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Discovery of *Papilio bianor* Cramer, 1777 (Lepidoptera: Papilionidae) in the Central Ryukyus, Japan: inference of the dispersal pathway based on morphological and genetic analyses

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Abstract

More than 120 species of migrated butterflies have been recorded in the Ryukyu Islands, Japan. Biogeographically, it is important to investigate the origins and characteristics of these species as they might establish populations after accidental migration. In August 2016, a *Papilio* (*Achillides*) butterfly was collected on Sesoko Island, approximately 600 m from Okinawa Main Island. Although *Papilio* (*Achillides*) *ryukyuensis* Fujioka is native to this area, the individual had different wing markings from this species. To identify the butterfly and infer its origin, both morphological and genetic analyses were conducted. Morphological analysis indicated that the identification was either *Papilio bianor thrasymedes* Fruhstorfer from Taiwan or *Papilio bianor bianor* Cramer from Mainland China. Subsequently, genetic analyses confirmed that the individual should be assigned as *P. b. thrasymedes*. This suggests that it was migrated from Taiwan, possibly influenced by Typhoon No. 10 of 2016.

Key words: *Achillides*, migrated butterfly, molecular phylogeny, NADH dehydrogenase subunit 5 (ND5), network analysis

Introduction

Over 120 species of migrated butterflies, which fly to areas where they are not originally distributed, have been recorded in the Ryukyu Islands, Japan (Shirôzu 2005a, 2005b; Shirôzu 2006a; Sugawara & Takahashi 2014). Based on their morphological characteristics, these butterflies are believed to originate from Taiwan, Mainland China, and Southeast Asia, and are influenced by weather phenomena such as typhoons and seasonal winds. There are some cases in which they were established in the Ryukyu Islands after accidental migration (Inoue & Ishii 2016; Fukuda 2020). Considering their potential impacts on ecosystems, understanding their origins and characteristics is crucial from a biogeographical perspective.

In August 2016, a *Papilio* (*Achillides*) butterfly was collected on Sesoko Island (Figs 1–2) located approximately 600 m west of the Motobu Peninsula in the north-central part of Okinawa Main Island, Japan (Fig. 3; 'Sesoko *Achillides*' hereafter). Although *Papilio* (*Achillides*) *ryukyuensis*, an endemic *Achillides* species to the Central Ryukyus, is distributed in Okinawa Main Island and its surroundings (Shirôzu 2006b; Inomata *et al.* 2013; Ohya 2018; Arata *et al.* 2021), the collected individual exhibited distinct wing markings from those of *ryukyuensis*. Furthermore, it differed from *Papilio bianor okinawensis* Fruhstorfer found in the Yaeyama Islands located further

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south in the Ryukyu Islands. Instead, it looked like *Papilio bianor bianor* Cramer, 1777 from Mainland China or *Papilio bianor thrasymedes* Fruhstorfer from Taiwan, but it was difficult to narrow down the candidates further, as *Achillides* has similar species and subspecies.

Taxonomic studies of East Asian *Achillides* have been conducted by many lepidopterists (ex. Igarashi 1979; Harada 1992; Izumi 1993; Shimogori 1997; Yoshimoto 1998; Gu & Chen 1999; Wang & Tang 2012; Wu & Hsu 2017; Hsu *et al.* 2018). Currently, 27 *Achillides* species are known to inhabit South Asia, Southeast Asia, East Asia, and Oceania (Nakae 2021). According to Yagi *et al.* (2006), the wing patterns of this group of butterflies have independently converged across species, making species/subspecies identification based only on morphology difficult, and resulting in some controversies exist regarding the scientific names of certain *Achillides* species. This study follows the taxonomy proposed by Inomata *et al.* (2013).

Several specimens morphologically resembling *P. b. bianor* from Mainland China, *P. b. thrasymedes* and *Papilio bianor kotoensis* Sonan from Taiwan have been collected in the Ryukyu Islands (ex. Arata 2015; Arita & Asahi 2022). However, few genetic analyses have been conducted to infer the origin of these *Achillides* butterflies. The only study in which an *Achillides* species was collected on Miyako Island, where the butterfly is not distributed, was identified to specific level as *P. b. bianor* by genetic analyses and morphological comparisons narrowed down it to subspecific level (Ogawa 2022). Thus, it has become possible to identify *Achillides* species/subspecies through combined genetic and morphological analyses.

This study employed phylogenetic and network analyses, as well as morphological analyses, to identify the species and subspecies of the individual collected on Sesoko Island and to infer its geographical origin. Based on the findings, the study discusses the possible factors that led to the arrival of the butterfly on Sesoko Island.

FIGURE 1. *Papilio* (*Achillides*) species collected on Seoko Island just after capture.

Material and Methods

Morphological analyses

Morphological analyses were conducted to identify the Sesoko *Achillides* and infer the origin of the individual. Morphological comparisons of three *Achillides* species including four subspecies of *P. bianor* distributed in nearby regions were conducted to identify the Sesoko *Achillides*. Many *Achillides* species present seasonal polyphenism and sexual dimorphism. Based on the wing markings and collection date, the Sesoko *Achillides* was identified as a summer-form female (Figs 2A–B).

