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Phylogenetic Separation of *Holotrichia* species (Insecta, Coleoptera, Scarabaeidae) Exhibiting Circadian Rhythm and Circa'bi'dian rhythm

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A unique two-day rhythm, circabidian rhythm, has been reported in the black chafer, *Holotrichia parallela*. However, it remains unknown how widely the circabidian rhythm appears in related species. We examined the activity rhythm and phylogeny of congeneric species inhabiting Japan to investigate the appearance of circabidian rhythms in a few subgenera of the genus *Holotrichia*. We found that *Holotrichia picea* also exhibited circabidian rhythm. In addition to the regular circabidian pattern, circabidian rhythms with day-switching or with a circadian activity component were also observed. In the day-switching pattern, *H. picea* switched appearance from odd to even days, or vice versa. In the circadian-like activity patterns, a major night activity and a minor dusk activity appeared alternately. *Holotrichia kiotonensis*, *Holotrichia convexopyga*, and *Holotrichia loochooana loochooana* exhibited a circadian rhythm. Two distinct clades, A and B, were recognized in the histone H3, cytochrome c oxidase subunit 1, and 16S ribosomal RNA phylogenetic trees. This phylogenetic separation was in accordance with the subgeneric classification based on external morphology in a previous study and with behavioral rhythm in the present study: clade A included *Nigrotrichia* group members, *H. kiotonensis*, *H. convexopyga*, *H. loochooana loochooana*, and *H. loochooana okinawana*, while clade B included *Pedinotrichia* group members, *H. paralella* and *H. picea*. We suggest that after separation into *Nigrotrichia* and *Pedinotrichia*, the behavioral trait of circabidian rhythm probably appeared once in an ancestral species of the *Pedinotrichia* group, including *H. parallela* and *H. picea*.

Key words: circabidian rhythm, circadian clock, molecular phylogeny, two-day rhythm, *Nigrotrichia*, *Pedinotrichia*

INTRODUCTION

A variety of endogenous rhythms, such as circatidal, circadian, and circalunar rhythms, have been reported in various animals. Organisms have acquired endogenous rhythmicity with a cycle similar to their environment to anticipate cyclic changes in their surroundings. However, a unique two-day rhythmicity has been reported in the black chafer, *Holotrichia parallela* (Insecta, Coleoptera, Scarabaeidae) (Yoshioka and Yamazaki, 1983; Leal et al., 1993; Kawasaki et al., 2017). Because two-day environmental cycles are not known in its habitat, *H. parallela* appears to exhibit a singular rhythm. In the field, adult beetles appear on the ground at sunset every two nights to feed and mate on deciduous trees, burrowing back into the soil at sunrise (Kawasaki et al., 2017). Even though *H. parallela* have only half the chance of eating and mating compared with species with circadian activity rhythm, they maintain their population size as an agricultural pest (Qin et al., 2019). Hence, there has been increasing interest in research on how the circabidian rhythm has evolved. Also, the biological significance of the circabidian rhythm remains a mystery.

The physiological mechanisms of the circabidian rhythm have been discussed previously (Kawasaki et al., 2017; Watanabe and Shiga, 2020). Under constant laboratory conditions, *H. parallela* adults exhibit a free-running rhythm with a period close to 48 h, and this circabidian rhythm entrains to every two cycles of 12 h light and 12 h darkness. Analysis of the phase responses of the rhythm to light pulses under constant darkness suggested that there are two circadian cycles, each consisting of a less responsive and more responsive phase in a phase-dependent manner, in one circabidian cycle (Kawasaki et al., 2017). Physiological studies on circabidian rhythms have revealed that the brain region called the optic lobe, known to furnish circadian clock cells in many species, is a prerequisite for the circabidian rhythm in *H. parallela* (Watanabe and Shiga, 2020). These reports suggest that the circadian clock system is involved in the circabidian rhythm, and there might be a mechanism dou-

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bling a circadian clock cycle to produce a circabidian rhythm (Watanabe and Shiga, 2020).

Another interesting point about the circabidian rhythm is when this behavioral trait emerged in evolutionary history. This may give us some clues regarding the appearance of this rhythm by examining activity rhythms in related species with reference to phylogenetic analysis. *Holotrichia* (Coleoptera, Scarabaeidae, Melonthianae, Rhizotrogina) is a large genus that includes heterogeneous species groups inhabiting Southeast and East Asia (Ward et al., 2002; Anitha et al., 2006; Matsumoto, 2016). Because *Holotrichia* species, including *H. serrata*, *H. consanguinea*, *H. reynaudi*, *H. loochooana loochooana*, and *H. parallela*, are known as serial pests of agricultural crops, studies on entomopathogens, chemical ecology, and olfactory mechanisms have been conducted for pest control (Leal et al., 1993; Arakaki et al., 2003; Ju et al., 2018; Chandel et al., 2019; Wang et al., 2019). However, chronobiological studies on these species have not been conducted, except for *H. parallela* (Kawasaki et al., 2017).

Matsumoto (2015a, b, 2016) separated *Holotrichia* species into five groups and proposed new genera: *Amphitrichia*, *Eotrichia*, *Pedinotrichia*, *Nigrotrichia*, and *Rufotrichia*, using data from the morphological features of external organs, including the male genitalia. However, a molecular phylogenetic study has not yet been conducted. According to Matsumoto (2016), *H. parallela* exhibiting circabidian rhythm belongs to the *Pedinotrichia* group. Then, we collected *Holotrichia picea*, which also belongs to the *Pedinotrichia* group, along with *Holotrichia kiotonensis*, *Holotrichia convexopyga*, and *H. loochooana loochooana*, which are classified into the *Nigrotrichia* group in Japan, to compare their activity rhythms. We also conducted a phylogenetic analysis of these *Holotrichia* species with *H. parallela* and *Holotrichia loochooana okinawana* using mitochondrial and nuclear DNA to investigate the appearance of the circabidian rhythm in *Holotrichia*. Although Matsumoto (2015a, b, 2016) proposed *Nigrotrichia* and *Pedinotrichia* as new genera from the *Holotrichia* group, we would like to use *Nigrotrichia* and *Pedinotrichia* as subgeneric groups in the present study and wait for another classification study with a larger-scale analysis of the *Holotrichia* group.

