixture, which was placed in an incubator at 37°C for 30 min. This DNA was diluted to 10 ng/µL distilled water (ddH_2O). Finally, 1 µL of this dilution was used for polymerase chain reaction (PCR) [10].

2.3 AFLP analysis

The AFLP technique followed the protocol described by [11]. Genomic DNA was digested with Eco*R*I and *Tru*9I primers. Pre-selective amplification was performed using primers with one selective base (E-A and M-C) (Bio Basic, Canada). The selective amplification step using 64 combinations of 8 *EcoR*I and 8 *Tru*9I primers (Table 2) was conducted with three selective bases at the 3' end of each primer. The conditions for pre-selective amplification were 25 cycles of denaturation at 94°C for 30 sec, annealing at 56°C for 60 sec, and extension at 72°C for 60 sec, followed by 5 min extension at 72°C. For the selective amplification step, touch-down PCR was carried out by denaturation at 94°C for 30 sec, annealing at 65°C for 30 sec, and extension for 60 sec at 72°C for the first cycle, followed by lowering the annealing temperature by 1°C for the next 24 cycles, then annealing at 56°C for the remaining 20 cycles with extension at 72°C for 60 sec. To analyze the DNA pattern, the PCR products were denatured at 95°C for 5 min and quickly cooled on ice. After the selective amplification step, 6% denaturing polyacrylamide gels in 1x Tris-borate (TBE) buffer was used to screen for suitable primer combinations, with polymorphic DNA bands and high resolution. The PCR products of suitable primer combinations were separated on 6% denaturing polyacrylamide gels in 1x TBE buffer, and electrophoresis was performed at constant power (55 W) and temperature (50°C) for 4 h. After electrophoresis, the bands were visualized by silver staining by adding 10% acetic acid for 20 min and then adding 1% nitric acid for 20 min. The gels were washed three times with double ddH_2O and stained with 0.2% silver nitrate solution for 30 min. After washing with ddH_2O, the gels were developed with 3% sodium carbonate supplemented with 0.02% formaldehyde until the DNA bands appeared. The reactions were stopped with 10% acetic acid for 2 min and washed again with ddH_2O. The gels were dried on filter paper at 55°C for 2 h under vacuum on a gel dryer.

2.4 Data analysis

The PCR products were electrophoresed and scored as 0 (absent) and 1 (present). Genetic similarity coefficients were calculated using unweighted pair group method with arithmetic averages (UPGMA), and a dendrogram representing the genetic relationship was created with the numerical taxonomy system (NTSYS)-pc version 2.20 program [12].

Table 1 Flower characteristics and source of thirty *Habenaria* and *Pecteilis* samples [13].