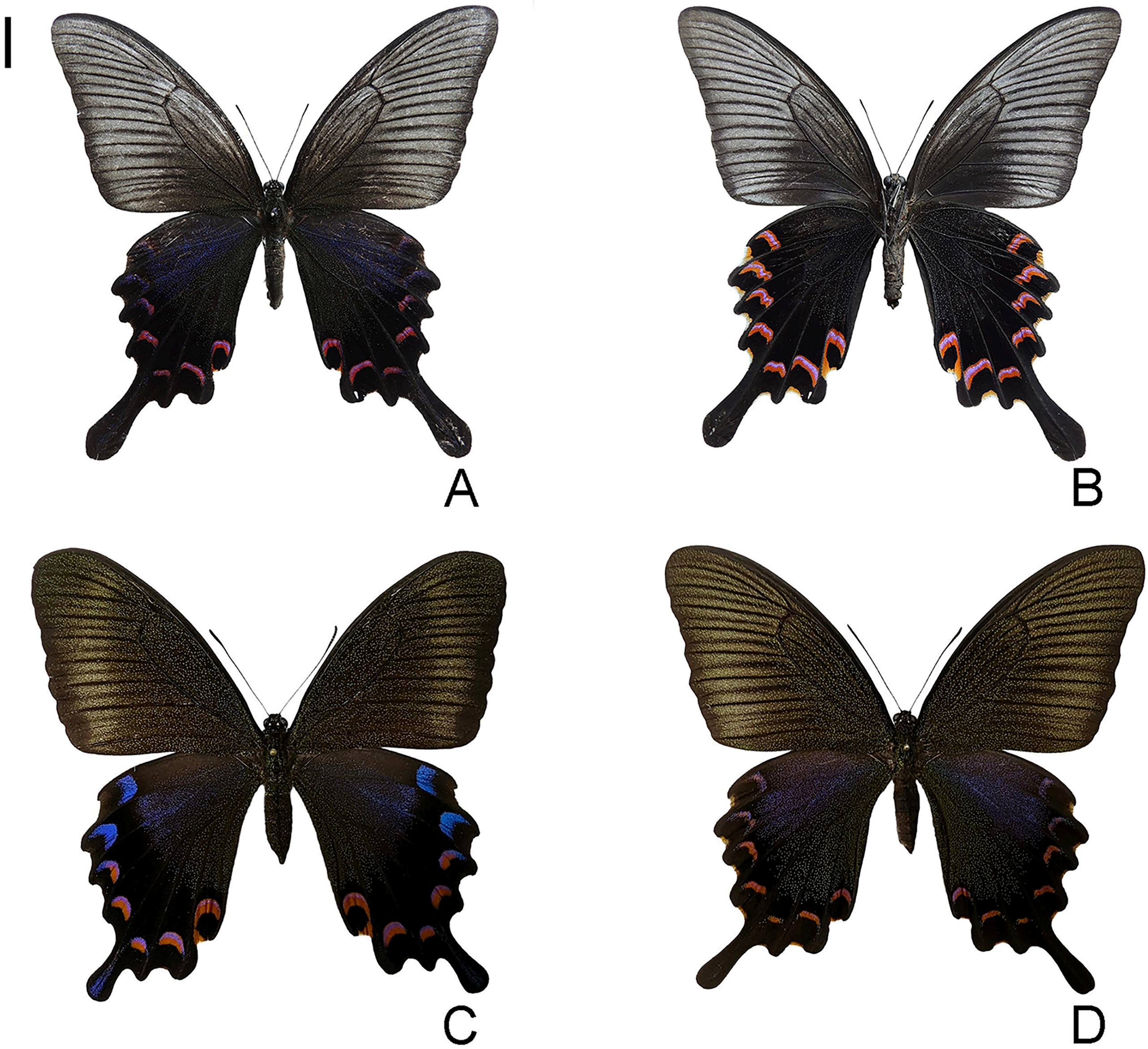

FIGURE 2. *Papilio* (*Achillides*) species in Ryukyus, Japan. (A) the Sesoko *Achillides* species (Sesoko Island, Okinawa Islands, Japan), upperside, (B) *Ditto*, underside, (C) *P.* (*A.*) *ryukyuensis ryukyuensis*, (Okinawa Main Island, Okinawa Islands, Japan; IGA-Pa1256-014), upperside, (D) *P.* (*A.*) *bianor okinawensis* (Ishigaki Island, Yaeyama Islands, Japan; IGA-Pa1257-020), upperside. Scale bar 10 mm.

For this purpose, this study used 123 dried female specimens (summer-form) of *P. bianor, P. ryukyuensis* and *Papilio dehaanii* C. Felder et R. Felder, sourced from the Ryukyu Islands and its surroundings: *P. bianor*: 19 from Yaeyama Islands, Japan, 18 from Taiwan, 45 from Mainland China, 2 from Vietnam, 4 from Laos, 1 from Thailand, 7 from Bhutan, 18 from Nepal, and 9 from India; *P. ryukyuensis*: 2 from Okinawa Main Island, and 1 from

Amami-Oshima Island; *P. dehaanii*: 1 from Nakanoshima Island, Tokara Islands. These specimens are part of the 'Suguru Igarashi' and 'Keiichi Omoto'. collections deposited in The University Museum, The University of Tokyo (UMUT) (Harada *et al.* 2012; Yago *et al.* 2021). In *Papilio dehaanii*, we examined only *P. d. tokarensis* because its distribution area is adjacent to the Central Ryukyus, while the other subspecies were excluded from the analyses, as they do not resemble the Sesoko *Achillides.*

The taxonomic classification of *Achillides* followed the criteria proposed by Yoshimoto (1998), Cotton & Racheli (2006), Inomata *et al.* (2013) and Cotton *et al.* (2021). Recently, Condamine *et al.* (2023) reviewed the systematics of *Papilio* based on molecular analyses, and treated *polyctor* and *bianor* as distinct species. However, we do not follow their classification because this study did not include enough samples to evaluate whether they are distinct species, and because genetic distance and reproductive ability are inherently different. In addition, according to Ae (1990), a hybridization experiment between Taiwanese *bianor* and Nepalese *polyctor* have been shown to produce F1 hybrids with reproductive ability. Harada (1992) also conducted a hybridization experiment between *bianor* from Sichuan Province, China, and *polyctor* from Nepal, resulting in F1 hybrids with reproductive ability. Furthermore, according to a breeding experiment by Harada (1992), a female from Sichuan Province, China, produced *bianor*, *polyctor*, and intermediate forms.