MATERIALS AND METHODS

Specimen sampling

For behavioral analysis, adult black chafer *H. kiotonensis* (Brenske, 1894; Fig. 1A), *H. convexopyga* (Moser, 1912; Fig. 1B), and *H. picea* (Waterhouse, 1875; Fig. 1C) were collected from the Toyonaka campus of Osaka University in Osaka, Japan (34°48′N, 135°27′E) from April to September 2016. Additional samples of *H. convexopyga* and *H. picea* were collected in May 2020. Adults of *H. loochooana loochooana* (Sawada, 1950) were collected from Nisi-Henna-Zaki (24°54′N, 125°15′E) and Hirara-Higashi-Nakasone (herein called Manabi-No-Mori, 24°48′N, 125°17′E) (Fig. 1E) Miyako Island, Okinawa, Japan, from June to August 2016 and 2018.

For phylogenetic analysis, in addition to the species mentioned above, *H. loochooana okinawana* (Nomura, 1964; Fig. 1D) were collected in Itoman City (26°7′N, 127°40′E), Okinawa, Japan, in June 2016; *H. parallera* (Motschulsky, 1854; Fig. 1F) on the bank of

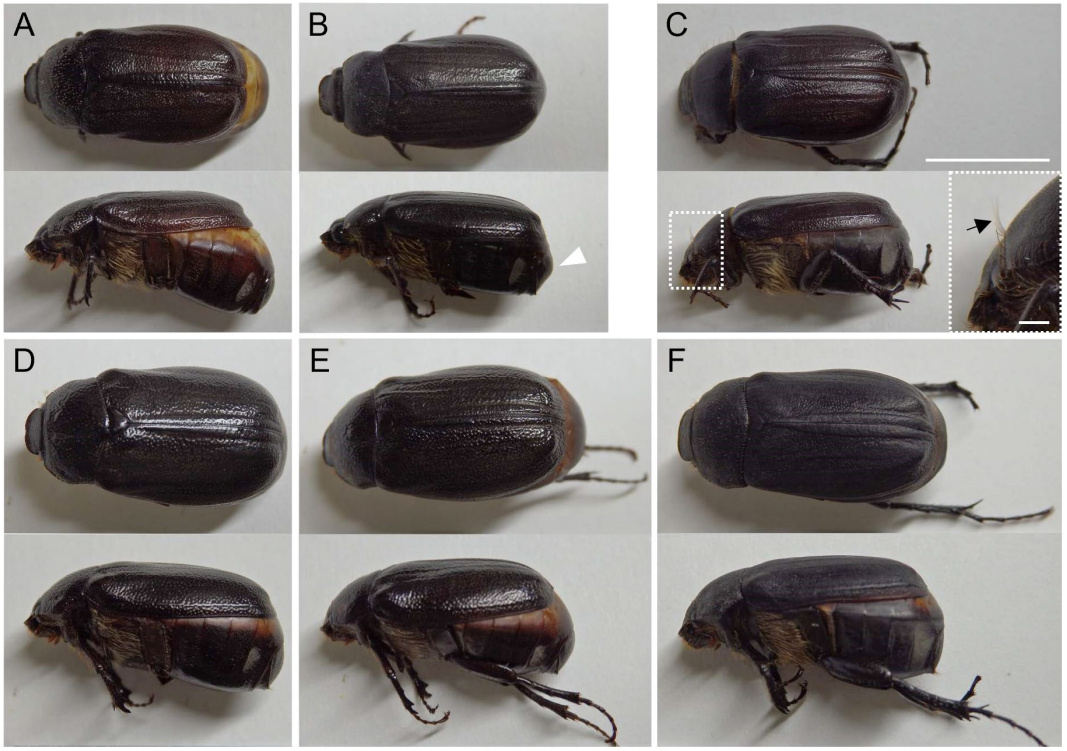

Fig. 1. Male habitus of *Holotrichia* species in Japan. The upper portion is the dorsal view and the lower portion is the sagittal view in each panel. **(A)** *Holotrichia kiotonensis*. **(B)** *Holotrichia convexopyga*. The arrowhead indicates the convex abdominal tip. **(C)** *Holotrichia picea*. The left dotted rectangle is enlarged as an inset. The anterior margin of the pronotum is arranged with hairs (arrow). **(D)** *Holotrichia loochooana okinawana*. **(E)** *Holotrichia loochooana loochooana* collected at Manabi-No-Mori (Hirara-Higashi-Nakasone). **(F)** *Holotrichia parallela*. All photos except for the inset of **(C)** are on the same scale. Scale bars for **(C)**: 10 mm for upper photo, 1 mm for inset.

Table 1. Collection and information of specimens used for phylogenetic analysis.

