

Pamphilius daisenus, an Oligophagous Sawfly Feeding on *Spiraea japonica*: The First Record of Pamphiliidae (Hymenoptera) Associated with *Spiraea*

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Abstract Based on rearing experiments and a molecular analysis using the mitochondrial cytochrome oxidase subunit 1 (COI) gene sequences, *Spiraea japonica* L.f. [Rosaceae] is recorded as a host plant of *Pamphilius daisenus* Takeuchi, 1938. This is the first record of *Spiraea* as the host plant of the Pamphiliidae. *Pamphilius daisenus* is an oligophagous species feeding also on *Aruncus dioicus* (Walter) Fernald var. *kamtschaticus* (Maxim.) H. Hara [Rosaceae].

Key words: Symphyta, new host record, *Spiraea*, *Aruncus*, Rosaceae, COI sequences.

Introduction

Pamphilius daisenus Takeuchi, 1938, is a rare species of leaf-rolling sawfly known to occur in Honshu, Japan, and Korea (Shinohara, 2001). In Honshu, adults are found mainly at higher altitudes (300–2000 m) in Aomori, Miyagi, Tochigi, Saitama, Nagano, Gifu, and Tottori Prefectures in June to August (Shinohara, 1985, 1988, 2001; Nakamura, 2003) and the larva is a solitary leaf-roller on *Aruncus dioicus* (Walter) Fernald var. *kamtschaticus* (Maxim.) H. Hara [Rosaceae] (Shinohara and Kojima, 2011).

On August 7, 2013, A. Shinohara and H. Kojima discovered two pamphiliid larvae solitarily rolling the margin of a leaf of *Spiraea japonica* L.f. [Rosaceae] in the vicinity of Takamine-onsen near the borders of Nagano and Gunma prefectures, central Honshu. This was a new discovery because no Pamphiliidae were known to be associated with *Spiraea* (Shinohara, 2002; Shinohara *et al.*, 2016). A larva of *Pamphilius daisenus* feeding on *A. dioicus* var.

kamtschaticus was also found in the same site on the same day. Specific identity of the *Spiraea*-feeding larvae remained unknown.

In mid and late August of the same year, H. Kojima tried to find the larvae on *S. japonica* again in the same and other localities but no additional larvae were found. However, he found several larvae of *P. daisenus* feeding on *A. dioicus* var. *kamtschaticus* and, because the larvae feeding on *S. japonica* closely resembled *P. daisenus* in general appearance, he made attempts to rear the larvae of *P. daisenus* with the leaves of *S. japonica*. These rearing experiments clearly showed that the larvae of *P. daisenus* also feed on *S. japonica* and grew to the adult stage without any problems under experimental conditions. Subsequent molecular analyses of the larvae collected on *S. japonica* and *A. dioicus* var. *kamtschaticus* in Tochigi and Nagano prefectures clarified that the larvae feeding on the two plants were quite similar genetically (almost identical in COI sequences). We conclude that *P. daisenus* is an oligophagous species feeding both on *A.*

dioicus var. *kamtschaticus* and *S. japonica*. Here we give details of the rearing experiments and molecular analyses.

Materials and Methods

Larvae were collected in the following localities in central Honshu: Hanaishi town, about 650 m alt., 36°45'N 139°35'E, Nikko city, Tochigi prefecture; Takamine-onsen, about 1950 m alt., 36°24'N 138°27'E, Komoro city, Nagano prefecture; Mt. Kasadake, about 1850 m alt., 36°40'N 138°28'E, Yamanouchi town, Nagano prefecture; Mt. Kotomiyama, about 1800 m alt., 36°39'N 137°48'E, Hakuba village, Nagano prefecture. Rearings were made by H. Kojima in an air-conditioned room in Kitanagaike, Nagano city, Nagano prefecture, at an altitude of 335 m. The temperature and day length of the rearing room were not rigidly controlled, except that the highest temperature was set at 25°C. In order to count the number of molts during the larval stage, each leaf-roll was examined every day and cast skins were collected when present. On maturity, each larva was released to an individual jar containing ordinary untreated soil. Oviposition experiments were done as follows: One female each was put in a plastic bag with one of the three species of plants (*A. dioicus* var. *kamtschaticus*, *S. japonica* or *S. salicifolia* L.) and left in the room from morning to late afternoon. The plants were then examined and the number of the eggs was counted. In one case, two different species of plants, *A. dioicus* var. *kamtschaticus* and *S. salicifolia*, were put with a female sawfly in the same bag to check if one of the plants was preferred for oviposition to the other.