Blue spots near the costal margin of the hindwings upperside are important characters for identifying *P. b. bianor, P. b. thrasymedes*, and other continental subspecies of *P. bianor* (Fujioka *et al.* 1997). However, as noted in the same study, the wing markings on the underside are unsuitable for identification due to significant individual variation. To evaluate these blue spots, we measured the average RGB values from cells 4 to 6 on the hindwings upperside using Adobe Photoshop 2025 for eight individuals each of *P. b. bianor* and *P. b. thrasymedes,* as well as the Sesoko *Achillides*. We then conducted Principal Component Analysis (PCA) using Past 4 (Hammer *et al*. 2001) (https://www.nhm.uio.no/english/research/resources/past/) to determine whether the Sesoko *Achillides* belongs to *P. b. bianor* or *P. b. thrasymedes*. Subsequently, we performed t-tests using JASP (JASP Team 2024) (https://jasp-stats.org/) to assess the significant differences between the two subspecies on PC1 and PC2 obtained from the PCA.

DNA Extraction, PCR amplification and sequencing

DNA extraction, PCR amplification and ND5 gene sequencing were performed following the protocols of Yago *et al.* (2011). The phenol–chloroform extraction method was employed using mid-legs from the Sesoko *Achillides*. The following primers were used for amplification and sequencing: V1 (forward) 5'-CCTGTTTCTGCTTTAGTTCA-3' and A1 (reverse) 5'-AATATDAGGTATAAATCATAT-3' (Yagi *et al.* 1999). Two internal primers were also used for sequencing: C2j (reverse) 5'-CTAAAATTAWATCYTTAGARTAGAAYCC-3' (Yago *et al.* 2009) and LepND5-Papilio-361F (forward) 5'-ATGTYTRAATATTTCAAATTTAG-3'. PCR was carried out in a 10 µL volume reaction containing Ampdirect Plus buffer (Shimadzu), 0.5 µM of each primer, 0.5 µL of DNA solution as template, and 0.25 units of NovaTaq Hot Start DNA polymerase (Shimadzu). The following PCR protocol was consisted of 40 cycles of denaturation at 94°C for 30 s, annealing at 45°C for 30 s, and extension at 72°C for 90 s. Automatic sequencing was performed using an Applied Biosystems 3730XL DNA Analyzer (Applied Biosystems, Waltham, MA, USA).

Alignment and phylogenetic reconstruction

Forty-three sequences of the ND5 gene from GenBank (www. ncbi.nlm.nih.gov/genbank/) were used in addition to the ND5 gene (782 bp) sequences of the Sesoko *Achillides* to elucidate its phylogenetic placement. The breakdown of 43 samples and their accession numbers are as follows: 1 *P. dehaanii dehaanii* from Russia (AB212919); 1 *P. d. dehaanii* from North Korea (AB212920); 1 *P. d. dehaanii* from South Korea (AB212921); 6 *P. dehaanii* from Mainland Japan (AB212922–AB212927); 1 *P. d. dehaanii* from Tsushima, Japan (AB212928); 5 *P. d. hachijonis* Matsumura from Izu Islands (AB212930 and AB223163–AB223166); 2 *P. d. tokaraensis* Fujioka from Tokara Islands (AB212929 and AB212931); 6 *P. ryukyuensis* from Central Ryukyus (AB013157, AB239775–AB239778 and AB239779); 3 *P. bianor bianor* from Mainland China (AB212916–AB212918); 1 *P. b. bianor* from Miyako Island, Japan (LC656924); 2 *P. b. thrasymedes* from Taiwan (AB223167–AB223168); 2 *P. b. kotoensis* Sonan from Lanyu and Hoshao Islands, Taiwan (AB223169–AB223170); 2 *P. b. triumphator* Fruhstorfer from Laos (AB239782–

AB239783); 1 *P. b. triumphator* from China (AB239784); 1 *P. b. stockleyi* Gabriel male from Thailand (AB239781); 1 *P. b. polyctor* Boisduval from India (AB239780). In addition, 1 *P. dialis* Leech from Taiwan (AB239827) and 1 *P. maackii* Ménétriès from Mainland China (AB239809) were designated as outgroups. These accession numbers can be obtained from Yagi *et al.* (2006) and Ogawa (2022). The mitochondrial ND5 gene sequence of the Sesoko *Achillides* was identified and stored in the GenBank database with accession number LC830741.

These sequences were aligned using the Clustal W algorithm implemented in the software MEGA11 (Tamura *et al.* 2021), and pairwise genetic distances were also measured with the Hasegawa-Kishino-Yano model for Maximum Likelihood (ML) and Kimura 2 para for Neighbor Joining (NJ) methods (Hasegawa *et al.* 1985). Phylogenetic tree construction was conducted using ML and NJ methods (Saitou & Nei 1987; Tamura *et al.* 2004; Tamura & Nei 1993). Bootstrap values in ML and NJ trees were obtained after 1000 and 10,000 replications, respectively.

Network analysis

Haplotype and nucleotide diversities were estimated using DnaSP version 6 (Rozas *et al.* 2017) (http://www.ub.edu/dnasp/DnaSP600.html). A haplotype network was constructed using the median-joining network method implemented in Network 4.6.1.0 (Bandelt *et al.* 1999) (http:// www.fluxus-engineering.com/). The median-joining algorithm with default setting was used for network construction (weight = 10; epsilon = 0).