Sample no.	Species	Sex	Date of collection	Locality
1	*Holotrichia kiotonensis*	M	2016.5.31	Toyonaka Campus, Osaka University (34°48′ N, 135°27′ E)
2	*Holotrichia kiotonensis*	M	2016.5.31	Toyonaka Campus, Osaka University (34°48′ N, 135°27′ E)
3	*Holotrichia kiotonensis*	F	2016.5.31	Toyonaka Campus, Osaka University (34°48′ N, 135°27′ E)
4	*Holotrichia kiotonensis*	F	2016.5.31	Toyonaka Campus, Osaka University (34°48′ N, 135°27′ E)
5	*Holotrichia convexopyga*	M	2016.5.31	Toyonaka Campus, Osaka University (34°48′ N, 135°27′ E)
6	*Holotrichia convexopyga*	M	2016.5.31	Toyonaka Campus, Osaka University (34°48′ N, 135°27′ E)
7	*Holotrichia convexopyga*	F	2016.5.31	Toyonaka Campus, Osaka University (34°48′ N, 135°27′ E)
8	*Holotrichia convexopyga*	F	2016.5.31	Toyonaka Campus, Osaka University (34°48′ N, 135°27′ E)
9	*Holotrichia picea*	M	2016.5.30	Toyonaka Campus, Osaka University (34°48′ N, 135°27′ E)
10	*Holotrichia picea*	M	2016.4.18	Toyonaka Campus, Osaka University (34°48′ N, 135°27′ E)
11	*Holotrichia picea*	F	2016.6.10	Toyonaka Campus, Osaka University (34°48′ N, 135°27′ E)
12	*Holotrichia picea*	F	2016.6.10	Toyonaka Campus, Osaka University (34°48′ N, 135°27′ E)
13	*Holotrichia parallela*	M	2016.7.4	Small hill by Sakuranomiya High School (34°43'N 135°31'E)
14	*Holotrichia parallela*	M	2016.7.4	Small hill by Sakuranomiya High School (34°43'N 135°31'E)
15	*Holotrichia parallela*	F	2016.7.4	Small hill by Sakuranomiya High School (34°43'N 135°31'E)
16	*Holotrichia parallela*	F	2016.7.4	Small hill by Sakuranomiya High School (34°43'N 135°31'E)
17	*Holotrichia loochooana loochooana*	M	2016.6.20	Nisihennazaki , Miyako island (24°54′ N, 125°15′ E)
18	*Holotrichia loochooana loochooana*	M	2016.6.20	Nisihennazaki , Miyako island (24°54′ N, 125°15′ E)
19	*Holotrichia loochooana loochooana*	F	2016.6.20	Nisihennazaki , Miyako island (24°54′ N, 125°15′ E)
20	*Holotrichia loochooana loochooana*	F	2016.6.20	Nisihennazaki , Miyako island (24°54′ N, 125°15′ E)
21	*Holotrichia loochooana loochooana*	M	2016.6.20	Manabinomori, Hirarahigashinakasone, Miyako island (24°48′ N, 125°17′ E)
22	*Holotrichia loochooana loochooana*	M	2016.6.20	Manabinomori, Hirarahigashinakasone, Miyako island (24°48′ N, 125°17′ E)
23	*Holotrichia loochooana loochooana*	M	2016.6.20	Manabinomori, Hirarahigashinakasone, Miyako island (24°48′ N, 125°17′ E)
24	*Holotrichia loochooana loochooana*	M	2016.6.20	Manabinomori, Hirarahigashinakasone, Miyako island (24°48′ N, 125°17′ E)
25	*Holotrichia loochooana okinawana*	M	2016.6.29	Forest behind Itoman City Central Library, Okinawa island (26°07'N 127°40'E)
26	*Holotrichia loochooana okinawana*	M	2016.6.29	Forest behind Itoman City Central Library, Okinawa island (26°07'N 127°40'E)
27	*Holotrichia loochooana okinawana*	M	2016.6.29	Forest behind Itoman City Central Library, Okinawa island (26°07'N 127°40'E)
28	*Holotrichia loochooana okinawana*	M	2016.6.29	Forest behind Itoman City Central Library, Okinawa island (26°07'N 127°40'E)
29	*Miridiba castanea*	M	2016.5.31	Toyonaka Campus, Osaka University (34°48′ N, 135°27′ E)

the Yodo River (34°43′N, 135°31′E), Osaka, Japan, in July 2016; and *Miridiba castanea* (Waterhouse, 1875) on the Toyonaka campus of Osaka University in May 2016. The collection sites, dates, and sex of all samples are shown in Table 1. *Miridiba castanea* was used as an outgroup. *Holotrichia loochooana loochooana*, *H. loochooana okinawana*, and their food, the beach cabbage, *Scaevola taccada*, were collected on Miyako Island, and transported by air. All collected beetles were kept in an air-conditioned room at 23–27°C under natural daylight before use. *Holotrichia loochooana loochooana* and *H. loochooana okinawana* were fed leaves of *S. taccada* and other species were fed the Japanese cherry *Prunus yedoensis* 'Somei-yoshino'.

Activity recording

The activity recording method was adopted from Kawasaki et al. (2017) and Watanabe and Shiga (2020). The activity of the beetles on the ground was individually recorded under periods of 12-h light and 12-h darkness (LD) for 10 days and subsequent constant darkness (DD) for another 14 days at 25 ± 1°C. A fluorescent lamp with an intensity of 1.35 W/m^2 (Panasonic FL15W; Panasonic, Osaka) was used as the light source. During the recording period, a leaf of *P. yedoensis* was provided to *H. kiotonensis*, *H. convexopyga*, and *H. picea*, and a leaf of *S. taccada* to *H. loochooana loochooana*, every 5 days. Approximately 20–25 mL of water was sprayed on the soil surface after it became dry.

The top of the container was covered with a transparent glass plate. Color or monochromatic images of the soil surface were taken every 1 min with a web camera (DC-NCR13U; Hanwha Japan, Tokyo). Beetle movements were detected automatically through differences in the total pixel values between two serial photos and eye inspections plotted on a double-plotted actogram (Kawasaki et al., 2017; Watanabe and Shiga, 2020). The presence of rhythmicity in beetle activity was determined separately in LD and DD using a chi-square periodogram analysis (Enright, 1965; Sokolove and Bushell, 1978).