Images of the larvae and the larval leaf rolls were taken with digital cameras, Ricoh GR Digital IV (Figs. 1, 2), Canon EOS Kiss Digital X (Fig. 3) and Nikon S5100 (Figs. 4–6), and the digital images were processed and arranged with Adobe Photoshop Elements 12.0 software. Scientific names of the host plants follow Yonekura and Kajita (2016). Definition of the concepts of monophagy and oligophagy follows Viitasaari (2002).

We sequenced a partial region of mitochondrial COI gene, which is generally used for DNA barcoding in various insects. Total genomic DNA was extracted using DNeasy® Blood & Tissue Kit (QIAGEN) by the standard protocol. Polymerase chain reaction (PCR) was conducted using the LCO1490 (5'-GGT CAA CAA ATC ATA AAG ATA TTG G-3') and HCO2198 (5'-TAA ACT TCA GGG TGA CCA AAA AAT CA-3') primers (Folmer *et al.* 1994) and the following reaction conditions: initial denaturation for 2 min at 96°C, followed by 35 cycles of 30 s at 96°C, 30 s at 45°C, and 1 min at 72°C, with final extension for 5 min at 72°C. All reactions were aided by ExTaq HS polymerase (TaKaRa), and the products were purified with Exo-SAP-IT (Affymetrix). Sequencing reaction was performed using an ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction Kit (Life Technologies) and electrophoresed on an ABI 3500xl sequencer (Life Technologies). The obtained COI sequences have been deposited in the DDBJ/EMBL/GenBank database under accession numbers LC158361–LC158363 and LC158365–LC158370.

The alignment of the COI gene was straightforward and required no gaps. Phylogenetic trees were obtained by maximum parsimony (MP) and maximum likelihood (ML) methods implemented in MEGA6 (Tamura *et al.* 2013). For MP analyses, heuristic searches were conducted with 10 random addition analyses using equal character weights and tree-bisection-reconnection (TBR) branch swapping. Prior to ML analysis, we determined an appropriate model of sequence evolution and model parameters using MEGA. As a result, GTR + G model was selected. Based on the selected model, ML analysis was performed with heuristic searches with subtree-pruning-regrafting-extensive (SPR) branch swapping. Nodal support for the MP and ML analyses was assessed using bootstrap analyses with 1000 replications.

Samples used for molecular analysis consist of eight larvae of *P. daisen* feeding on *A. dioicus* var. *kamtschaticus* or *S. japonica* found in Toch-



Figs. 1–6. Larval abodes and immature stages of *Pamphilius daisenus*.—1, Larval abode on *Spiraea japonica*, near Takamine-onsen, Nagano pref., August 6, 2014 (remains of egg shell arrowed); 2, larval abode on *S. japonica*, Hanaishi town, Tochigi pref., June 15, 2014 (remains of egg shell arrowed); 3, late instar larva in the abode shown in Fig. 2, June 19, 2014; 4, egg on a leaf of *Spiraea salicifolia* (arrowed) deposited by the female C 4 in Table 1, Kitanagaike, Nagano pref., June 1, 2014; 5, larval abode on *S. salicifolia* made by the same individual shown in Fig. 4 (4th instar larva), July 2, 2014; 6, mature larva, C 2 in Table 1, Mt. Kotomiyama, reared with *Aruncus dioicus* var. *kamtschaticus* and *S. japonica* (after September 1), September 10, 2013. Photographed by A. Shinohara (Figs. 1–3) and H. Kojima (Figs. 4–6).

igi and Nagano prefectures and five outgroup species of the genera *Neurotoma* and *Pamphilius* as listed below. As outgroup species of *Pamphilius*, we selected one species each of the *P. sylvaticus*, *P. sulphureipes*, *P. alternans* and *P. histrio* groups. *Pamphilius daisen* belongs to the *P. sylvaticus* group (Shinohara, 2002). All the specimens are kept in the National Museum of Nature and Science, Tsukuba.