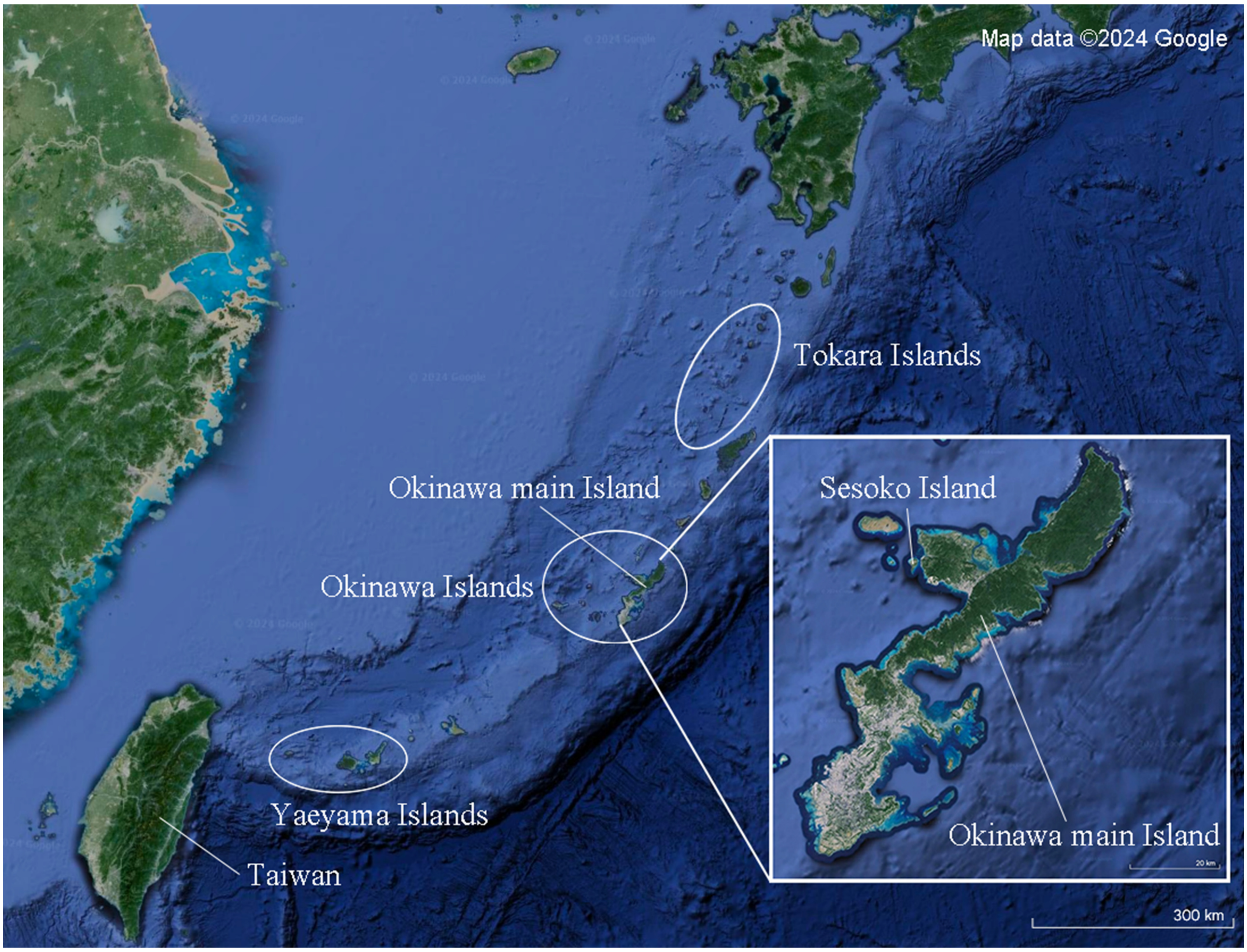

FIGURE 3. Location of Sesoko Island in the Ryukyu Islands.