Number	Scientific name	Province/Source	Features of flowers
1	*H. roebelenii*	Nongkhai	red flower, red sepals and petals with red lip
2	*H. rhodocheila*	Udon Thani	orange flower, orange sepals and petals with orange lip
3	*H. janellehayneiana*	Phitsanulok	pink flower, pink sepals and petals with pink lip
4	*H. rhodocheila*	Phangnga	red flower, greenish sepals and petals with red lip
5	*H. rhodocheila*	Ubon Ratchathani	orange flower, greenish sepals and petals with orange lip
6	*H. rhodocheila*	Satun	pink-red flower, greenish sepals and petals with pink lip
7	*H. rhodocheila*	Nakhon Si Thammarat	yellow flower, greenish sepals and petals with yellow lip
8	*H. erichmichaelii*	Flower Market, Chiang Mai	pink flower, brown sepals and petals with pink lip
9	*H. carnea*	Phrao Orchids Nursery, Chiang Mai	pink flower, pink sepals and petals with pink lip
10	*H. rhodocheila*	Chanthaburi	yellow, orange, red flower, greenish sepals and petals with yellow, orange, red lip
11	*H. xanthocheila*	Flower Market, Chiang Mai	yellow flower, yellow-orange sepals and petals with yellow lip
12	*H. dentata*	Flower Market, Chiang Mai	white flower, white sepals and petals with white lip
13	*H. myriotricha*	Flower Market, Chiang Mai	white flower, green sepals and petals with white lip
14	*H. porphyricola*	Flower Market, Chiang Mai	white flower, pale greenish sepals and petals with white lip
15	*H. lindleyana*	Flower Market, Chiang Mai	white flower, white sepals and petals with white lip
16	*H. hosseusii*	Flower Market, Chiang Mai	white flower, white sepals and petals with white lip, greenish distal parts of petals
17	*H. malintana*	Phrao Orchids Nursery, Chiang Mai	white flower, white sepals and petals with white lip
18	*H. vidua*	Flower Market, Chiang Mai	white or whitish yellow flower, white sepals and petals with green lip
19	*H. lucida*	Flower Market, Chiang Mai	green flower, yellow-green sepals and petals with yellow-green lip
20	*H. humistrata*	Flower Market, Chiang Mai	white or greenish white flower, greenish-brown or green sepals, whitish lateral sepal, light green petals, green and white lip
21	*H. thailandica*	Phrao Orchids Nursery, Chiang Mai	green or greenish white flower, green sepals and petals with greenish-white lip
22	*H. limprichtii*	Flower Market, Chiang Mai	green flower, yellow-green sepals and petals with yellow-green lip
23	*H. austrosinensis*	Flower Market, Chiang Mai	greenish or white flower, green sepals and petals with greenish lip
24	*H. reflexa*	Flower Market, Chiang Mai	light green or whitish flower, light green sepals and petals with green lip
25	*H. chlorina*	Flower Market, Chiang Mai	yellow flower, greenish with brown spots or brown sepals and yellow petals
26	*H. marginata*	Phrao Orchids Nursery, Chiang Mai	orange-yellow or pale yellow flower, yellow sepals, petals and lip
27	*H. rostellifera*	Flower Market, Chiang Mai	pink flower, pink or brown lateral sepals, dorsal sepal, petal with pink lip
28	*P. hawkesiana*	Flower Market, Chiang Mai	white flower, white sepals and petals with white lip
29	*P. sagarikii*	Flower Market, Chiang Mai	white flower, white sepals and petals with yellow lip
30	*P. susannae*	Flower Market, Chiang Mai	creamy white flower, creamy white sepals, petals and white lip

Figure 1 Flowers of *Habenaria* and *Pecteilis* samples for AFLP analysis (A) *H. roebelenii* (Nongkhai), (B) *H. rhodocheila* (Udon Thani), (C) *H. janellehayneiana* (Phitsanulok), (D) *H. rhodocheila* (Phangnga), (E) *H. rhodocheila* (Ubon Ratchathani), (F) *H. rhodocheila* (Satun), (G) *H. rhodocheila* (Nakhon Si Thammarat), (H) *H. erichmichaelii*, (I) *H. carnea*, (J) *H. rhodocheila* (Chanthaburi), (K) *H. xanthocheila*, (L) *H. dentata*, (M) *H. myriotricha*, (N) *H. porphyricola*, (O) *H. lindleyana*, (P) *H. hosseusii*, (Q) *H. malintana*, (R) *H. vidua*, (S) *H. lucida*, (T) *H. humistrata*, (U) *H. thailandica*, (V) *H. limprichtii*, (W) *H. austrosinensis*, (X) *H. reflexa*, (Y) *H. chlorina*, (Z) *H. marginata*, (AA) *H. rostellifera*, (BB) *P. hawkesiana*, (CC) *P. sagarikii* and (DD) *P. susannae.*

Table 2 Sixty-four primer combinations used in the AFLP technique.