Pamphilius daisen: 2 larvae (sample no. 629, 630), Hanaishi town, Nikko city, Tochigi pref., 14. VI. 2014, matured and fixed 19. VI., host: *Spiraea japonica*, A. Shinohara; 1 larva (sample no. 701), near Takamine-onsen, Nagano pref., 7. VIII. 2013, matured and fixed 15. VIII., host: *Spiraea japonica*, A. Shinohara & H. Kojima; 1 larva (sample no. 631), same locality, 6. VIII. 2014, matured and fixed 6. VIII., host: *Spiraea japonica*, A. Shinohara; 1 larva (sample no. 628), same locality, 10. VIII. 2013, matured and fixed 20. VIII., host: *Aruncus dioicus* var. *kamtschaticus*, A. Shinohara; 2 larvae (sample no. 632, 633), same locality, 18. VIII. 2014, matured and fixed 22. VIII., host: *Aruncus dioicus* var. *kamtschaticus*, A. Shinohara; 1 larva (sample no. 702), same locality, 11. VIII. 2015, matured and fixed 12. VIII., host: *Aruncus dioicus* var. *kamtschaticus*, A. Shinohara.

Outgroups: *Neurotoma sibirica* Gussakovskij, 1935: GenBank accession no. LC126728. *Pamphilius histrio* Latreille in Olivier and Latreille, 1812: GenBank accession no. LC126714. *Pamphilius komonensis* Takeuchi, 1930: GenBank accession no. LC126715. *Pamphilius ishikawai* Shinohara, 1979: GenBank accession no. LC126719. *Pamphilius volatilis* (Smith, 1874): 1 ♂ (sample no. 276), Asahidake-onsen, Hokkaido, Japan, 27. VI. 2006, A. Shinohara.

Results

Field observations, rearing records and oviposition experiments

Table 1 summarizes rearing records of the larvae collected in the field in 2013 and 2014. On August 7, 2013, A. Shinohara and H. Kojima col-

lected three larvae, two on *S. japonica* and one on *A. dioicus* var. *kamtschaticus* in Takamine-onsen. They matured on August 15 and 17, one on *S. japonica* was fixed in ethanol for examination (sample no. 701), the other on *S. japonica* died in the earthen cell, and the larva on *A. dioicus* var. *kamtschaticus* became an adult and emerged on July 10, 2014. In the same site, A. Shinohara found one larva feeding on *A. dioicus* var. *kamtschaticus* on August 10, 2013, and fixed it in ethanol for molecular work (sample no. 628).

In 2013, H. Kojima also collected two larvae in the same area on August 18, two larvae on Mt. Kasadake on August 20, and four larvae on Mt. Kotomiyama on August 28, all from *A. dioicus* var. *kamtschaticus*. To determine if the larvae can also feed and grow on *S. japonica*, H. Kojima removed all the larvae from *A. dioicus* var. *kamtschaticus* leaves and put them on *S. japonica* leaves one to four days after collection. All the larvae, except for the one that died before the change of the diet (C 3 in Table 1), fed on *S. japonica* and matured apparently without problems (one of the mature larva shown in Fig. 6). Of the seven prepupae, two died in the soil, one was killed by a parasitoid, and four emerged as female adults in May and June, 2014. On August 4, 2014, H. Kojima collected two larvae feeding on *A. dioicus* var. *kamtschaticus* in Takamine-onsen and reared them with *S. japonica*. The two larvae fed and grew on *S. japonica* and matured on August 18 and 23, and the adults emerged in 2015, a female on June 10 and a male on June 19.

In May and June, 2014, H. Kojima kept two of the emerged females alive in containers with three different plants, *A. dioicus* var. *kamtschaticus*, *S. japonica* and *Spiraea salicifolia* to check if they oviposit and their offspring grow without problems. The two mother females were those that emerged on May 23 (B 2 in Table 1) and May 24 (C 4 in Table 1), respectively. As noted above, they were collected as larvae feeding on *A. dioicus* var. *kamtschaticus* and later reared with *S. japonica*. A total of 11 oviposition experi-

Table 1. Rearing records of *P. daisenus* from larvae collected in three localities in Nagano prefecture in 2013 (A–C) and 2014 (D) by H. Kojima. See text for explanation.