DNA sequencing

Collected beetles were sacrificed, stored in 99.5% ethanol, and placed in a freezer until subsequent experiments. Genomic DNA was extracted from the hind leg, and purification of total DNA from insects was performed using a DNeasy® Blood & Tissue Kit (QIAGEN, Hilden, Germany) following the manufacturer's instructions. Polymerase chain reaction (PCR) was conducted to amplify the cytochrome c oxidase subunit 1 (CO1) (Former et al., 1994) and 16S ribosomal RNA (16S rRNA) (Hosoya et al., 2001) genes in the mitochondrial DNA and the histone 3 gene (H3) (Ogden and Whiting, 2003) in the nuclear DNA using a GoTaq® Green Master Mix (Promega, Madison, Wisconsin, United States). The primer sequences are listed in Table 2. The PCR amplification temperatures were as follows: for CO1, initial denaturation at 94°C for 2 min,

Table 2. Primer sequences used in the study.

Gene		Sequence name in reference	Sequence	Tm	Reference
CO1	F	LCO1490	5′-GGTCAACAAATCATAAAGATATTGG-3′	47.3	Former et al. (1994)
	R	HC02198	5′-TAAACTTCAGGGTGACCAAAAAATCA-3′	49.4	Former et al. (1994)
16S rRNA	F	16SA	5′-CGCCTGTTTAACAAAAACATGT-3′	45.4	Hosoya et al. (2001)
	R	16SB	5′-CCGGTTTGAACTCAGATCATGT-3′	49.2	Hosoya et al. (2001)
H3	F	HexAF	5′-ATGGCTCGTACCAAGCAGACGGC-3′	56.8	Ogden and Whiting (2003)
	R	HexAR	5′-ATATCCTTGGGCATGATGGTGAC-3′	51.5	Ogden and Whiting (2003)

followed by 30 cycles at 94°C for 15 s, 45°C for 30 s, and 72°C for 1 min; for 16S rRNA and H3, initial denaturation at 94°C for 2 min, and then 30 cycles at 94°C for 15 s, 50°C for 30 s, and 72°C for 1 min. The product yield was monitored via electrophoresis using a 1.5% agarose gel (code No. 312-01193, Nippon Gene, Toyama), and the amplicons were purified using a Monarch PCR & DNA Cleanup Kit (New England Biolabs Inc., Ipswich, MA).

Purified samples were prepared as 10–40 ng of CO1 and 16S rRNA PCR products and 6–20 ng of H3 PCR products using a NanoDrop 2000 (Thermo Fisher Scientific, Waltham, MA). Sequencing was outsourced (Fasmac Co., Ltd., Kanagawa).

Phylogenetic analysis

We used three datasets for phylogenetic analysis: H3, CO1, and 16S rRNA. After removing data of beetles that lacked any of the three sequences, the H3, CO1, and 16S rRNA sequences were concatenated and aligned using T-Coffee (http://tcoffee.crg.cat/apps/tcoffee/do:mcoffee; Notredame et al., 2000) and trimmed using TrimAl (Capella-Gutiérrez et al., 2009) to obtain 29 1527-bp long sequences. Aligned sequences were checked manually to find the 1st to 3rd codon positions of H3 and CO1. The optimal partitioning strategy was assessed using PartitionFinder (Lanfear et al., 2017) to obtain seven partitioned datasets for maximum likelihood estimation and the Bayesian inference (see Supplementary Table S1).

Using partitioned datasets, a maximum likelihood estimation was conducted using the IQ-Tree online version (http://iqtree.cibiv.univie.ac.at/; Trifinopoulos et al., 2016) with 1000 bootstrap replications (Efron, 1982; Felsenstein, 1985). Models were selected using the Bayesian Information Criterion with the ModelFinder subprogram in IQ-Tree for partitioned sequences (see Supplementary Table S1). Phylogenetic trees and bootstrap values were then obtained. The Bayesian inference method was performed using Bayesian MrBayes 3.2.7 (Ronquist et al., 2012). The selection of rate-setting models was made using PartitionFinder (Lanfear et al., 2017) (see Supplementary Table S1). Markov chain Monte Carlo analyses were run for 1,000,000 generations with tree sampling every 5000 generations, and convergence was obtained. The first 25% of the sampled trees were discarded as burn-in trees.

Statistical analyses

The Shapiro-Wilk test was used to examine if the data set was drawn from a normal distribution. Bartlett's test was used to test whether the samples had equal variances. For nonparametric tests, the Exact Wilcoxon rank-sum test (for data sets with homoscedasticity), Fligner-Policello test (for datasets with heteroscedasticity), or the Steel-Dwass test was used. All tests were conducted using R software ver. 4.0.3 (http://cran.r-project.org, Retrieved 10 October 2020) with an additional package ("http://aoki2.si.gunma-u.ac.jp/R/src/Steel-Dwass.R", encoding = "euc-jp", Retrieved 26 November 2021).

RESULTS

Activity rhythm

We recorded the activity rhythms of 13 *H. kiotonensis*, 14 *H. convexopyga*, and 25 *H. loochooana loochooana* collected at Manabi-No-Mori, 17 *H. loochooana loochooana* collected at Nishi-Henna-Zaki, and 14 *H. picea*. The collection sites, sex, and behavior types of the beetles used in activity recording are listed in Supplementary Table S2. In *H. loochooana loochooana*, there was no significant difference in the free-running period between males and females (*H. loochooana loochooana* Manabi-No-Mori: Fligner–Policello test, $P = 0.369$; *H. loochooana loochooana* Nishi-Henna-Zaki: Wilcoxon–Mann–Whitney test, $P = 0.832$). In other species, no noticeable difference of the activity rhythm between sexes was observed, although statistical tests were not applicable because of the small sample size in this study. Therefore, we showed the results for males and females together in Figs. 2 and 3.