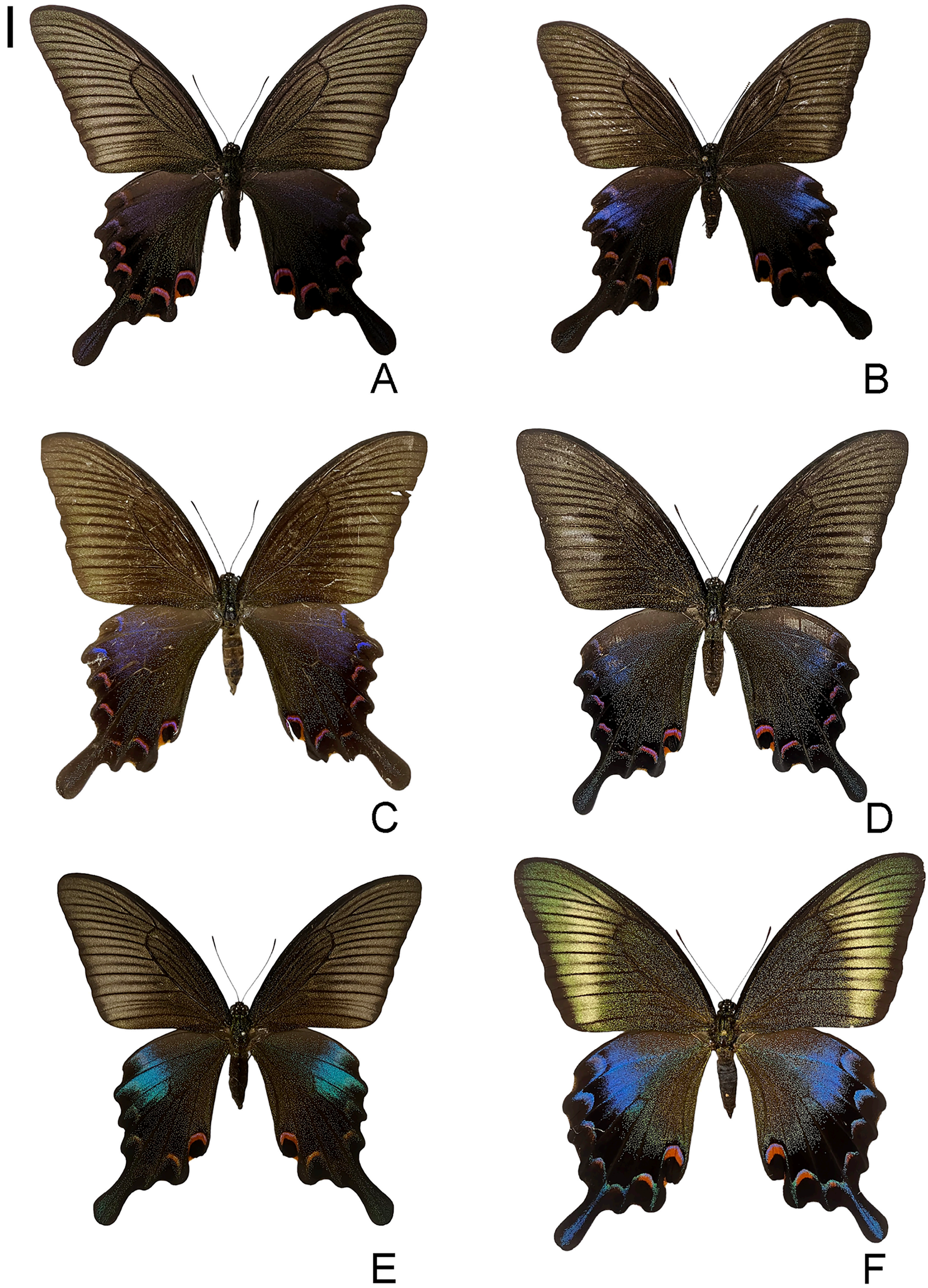

FIGURE 4. Summer form females of *Papilio* (*A.*) *bianor* and *P.* (*A.*) *dehaanii,* upperside. (A) *P. b. bianor* (Sichuan, China; IGA-Pa1261-007), (B–D) *P. b. thrasymedes* (Taiwan; IGA-Pa1258-017, OMT-PAP093-013 and OMT-PAP092-019, respectively), (E) *P. b. triumphator* (Laos; IGA-Pa1265-006), (F) *P. dehaanii tokaraensis* (Nakanoshima Island, Tokara Islands, Japan; IGA-Pa1260-005). Scale bar 10 mm.

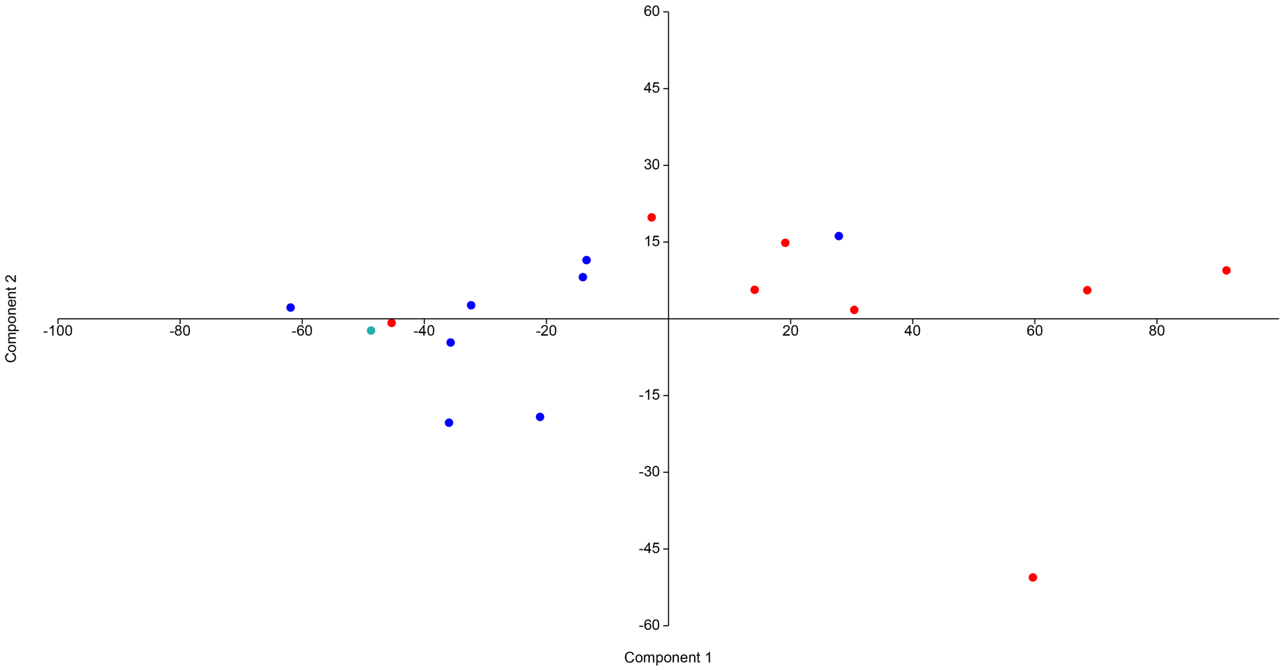

FIGURE 5. A two-dimensional plot of principal component analysis (PCA) based on the average RGB values of cells 4 to 6 of the left hindwing upperside for 17 individuals (Blue: *Papilio bianor bianor*, Red: *P. b. thrasymedes*, Lightseagreen: The Sesoko *Achillides*). X and Y axes show PC1 and PC2, respectively. The t-values for PC1 and PC2 between the two subspecies were -2.295 and -0.097, respectively, indicating no significant difference.