Localities	August																		September							Adult emergence													
	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28		29	30	31	1	2	3	4	5	6	7	8	9	10
A	1				L	L	L	L	L	L	L	L	M																										fixed in ethanol
	2				L	L	L	L	L	L	L	L	L	M																							dead in soil		
	3				L	L	L	L	L	L	L	L	L																								♀, July 10, 2014		
	4				L	L	L	L	L	L	L	L	L		L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	M					dead in soil			
	5														L	L	L	L	L	L	M																♀, June 10, 2014		
B	1																																				killed by parasitoid		
	2																																				♀, May 23, 2014		
C	1																																				dead in soil		
	2																																				♀, June 18, 2014		
	3																																						
	4																																				♀, May 24, 2014		
D	1	L	L	L	L	L	L	L	L	L	L	L	L	L	M																						♂, June 19, 2015		
	2	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	M																♀, June 10, 2015			

Localities A, D: Takamine-onsen. B: Mt. Kasadake. C: Mt. Kotomiyama.

Host plants A 1, 2: *Spiraea japonica*. A 3: *A. dioicus* var. *kamtschaticus*. A 4, 5: *A. dioicus* var. *kamtschaticus* and *Spiraea japonica* (after August 19). B: *A. dioicus* var. *kamtschaticus* and *Spiraea japonica* (after August 21). C: *A. dioicus* var. *kamtschaticus* and *Spiraea japonica* (after September 1). D: *A. dioicus* var. *kamtschaticus* and *Spiraea japonica* (after August 6).

L: larva (molt in bold letter). M: matured. D: dead.

ments were made following the methods described above. During the period from May 25 to June 7, the female B 2 deposited 16 eggs on *A. dioicus* var. *kamtschaticus*, six eggs on *S. japonica* and five eggs on *S. salicifolia* (Table 2). During the same period, the female C 4 deposited eight eggs on *A. dioicus* var. *kamtschaticus*, seven eggs on *S. japonicus* and nine eggs on *S. salicifolia* (one of them shown in Figs. 4, 5) (Table 2). In other words, a total of 51 eggs were deposited by the two females, namely, 24 eggs on *A. dioicus* var. *kamtschaticus*, 13 eggs on *S. japonica* and 14 eggs on *S. salicifolia*. In all four experiments where *A. dioicus* var. *kamtschaticus* or *S. japonicus* were given, the sawflies laid eggs. In the six experiments where the females were kept only with *S. salicifolia*, the sawflies oviposited in three cases and did not oviposit in other three cases. In one case where *A. dioicus* var. *kamtschaticus* and *S. salicifolia* were put in one plastic bag with the B 2 female, 16 eggs were laid on *A. dioicus* var. *kamtschaticus* while only one egg was laid on *S. salicifolia*.

Of the 51 eggs laid, only 16 eggs (eight on *A. dioicus* var. *kamtschaticus*, six on *S. japonicus* and two on *S. salicifolia*) grew to the third or later larval instars. Others died at the egg or early larval stages. Table 3 shows details of the rearing records of those 16 individuals and Table 4 summarizes the duration of the egg and larval instars for each individual. The egg period is seven days for all individuals, whereas the larval period was 17 to 21 days for those feeding on *A. dioicus* var. *kamtschaticus*, 15 to 23 days for those feeding on *S. japonica*, and 47 days for one feeding on *S. salicifolia* (Fig. 5; another larva feeding on the same plant had 36 days before it died in the 7th instar).

In 2014, A. Shinohara collected three larvae feeding on *S. japonica*, two in Hanaishi town, about 650 m alt., 36°45'N 139°35'E, Nikko city, Tochigi prefecture, on June 15 (Figs. 2, 3) and one in Takamine-onsen on August 6 (Fig. 1), and two larvae feeding on *A. dioicus* var. *kamtschaticus* in Takamine-onsen on August 18. On August 11, 2015, A. Shinohara collected one larva on *A.*

Table 2. Number of eggs laid by two females on three different plant species in 11 oviposition experiments.