Holotrichia kiotonensis were mainly active during the night period, but their rhythmicity was unclear compared with other species. Sometimes their activity occurred during the light period of the LD cycles (Fig. 2A). During DD only 38% of beetles showed circadian rhythms, with a free-running period of 22.6 ± 0.8 h (mean ± S.D.) (Fig. 3). The other beetles exhibited arrhythmicity in DD.

Holotrichia convexopyga showed nocturnal activity in LD cycles. They walked around and ate leaves on the ground during the dark period but stayed mostly underground in the light period. All beetles exhibited circadian rhythm in DD with a period of 23.2 ± 0.6 h (Figs. 2B, 3).

Holotrichia loochoana loochoana collected from Manabi-No-Mori and Nishi-Henna-Zaki both exhibited circadian rhythms in DD (Fig. 3A). However, differences between the two populations were found in the light period of the LD cycles. *Holotrichia loochoana loochoana* from Manabi-No-Mori started major activities at the onset of darkness (Fig. $2C_1$, and see Supplementary Figure S1A), whereas those from Nishi-Henna-Zaki started to increase activities approximately 4–5 hours before light-off (Fig. $2C_2$, and see Supplementary Figure S1B). The free-running period under DD of the Manabi-No-Mori population (23.4 ± 0.4 h) was slightly but significantly longer than that of the Nishi-Henna-Zaki population (22.8 ± 0.8 h) (Steel-Dwass test, $P = 0.038$; Fig. 3B).

In *H. picea*, most beetles exhibited nocturnal activity every 2 days under LD, with 92.9% of them ($n = 14$) showing circabidian rhythm in DD (Figs. 2D, 3A). In beetles with circabidian rhythms in DD, some switched appearance from the odd days (day 1, 3, 5, and so on) to the even days (day 2, 4, 6, and so on), or vice versa, as counted from the start of the recording (day-switching). One beetle switched appearance from odd to even days by emerging in two consecutive nights (day 15 and day 16) in DD (Fig. $2D_2$). Another beetle switched twice in DD (Fig. $2D_3$). During DD, 50.0% of the

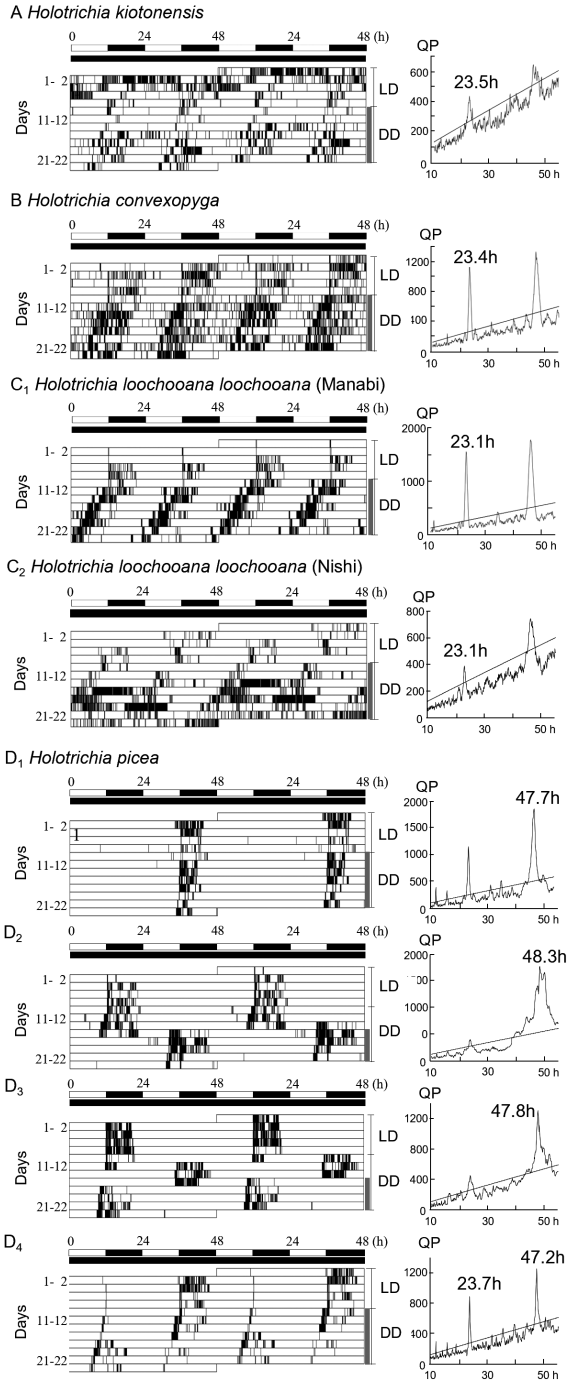

Fig. 2. Double-plotted actogram (left) and the associated chi-square periodogram analysis (right) showing locomotor activity rhythm on the ground in adult *Holotrichia* species. **(A)** *Holotrichia kiotonensis* (female) showing circadian rhythm. **(B)** *Holotrichia convexopyga* (male) showing circadian rhythm. **(C_1)** *Holotrichia loochooana loochooana* (male) from Manabi-No-Mori and **(C_2)** *Holotrichia loochooana loochooana* (male) from Nishi-Henna-Zaki showing circadian rhythm. **(D_1)** *Holotrichia picea* (female) showing regular circadian rhythm, **(D_2)** *Holotrichia picea* (female) showing circadian rhythm with day switching (once), **(D_3)** *Holotrichia picea* (sex not determined) showing circadian rhythm with day switching (twice), **(D_4)** *Holotrichia picea* (female) showing circadian rhythm with a circadian-like pattern. Periodogram analysis was conducted for the activity during the DD period indicated with a gray vertical bar on the actogram. The peak values are indicated.