Results

Morphological analyses

The wings of *P. ryukyuensis* (Fig. 2C) distributed in the Central Ryukyus were squarer than those of *P. bianor* (Figs 2D, 4A–E). Furthermore, *P. d. tokaraensis*, the population of *P. dehaanii* distributed closest to Okinawa Main Island, had a broad white band on the forewing upperside (Fig. 4F). Therefore, the Sesoko *Achillides* distinctly differed from *P. ryukyuensis* and *P. d. tokaraensis*.

Papilio bianor generally exhibits regional variation in upperside wing markings. This study focused on the hindwing upperside. Most *P. b. triumphator* and some *P. b. thrasymedes* had a large blue patch on the subapical area (cells 5–7) of the hindwings (Figs 4B, 4E), whereas some *P. b. thrasymedes* had a small or faint patch (Figs 4C–D), and *P. b. bianor* tended to have little or no patches (Fig. 4A). *Papilio b. okinawensis* (Fig. 2D) quite resembled *P. b. thrasymedes,* but as indicated by Fujioka *et al*. (1997), it was usually distinguished by its denser and wider deep blue scales, and additionally, *P. b. okinawensis* showed an angular forewing shape and a linear outer margin of the forewings.

Although the Sesoko *Achillides* was similar to some *P. b. thrasymedes* (Figs 4C–D) in having a faint blue patch on the subapical area of the hindwing upperside, the density of blue lunar spots forming wave patterns on the submarginal area of the hindwings is low as in *P. b. bianor* (Fig. 4A), indicating that it was difficult to identify at subspecies level only based on the wing markings. As a result of principal component analyses using the RGB values (Fig. 5), it also indicated that it was difficult to determine whether the Sesoko *Achillides* should be included in *P. b. bianor* or *P. b. thrasymedes.* The t-tests for both PC1 and PC2 to evaluate significant differences between the two subspecies showed t-values of -2.295 and -0.097, respectively, indicating no significant difference.

Phylogenetic trees

Phylogenetic trees with similar topologies were obtained using the ML and NJ methods (Fig. 6). Although the trees are not highly reliable as a result of several nodes with low bootstrap values, each of the three species, *P. dehaanii*, *P. ryukyuensis* and *P. bianor,* formed monophyletic groups with relatively high bootstrap values of 99 %, 99 % and 59–73 % (ML and NJ), respectively. The Sesoko *Achillides* was included in the *P. bianor* clade. Furthermore, two *P. b. thrasymedes* from Taiwan and the Sesoko *Achillides* diverged from the other OTU of *P. bianor* with high values of 94 % (ML and NJ)*,* indicating they belong to the same clade.