Mother sawfly	Date	Plant		
		<i>Aruncus dioicus</i> var. <i>kamtschaticus</i>	<i>Spiraea japonica</i>	<i>Spiraea salicifolia</i>
C 4	May 25		7	
	May 28			2
	June 1			7
	June 5	8		
B 2	May 25		1	
	May 28			4
	May 29			0
	May 30		5	
	June 1			0
	June 5			0
	June 7	16		1

dioicus var. *kamtschaticus* in Takamine-onsen. All the six larvae were fixed in ethanol for molecular analysis (sample nos. 629–633, 702).

Molecular analysis

The COI data matrix consisted of 669 nucleotide sites, of which 61 were parsimony-informative. The MP analysis resulted in five most parsimonious trees (191 steps, consistency index excluding uninformative sites [CI] = 0.6696, retention index [RI] = 0.6807). The result of ML analysis was consistent with the MP analysis (Fig. 7). The sequences of *P. daisenus* specimens formed a cluster with 100% MP and ML bootstrap supports with only two variable sites among eight individuals analyzed.

The analysis included one species of *Neurotoma* and four species of *Pamphilius* as outgroups. Of these, *P. volatilis* formed a clade with *P. daisenus* with 78% MP and 67% ML bootstrap supports. The relationship of this clade with the other three *Pamphilius* samples was not well resolved in our analysis.

Discussion

Host plants of *Pamphilius daisenus*

The observations given above indicate that *A. dioicus* var. *kamtschaticus* and *S. japonica* are the host plants of *P. daisenus*. This is the first record of *Spiraea* as a host plant of the Pamphiliidae. The larval periods of the individuals feed-

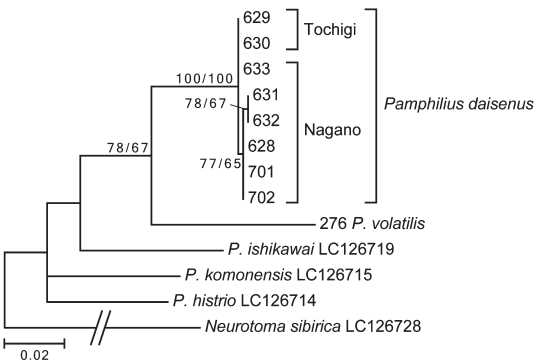


Fig. 7. The maximum likelihood tree showing phylogenetic relationships among *Pamphilius daisenus* and five pamphiliine species (-ln likelihood = 1665.6824). Numbers on branches indicate bootstrap values for MP and ML analyses (shown only for higher nodes with > 50). Each terminal label represents the sample number (upper nine individuals) or GenBank accession number (lower four).

ing on the two plant species do not differ significantly (17 to 21 days and 15 to 23 days). The larval periods of 15 individuals reared with *A. dioicus* var. *kamtschaticus* by Shinohara and Kojima (2011) ranged from 13 to 18 days.

The female of *P. daisenus* also laid eggs on *S. salicifolia* under experimental conditions when this plant only was available for oviposition. However, when both *A. dioicus* var. *kamtschaticus* and *S. salicifolia* were given, the female apparently preferred the former to the latter (Table 2). Two individuals that fed on *S. salicifo-*

Table 3. Rearing records of *P. daisenus* from eggs deposited on three different species of plants by two females (B 2 and C 4 in Table 1) that emerged in 2014 from larvae collected in Nagano prefecture in 2013 by H. Kojima. See text for explanation.

[illegible]

Diet AD: *Aruncus dioicus* var. *kamtschaticus*. SI: *Spiraea japonica*. SS: *Spiraea salicifolia*.

E: egg. Numerals: larval instars (molt in bold letter). M: matured. D: dead.

*: This individual became 6th instar on July 10, 7th instar on July 13, matured on July 25, but died in soil.

***. This individual became 6th instar on July 8, 7th instar on July 14, and died on July 20 at 7th instar. This individual became 6th instar on July 16, 7th instar on July 17, and died on July 23, but died in 5th instar.

: This individual became ill instar 6, / ill instar on July 14, and died on July 20 at / ill instar.

lia spent unusually long time as larvae (47 and 36+ days, respectively) and eventually did not survive (Tables 3, 4). Although the sample size is very small, *S. salicifolia* may not serve as a host.