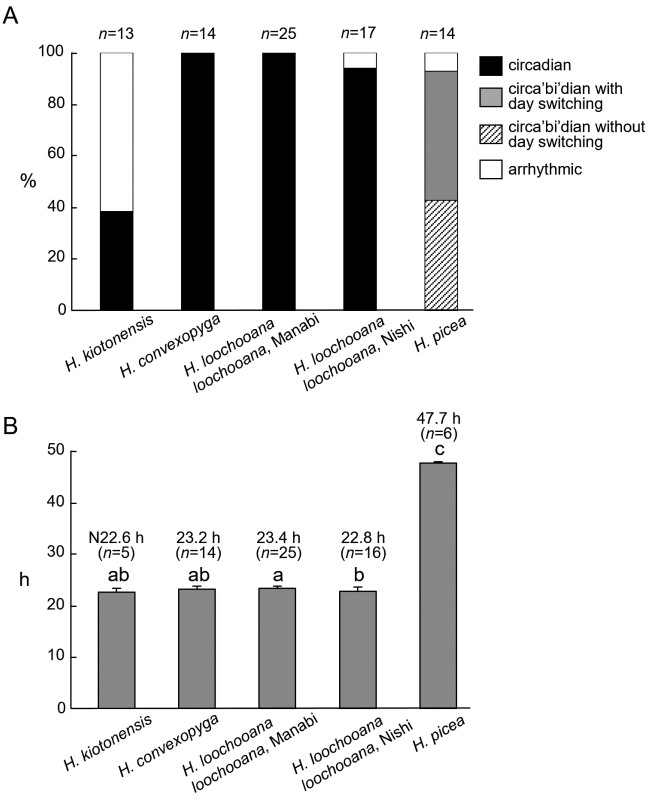

Fig. 3. Summary of activity rhythms in *Holotrichia kiotonensis*, *Holotrichia convexopyga*, *Holotrichia loochooana loochooana* Manabi-No-Mori (Manabi), *Holotrichia loochooana loochooana* Nishi-Henna-Zaki (Nishi), and *Holotrichia picea*. **(A)** Proportion of beetles exhibiting circadian rhythm, circabidian rhythm without day-switching, circabidian rhythm with day-switching, and arrhythmic patterns in their locomotor activity during constant darkness. **(B)** Average and standard deviation of the free-running period during constant darkness. Columns with different letters show significant differences in the free-running period (Steel-Dwass test, $P < 0.05$).

beetles showed circabidian rhythms with day-switching (Fig. 3A). Day-switching was also observed during the LD cycles in four of the 14 beetles (see Supplementary Table S2). Interestingly, among the beetles showing circabidian rhythms, two showed a circadian-like activity component. In one beetle (Fig. $2D_4$), major activity was observed in the even days of the LD cycles and in DD until day 16, while a minor activity component appeared on the odd days in the same period. In the minor activity, the beetle emerged on the ground briefly at the beginning of the subjective nights, followed by burrowing back into the soil. From day 17, the occurrence of major and minor activities switched (Fig. $2D_4$). This beetle was classified as circabidian with day-switching (Fig. 3A). In the other beetle, a minor component appeared only in the last four circabidian cycles during DD and was classified as circabidian without day-switching in Fig. 3A. The free-running period of the circabidian rhythm without day-switching ($n = 6$) was 47.7 ± 0.3 h, which was significantly longer than that of other species (Steel-Dwass test, $P < 0.05$, Fig. 3B).