Primer	M-CAA	M-CAC	M-CAG	M-CAT	M-CTA	M-CTC	M-CTT	M-CTG
E-ATT	1	2	3	4	5	6	7	8
E-AGC	9	10	11	12	13	14	15	16
E-ACG	17	18	19	20	21	22	23	24
E-AGT	25	26	27	28	29	30	31	32
E-ATA	33	34	35	36	37	38	39	40
E-AGG	41	42	43	44	45	46	47	48
E-ACA	49	50	51	52	53	54	55	56
E-ACC	57	58	59	60	61	62	63	64

3. Results and discussion

3.1 AFLP analysis

The results revealed genetic diversity in *Habenaria* and *Pecteilis* genera, similar to other orchid species, e.g., *Dendrobium thyrsiflorum* [14], *Liparis japonica* [15] and *Phalaenopsis* [16]. Four selected AFLP primer pairs generated 236 reproducible amplicons ranging in size from 100 to 311 base pair (bp); 233 bands were polymorphic, and the polymorphism percentage was 98.72. The number of AFLP bands per primer combination ranged from 51 to 67 with an average of 59 bands, while the range of polymorphic bands per primer pair combination varied from 50 to 67, with an average of 58.25 bands (Table 3).

Table 3 Numbers of AFLP detected with four primer pairs for 30 samples of *Habenaria* and *Pecteilis* species.

Primer pair	Number of amplified bands	Number of polymorphic bands	Polymorphism (%)
E-ATT/M-CAC	54	53	98.14
E-AGT/M-CTG	51	50	98.03
E-ACA/M-CAC	67	67	100
E -ACC/M-CAC	64	63	98.43
Total	236	233	98.72
Average	59	58.25	98.65

3.2 Genetic relationship of thirty Habenaria and Pecteilis species by AFLP

Two hundred and thirty-six polymorphic bands from four primer combinations were used to calculate the similarity coefficients by the NTSYS-pc version 2.2 program. Similarity coefficients among species indicated that H. erichmichaelii and H. carnea had the closest genetic relationship, with similarity coefficients greater than 0.60 (Figure 2). It indicated that differences among studies species were found. A coefficient value of 0.60 could distinguish these two genera. Dendrogram analysis by UPGMA (Figure 2) and PCoA (Figure 3) showed that the *Habenaria* and *Pecteilis* could be separated into two main groups. Group 1 consisted of all 27 samples of *Habenaria* with small to large colorful flowers such as *H. roebelenii* (Nongkhai), *H. rhodocheila* (Udon Thani), *H. janellehayneiana* (Phitsanulok), *H. rhodocheila* (Phangnga), *H. rhodocheila* (Ubon Ratchathani), *H. rhodocheila* (Satun), *H. rhodocheila* (Nakhon Si Thammarat), *H. erichmichaelii, H. carnea, H.rhodocheila* (Chanthaburi), H. *xanthocheila, H. dentata, H. myriotricha, H. porphyricola, H. lindleyana, H. hosseusii, H. malintana, H. vidua, H. lucida, H. humistrata, H. thailandica, H. limprichtii, H. austrosinensis, H. reflexa, H. chlorina, H. marginata and H. rostellifera*. Group 2 consisted of 3 samples of *Pecteilis* with large white and yellow flowers, including *P. hawkesiana, P. sagarikii* and *P. susannae*. AFLP supports the separation of *Habenaria* and *Pecteilis*, a descripted by botanists [17]. Group 1 was further divided into 3 subgroups. Subgroup 1.1 consisted of large and colorful flowers, called “Lin-mang-gon” in Thai. This subgroup has a spheroid tuber underground with leaves that are circular, ovate, succulent, and rosette in a spiral leaf arrangement and spiral leaf cover ground. It typically has 3 to 8 leaves per plant. The inflorescence is 12-90 cm long. There are typically 8 to 13 flowers per plant. These include *H. roebelenii* (Nongkhai), *H. rhodocheila* (Udon Thani), *H. janellehayneiana* (Phitsanulok), *H. rhodocheila* (Phangnga), *H. rhodocheila* (Ubon Ratchathani), *H. rhodocheila* (Satun), *H. rhodocheila* (Nakhon Si Thammarat), *H. erichmichaelii, H. carnea, H.rhodocheila* (Chanthaburi) and *H. xanthocheila.* In the past, all of these were classified under *H. rhodocheila* except for *H. carnea*, which had a unique characteristic i.e., white spots on green leaves and pinkish-orange spots on orange-brown flowers. Over the years, several new species have been proposed. *H. xanthocheila* was separated a long time ago as the color of this species is bright yellow as well as *H. roebelenii* whose flower color is bright to deep red, and *H. erichmichaelii* whose flower color is bright pink. Recently, *H janellehayneiana* was separated from *H. rhodocheila* due to its bright pink flower color and pure green leaf. In addition, reports by Kawchadee [18] and Saikum [19] on the crossability of some terrestrial orchid species of the genus Habenaria and Pecteilis used the same. Habenaria was in subgroup 1.1 and showed that the number of fruit sets of interspecific hybridization was more than 80-100%. This indicated a close relationship in subgroup 1.1, though some were separated into different species.