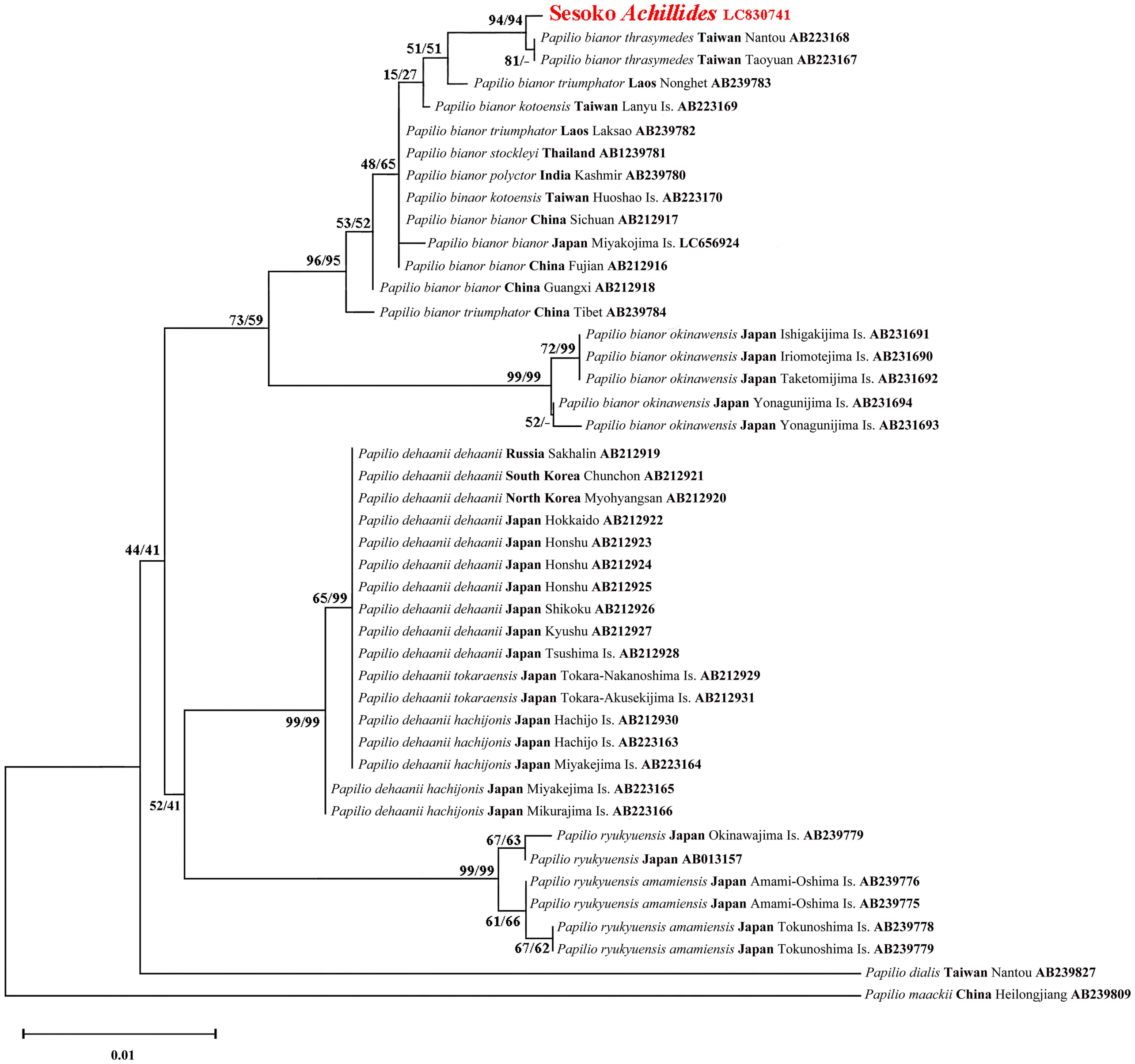

FIGURE 6. Phylogenetic tree constructed using ML methods with bootstrap values (ML/NJ). In four nodes indicated as "-", the bootstrap value of NJ was not obtained since the topology of the NJ tree differed from that of ML.

Haplotype network

A network analysis of 44 individuals was performed using the median-joining network method. Our analysis identified a total of 18 haplotypes, grouped into five partitions (Fig. 7). The network analysis showed that the Sesoko *Achillides* was grouped with *P. bianor thrasymedes,* as well as in the phylogenetic trees.

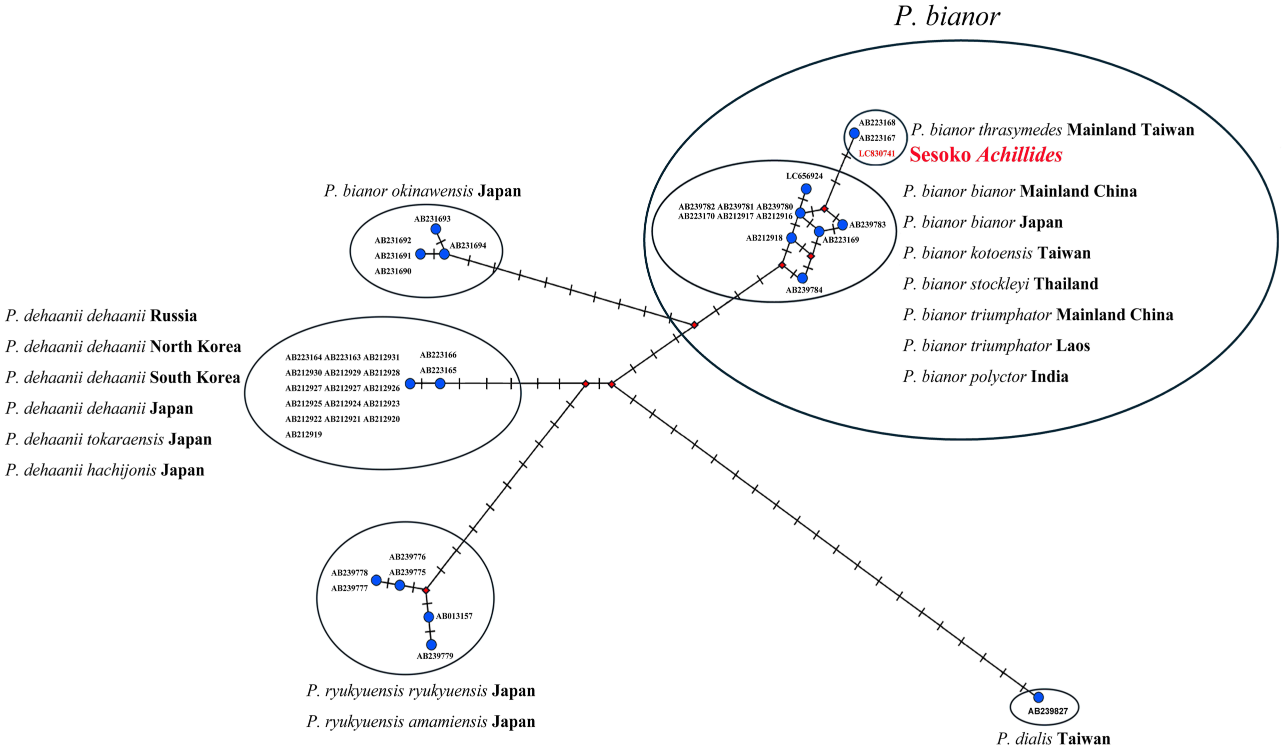

FIGURE 7. Median-joining network derived from ND5 sequences of *Achillides* species.

Discussion

The Sesoko *Achillides* was identified as *P. bianor thrasymedes* based on genetic analyses, although it was uncertain which subspecies it belongs to based only on morphology. This individual was presumed to be a migrated butterfly that flew from Taiwan, where this subspecies is distributed (Hsu *et al.* 2018). According to a previous study, the migration of *P. bianor* to Miyako Island, not inhabited by *Achillides* species, was suggested (Ogawa 2022). However, based on both morphological and molecular evidence, this study is the first to document the arrival of *P. bianor* around Okinawa Main Island, which is inhabited by another *Achillides* species. Contrary to Ogawa (2022), we could not narrow down the identification with morphological traits. Instead, molecular data was useful for that purpose.