Phylogenetic analysis

We obtained a 682.83 ± 0.35 (average ± S.E.)-bp

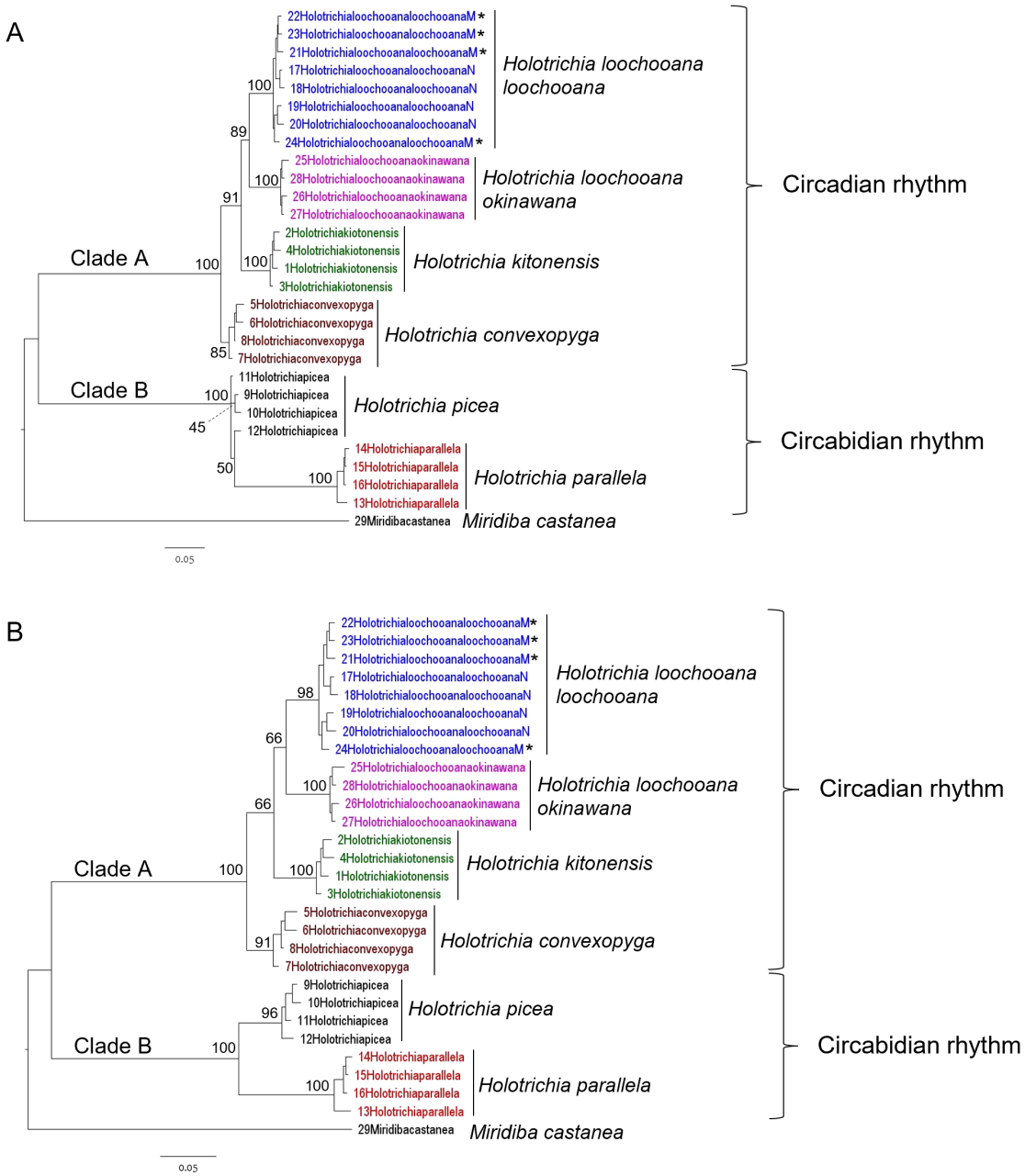

Fig. 4. Phylogenetic relationships among *Holotrichia* species reconstructed using the maximum likelihood estimation **(A)** and Bayesian inference method **(B)** from concatenated sequences of the histone H3, cytochrome c oxidase subunit 1, and 16S ribosomal RNA genes. Numbers above the branches show bootstrap values in **(A)** and Bayesian posterior inferences in **(B)**. *Holotrichia loochooana loochooana* with and without an asterisk are collected at Manabi-No-Mori and Nishi-Henna-Zaki, respectively. Scale bars indicate 5% nucleotide mutation rate.

sequence for CO1, 521.24 ± 0.26-bp sequence for 16S rRNA, and 344.14 ± 0.35-bp sequence for H3 from 29 individuals (Table 1, Accession nos. LC677229–LC677315). Lee et al. (2015) showed that the nodal supporting values on the phylogenetic tree, bootstrap values, and Bayesian posterior probabilities were improved using the concatenated sequences of CO1 and 16S rRNA compared with those based on their respective single sequences. Therefore, we employed concatenated sequences of the H3, CO1, and 16S rRNA genes for phylogenetic analysis. There was distinct separation into two major clades, clades A and B, in both the maximum likelihood estimation analysis and Bayesian inference analysis with strong supporting values in the *Holotrichia* group (Fig. 4). Six ingroup taxa representatives were clustered into six monophyletic groups corresponding to nominal species, except for *H. picea*, according to the maximum likelihood estimation (Fig. 4A). In the Bayesian inference method, *H. picea* was clustered in one group (96%, Fig. 4B). Clade A included *H. loochooana loochooana*, *H. loochooana okinawana*, *H. kiotonensis*, and *H. convexopyga*. Two subspecies of *H. loochooana* were separated into distinct groups. They formed one cluster with moderate supporting

values, a bootstrap value of 89, and a Bayesian inference probability of 66. The Manabi-No-Mori and Nishi-Henna-Zaki groups were not separated in the *H. loochooana loochooana* cluster (Fig. 4). *Holotrichia kiotonensis* clustered with the *H. loochooana* clade (bootstrap value of 91 and Bayesian inference probability of 66). Clade B was composed of *H. picea* and *H. paralella*. High nodal supporting values in clade B were obtained using the Bayesian inference method.

DISCUSSION

In addition to *H. parallela* (Kawasaki et al., 2017), we found that *H. picea* also exhibited circabidian rhythms in their activity on the ground. However, in contrast to *H. parallela*, there were variations in the activity patterns of *H. picea*. In addition to their regular circabidian rhythm, day-switching and circadian-like patterns were also observed (Fig. 2D). Because the major activity component occurred every two nights of the LD cycles or two subjective nights of DD in both patterns, we concluded that *H. picea* exhibited circabidian rhythm. The appearance of the day-switching and circadian-like patterns in the experimental LD and DD conditions is a distinct feature of *H. picea*.

In the circadian-like pattern, a major activity during the dark period and a brief minor activity at dusk occur alternately. Although this pattern was only observed in a few beetles, the appearance of the minor activity component supports the idea that the circadian clock is oscillating and is possibly involved in producing circabidian rhythms (Kawasaki et al., 2017; Watanabe and Shiga, 2020). In *H. parallela*, circadian-like activity was observed in a few beetles only when light pulses were given under constant dark conditions (Kawasaki et al., 2017). The detection of circadian-like patterns in *H. picea* suggests that circadian clock output might be suppressed every two cycles in the circabidian behavior, and minor activity components would appear when the suppression was moderate. The degree of activity suppression every two nights might depend on the species and environmental signals.

We hypothesized that there should be a mechanism that counts two circadian clock cycles. Another possibility is the flip-flop mechanism. In moth olfactory processing systems, flip-flopping interneurons have been reported, and the flip-flop signal is thought to underlie the locomotion occurring during pheromone-triggered orientation behavior (Olberg, 1983; Namiki and Kanzaki, 2016). This type of neuron switches back and forth between long-lasting high and low firing rates in response to repeated stimuli (Olberg, 1983; Kanzaki et al., 1994). A similar type of neuron might also be in the neural circuits for the circabidian rhythm, and switch back and forth between “true” and “false” outputs responding to the repeated input signals from the circadian clock every day. Then circabidian rhythms could be produced in both *H. parallela* and *H. picea*.