Subgroup1.2 consisted of *Habenaria* with small greenish, bright green, white and yellow flowers, including *H. dentata, H. myriotricha, H. porphyricola, H. lindleyana, H. hosseusii, H. malintana, H. vidua, H. lucida, H. humistrata, H. thailandica, H. limprichtii, H. austrosinensis, H. reflexa and H. marginata*. This subgroup has spheroid and slender tuber underground with leaves that are elliptic oblanceolate or obovate shapes. It typically has 3 to 8 leaves per plant with green leaves in, a spiral leaf arrangement. The inflorescence is 10-50 cm long. Subgroup 1.3 consisted of small and colorful flowers such as *H. chlorina* and *H. rostellifera*, which have sub-spheroid tuber underground and leaves that are lanceolate in a spiral leaf arrangement. The inflorescence is 10-20 cm long. There are typically 10 to 25 flowers per plant (Figure 2). A similar study on the genetic relationship

between the genus *Habenaria* and *Pecteilis* used the RAPD technique. 9 samples of *Habenaria* and *Pecteilis* were separated using the RAPD technique into 2 groups. Group 1 comprised colored flowers of *H. rhodocheila* (red and pink flowers) and *H. xanthocheila*, while group 2 comprised white flowers of *H. myriotricha* and *H. lindleyana* as well as *P. hawkesiana* (white flower with yellow lip) [20]. A similar study on colored flower patterns using AFLP was found in *Phalaenopsis cornu-cervi.* The AFLP technique distinguished reddish-brown flowers and yellow flowers with reddish-brown bars and spots [21]. The cDNA-AFLP markers linked to the gene expression of flower buds were studied in *Phalaenopsis equestris*. It was found that the gene expression of flower buds of two F2 progenies was derived from a cross between *P. equestris* "W9-52" and "W9-17" using the cDNA-AFLP method. Two fragments of AM1-3 and AM4-1 were found to correspond to flower color [22]. These results were consistent with a preliminary study for *Spathoglottis species*, which showed that, out of 56 AFLP primer combinations tested on *Spathoglottis plicata*, only eight provided scorable polymorphic bands. A total of 60 *Spathoglottis* samples were analyzed using eight primer combinations, generating 891 polymorphic bands with an average of 98.41% from 905 present bands [23]. AFLP markers based on genomic DNA can be used for orchid genome mapping, genetic relationships, and the identification of different orchid cultivars, hybrids and diversity studies.

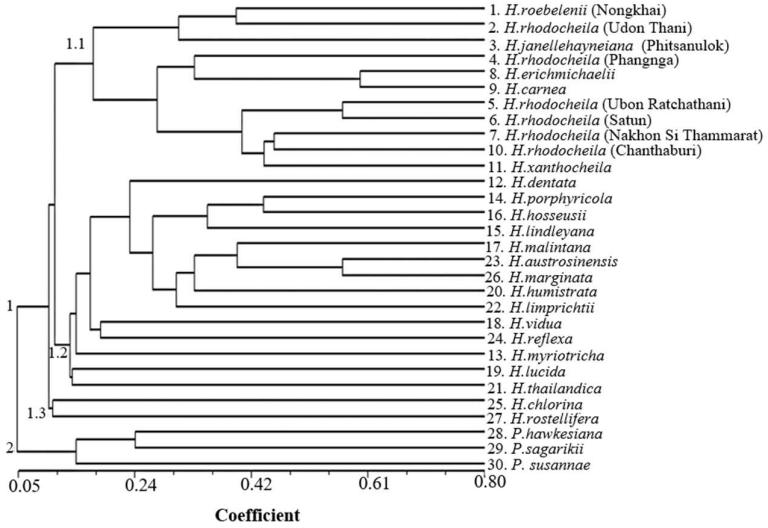

Figure 2 Dendrogram of 30 *Habenaria* and *Pecteilis* samples using four AFLP primer combinations (E-ATT/M-CAC), (E-AGT/M-CTG), (E-ACA/M-CAC) and (E-ACC/M-CAC).