Spiraea is a speciose genus of shrubs and *Aruncus* is a small genus of herbaceous perennial plants. Superficially they look quite different, but they are actually closely related phylogenetically and classified together in the small tribe Spiraeae of the rosaceous subfamily Amygdaloideae (Potter *et al.*, 2007). Plants of the two genera, at least *A. dioicus* var. *kamtschaticus* and *S. japonica*, should have something in common which attracts the females of this sawfly for oviposition and provide sufficient and suitable diet for the development of the larvae. It is interesting that such traits are apparently missing or insufficient in certain species of *Spiraea*, at least in *S. salicifolia*.

So far as is known, P

commonly monophagous, though this observation may at least partly reflect the insufficient knowledge on the host plant associations. Host plants are known for 29 species of *Pamphilius* occurring in Japan (Shinohara, 2002, 2003, 2005; Shinohara and Hara, 1999, 2006, 2009, 2011; Shinohara and Kojima, 2009, 2011; Shinohara *et al.*, 2016), and only two of them are oligophagous: *Pamphilius volatilis* (Smith, 1874) feeds on *Cerasus* spp. and *Crataegus chlorosarca* Maxim. (Rosaceae, Shinohara and Hara, 1999) and *P. masao* Shinohara, 2005, feeds on *Betula ermanii* Cham. and *Alnus viridis* (Chaix) Lam. et DC. subsp. *maximowiczii* (Callier) D.Löve (Betulaceae, Shinohara, 2005). Of the 24 German *Pamphilius* species whose hosts are known, ten species are reported as oligophagous (Taeger *et al.*, 1998), whereas some of the previous host records cited therein may need confirmation. Present discovery of the oligophagy in *P. daisenensis* is a well documented additional example of oligophagy in *Pamphilius*.

Table 4. Duration of egg and feeding larval periods (days), number of instars, and lengths of mature larvae of 16 male individuals of *P. daisenus*. See text for more explanation.

Host plant	Mother sawfly	Egg	Larva, instars I–VII, and total								Number of instars	Length of mature larva (mm)	Adult emergence in 2015
			I	II	III	IV	V	VI	VII	total			
<i>Aruncus dioicus</i> var. <i>kamtschaticus</i>	C 4	7	4	2	3	3	3	5		20	6	15	May 11
	C 4	7	3	2	5	3	7			20	5	12	dead in soil
	C 4	7	3	2	4+					9+			dead in 3rd instar
	B 2	7	3	2	2	2	3	5		17	6	16	May 3
	B 2	7	3	2	4	2	4	6		21	6	14	May 2
	B 2	7	3	2	3	3	3	4		18	6	15	April 14
	B 2	7	3	2	3	2	4	6		20	6	15	dead in soil
	B 2	7	3	2	2	3	3	5		18	6	15	April 22
<i>Spiraea japonica</i>	C 4	7	3	2	5	3	3	7		23	6	14	May 2
	C 4	7	3	2	5+					10+			dead in 3rd instar
	C 4	7	3	2	2	3	6			16	5	13	May 11
	C 4	7	3	2	2	4	5			16	5	14	May 2
	C 4	7	2	2	2	3	5			15	5	14	May 3
	B 2	7	2	1	2	2	3	5		16	6	15	April 28
<i>Spiraea salicifolia</i>	C 4	7	10	7	4	4	7	3	12	47	7	11	dead in soil
	B 2	7	4	4	6	7	3	6	6+	36+			dead in 7th instar

Implications of the results of molecular analysis

The eight specimens of *P. daisenus* used for molecular analysis were quite uniform in COI data, with only 0.3% intraspecific variability. The individuals feeding on *S. japonica* (629, 630, 631, 701) were not distinguishable from those feeding on *A. dioicus* var. *kamtschaticus* (628, 632, 633, 702) and we detected no geographical differences between the Tochigi and Nagano samples.

The samples of *P. daisenus* were clustered with *P. volatilis* with 78% MP and 67% ML bootstrap supports. These two species belong to the *P. sylvaticus* group, which was regarded as monophyletic on the basis of morphology (Shinohara, 1985, 1988, 2001, 2002). The present result based on COI sequences may support, though not very strongly, the hypothesis of the monophyly of the *P. sylvaticus* group.

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