The occurrence of day-switching under laboratory conditions is another feature of *H. picea*. Day-switching might be caused by the weak robustness of the counter or the flip-flop circuit. In *H. parallela*, a previous mark-and-recapture study showed that day-switching occurred in the field in circumstances such as heavy rain (Kawasaki et al., 2017), but only rarely in laboratory conditions (Watanabe and Shiga, 2020). This evidence suggests that the robustness of the two-day cycles also depends on the species and environmental signals. Finding behavioral variations in *H. picea* can provide clues for future research in searching for neuronal mechanisms that produce a unique rhythm.

Other species, including *H. kiotonensis*, *H. convexopyga*, and *H. loochooana loochooana*, all exhibited circadian rhythms with nocturnal activity. However, more than half of the *H. kiotonensis* beetles showed arrhythmic patterns in DD. *Holotrichia kiotonensis* and *H. convexopyga* are sympatric species and are both collected from bushy plants; we did not find differences in their habitats or diets. Rhythmicity in *H. kiotonensis* may be unstable under our experimental conditions compared to other species. However, the sample size was small in the present study, and more samples may be required to make conclusions about differences in rhythmicity between *H. kiotonensis* and *H. convexopyga*.

In *H. loochooana loochooana*, LD activity patterns differed between the Nishi-Henna-Zaki and Manabi-No-Mori populations, as reported by Arakaki et al. (2021). The Nishi-Henna-Zaki population appeared on the ground during the light period, which has also been reported in the mating and female aggregation behavior of this population (Kawamura et al., 2001; Yasui et al., 2007). In contrast, the emergence of the Manabi-No-Mori population on the ground was synchronous at the time of light-off. Interestingly, the daily allochronicity of pheromone attraction in male beetles and body size difference have also been shown between these two populations (Arakaki et al., 2021). However, no molecular phylogenetic separation between the two populations was observed in our study. The different free-running periods under DD also suggest differences in the chronobiological traits of the same species. However, further analysis is needed to determine the significance and mechanisms of this difference.

In this study, two clades were recognized from the H3, CO1, and 16S rRNA phylogenetic trees for the *Holotrichia* species. Matsumoto (2016) also proposed the separation of *Holotrichia* into *Pedinotrichia*, including *H. parallela* and *H. picea*, and *Nigrotrichia*, including *H. kiotonensis*, *H. convexopyga*, *H. loochooana loochooana*, and *H. loochooana okinawana*. According to Matsumoto (2016), *Pedinotrichia* species have slender legs in both males and females, and the male genitalia is curved and rounded at the apices. *Nigrotrichia* have a less occiput punctuation with a blackish body, and the male genitalia is generally pointed at the apices. Our phylogenetic analysis confirmed the morphological classification by Matsumoto (2016). *Holotrichia* is a genus of the subfamily Melolonthinae. The lack of taxonomic studies in Melolonthinae has led to erroneous identifications and inappropriate taxonomic assignments of its species, many of which are included in *Holotrichia* (Coca-Abia, 2008). Our study supports the classification of *Pedinotrichia* and *Nigrotrichia* by Matsumto (2016) at least for species *Holotrichia* (*Pedinotrichia) parallela*, *Holotrichia* (*Pedinotrichia) picea*, *Holotrichia* (*Nigrotrichia*) *kiotonensis*, *Holotrichia* (*Nigrotrichia*) *convexopyga*, *Holotrichia* (*Nigrotrichia*) *loochooana loochooana*, and *Holotrichia* (*Nigrotrichia*) *loochooana okinawana*.

The phylogenetic separation between *Nigrotrichia* and *Pedinotrichia* accords well with the behavioral distinctions observed. Circadian behavioral rhythms have been reported in coleopteran species (Abe et al., 2021; Nisimura et al.,

2005), but the circabidian rhythm has not. In *Dasylepida ishigakiensis* (Coleoptera: Scarabaeidae), circadian-like behavior has also been reported (Harano et al., 2010). Considering the universality of the circadian rhythm, common ancestors of *the Holotrichia* group had a circadian rhythm. After the separation into *Nigrotrichia* and *Pedinotrichia*, the behavioral trait of circabidian rhythm appeared probably once in an ancestral species of the *Pedinotrichia* group (Clade B in Fig. 4). It is difficult to understand why circabidian rhythm has appeared or on which occasion(s) this unique rhythm has emerged. Some harmful or severe environmental conditions or events might have given an advantage to the disappearance on alternate days, giving rise to the circabidian rhythm. By acquiring a compensating mechanism for the reduced chance of mating or foraging, this rhythm could be fixed in a species in *Pedinotrichia* descending to *H. parallela* and *H. picea*. It might be interesting to compare mating or reproductive strategies between species with circabidian and circadian rhythms in future studies.

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COMPETING INTERESTS

All authors declare that they have no conflicts of interest.

AUTHOR CONTRIBUTIONS

YO designed the experiment, and conducted the behavioral analyses of all species and the molecular phylogeny analysis. YK recorded the behavioral activity of *H. convexopyga* and *H. picea*. KW conducted behavioral recordings of *H. kiotonensis* and *H. picea*, as well as behavioral analysis. SS designed the experiments, conducted data analysis, and wrote the manuscript. All authors read, edited, and approved the final manuscript.

SUPPLEMENTARY MATERIALS

Supplementary materials for this article are available online. (URL: https://doi.org/10.2108/zs210091)

Supplementary Figure S1. Diel activity amounts of *Holotrichia loochooana loochoana* under 12-h light and 12-h dark cycles.

Supplementary Table S1. Partitioning strategy and evolutionary models.

Supplementary Table S2. Collection site, sex, and periodicity of beetles used for behavioral recording.

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