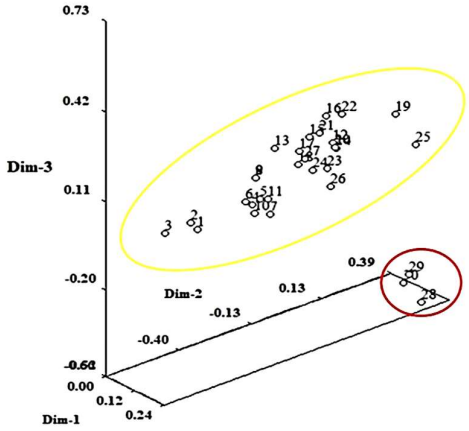

Figure 3 Grouping of relationships among *30 Habenaria* and *Pecteilis* sample accessions revealed by principal coordinate analysis (PCoA) based on AFLP 1. *H. roebelenii* (Nongkhai), 2. *H. rhodocheila* (Udon Thani), 3. *H. janellehayneiana* (Phitsanulok), 4. *H. rhodocheila* (Phangnga), 5. *H. rhodocheila* (Ubon Ratchathani), 6. H. rhodocheila (Satun), 7. H. rhodocheila (Nakhon Si Thammarat), 8. H. erichmichaelii, 9. H. carnea, 10. *H. rhodocheila* (Chanthaburi), 11. *H. xanthocheila*, 12. *H. dentata*, 13. *H. myriotricha*, 14. *H. porphyricola*, 15. *H. lindleyana*, 16. *H. hosseusii*, 17. *H. malintana,* 18. *H. vidua*, 19. *H. lucida*, 20. *H. humistrata*, 21. *H. thailandica*, 22. *H. limprichtii*, 23. *H. austrosinensis*, 24. *H. reflexa,* 25. *H. chlorina*, 26. *H. marginata*, 27. *H. rostellifera*, 28. *P. hawkesiana*, 29. *P. sagarikii* and 30. *P. susannae*.

4. Conclusion

The AFLP technique could be used to distinguish *Habenaria* and *Pecteilis* species in accordance with morphology, especially in terms of flower color and flower form. In this study, *Habenaria* and *Pecteilis* were divided into 2 groups. Group 1 consisted of 27 samples of *Habenaria,* which had small to large and colorful flowers. Further, group 1, could be separated into 3 subgroups with subgroup 1.1 consisting of *Habenaria*, which has big and colorful flowers i.e. *H. roebelenii* (Nongkhai), *H. rhodocheila* (Udon Thani), *H. janellehayneiana* (Phitsanulok), *H. rhodocheila* (Phangnga), *H. rhodocheila* (Ubon Ratchathani), *H. rhodocheila* (Satun), *H. rhodocheila* (Nakhon Si Thammarat), *H. erichmichaelii, H. carnea, H. rhodocheila* (Chanthaburi), and *H. Xanthocheila.* Subgroup 1.2 consisted of white, light green or green flower colors of *H. dentata, H. myriotricha, H. porphyricola, H. lindleyana, H. hosseusii, H. malintana, H. vidua, H. lucida, H. humistrata, H. thailandica, H. limprichtii, H. austrosinensis, H. reflexa,* and *H. Marginata*, while subgroup 1.3 consisted of *H. chlorina,* which had small and yellow flowers, and *H. rostellifera*, which had small and pink flowers. Group 2 consisted of 3 samples of *Pecteilis,* which had large, white- and yellow-colored flowers of *P. hawkesiana, P. sagarikii*, and *P. susannae.*

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