The closest habitat of the subspecies, New Taipei City, Taiwan, is approximately 650 km away from Sesoko Island, suggesting that the individual could not have reached the island solely by its own flight. Since it was collected in August, it is highly possible that it came from the continental island with winds generated by typhoons or monsoons. According to the Japan Meteorological Agency (2016), 11 typhoons occurred from January to August 2016. Among them, Typhoon No. 10, *Lekima* (*Dindo*; 201610 12W), developed around the Philippine Sea from August 21 to 31, just before the Sesoko *Achillides* was collected. It was inferred that it most likely flew from Taiwan from the night of August 30 to the morning of August 31, riding on southwestern winds generated by Typhoon No. 10 (Fig. 8). Considering the wind speeds at that time, there is a possibility that it came from the highlands of approximately 3,000 m, such as Mt. Kalahei and Mt. Chiyou, due to southwestern winds at 700 hPa in northern Taiwan at 6 pm on August 30 (Figs 8A–B). Shouji (1995) mentioned that migrated butterflies tend to fly from south to north when typhoons are located north of the area, and our inference was consistent with this pattern. As Orui (2019) reported that many *Vanessa cardui* (Linnaeus) migrated at night, it would not be surprising to assume that this individual also flew during the night. Although the possibility that the Sesoko *Achillides* was introduced artificially cannot be ruled out, we considered it unlikely, as no ships from Taiwan called at any port around the island.

Since the wings of the Sesoko *Achillides* are relatively in good condition, it is also necessary to consider the possibility that *P. b. thrasymedes* already established on Sesoko Island. Although strong winds do not blow from Taiwan to Okinawa in the spring, it is possible that the specimen is the descendant of a female that arrived in June or early July, brought by strong winds later in the season, and laid one or more eggs at that time. Previous studies have

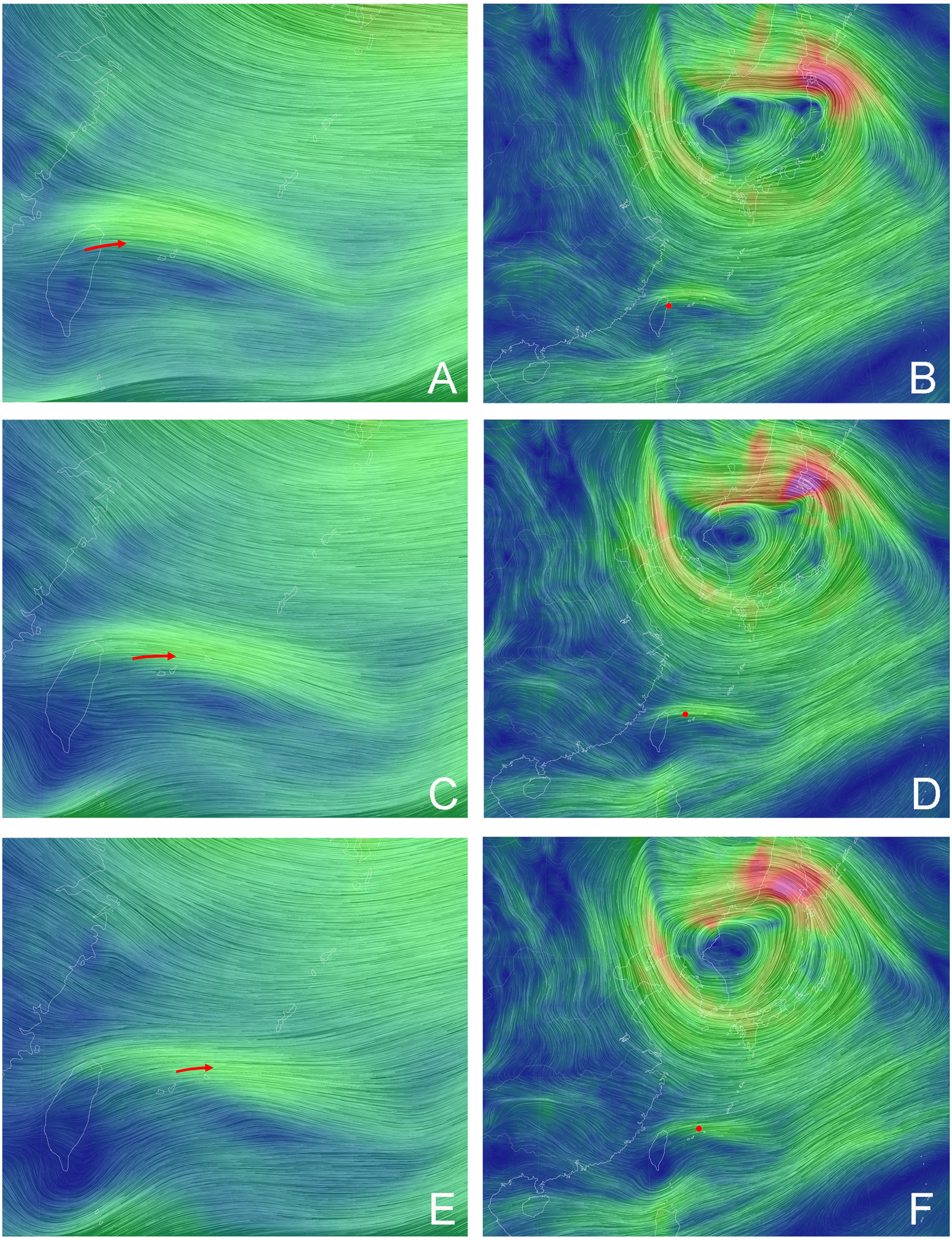

FIGURE 8. Animated virtual maps indicating the wind directions from the night of August 30 to the morning of August 31, 2016, by Beccario (2016) (available at https://earth.nullschool.net) (A, B) At 700 hPa at 6 pm on August 30, (C, D) At 700 hPa at 9 pm on August 30. (E, F) At 700 hPa at midnight on August 31. (A, C, E) Around Taiwan and the Okinawa Main Island. (B, D, F) Around the Japanese archipelago. © 2024 Cameron Beccario. Red arrows and dots indicate the dispersal pathways and locations of the Sesoko *Achillides* for each period, respectively.

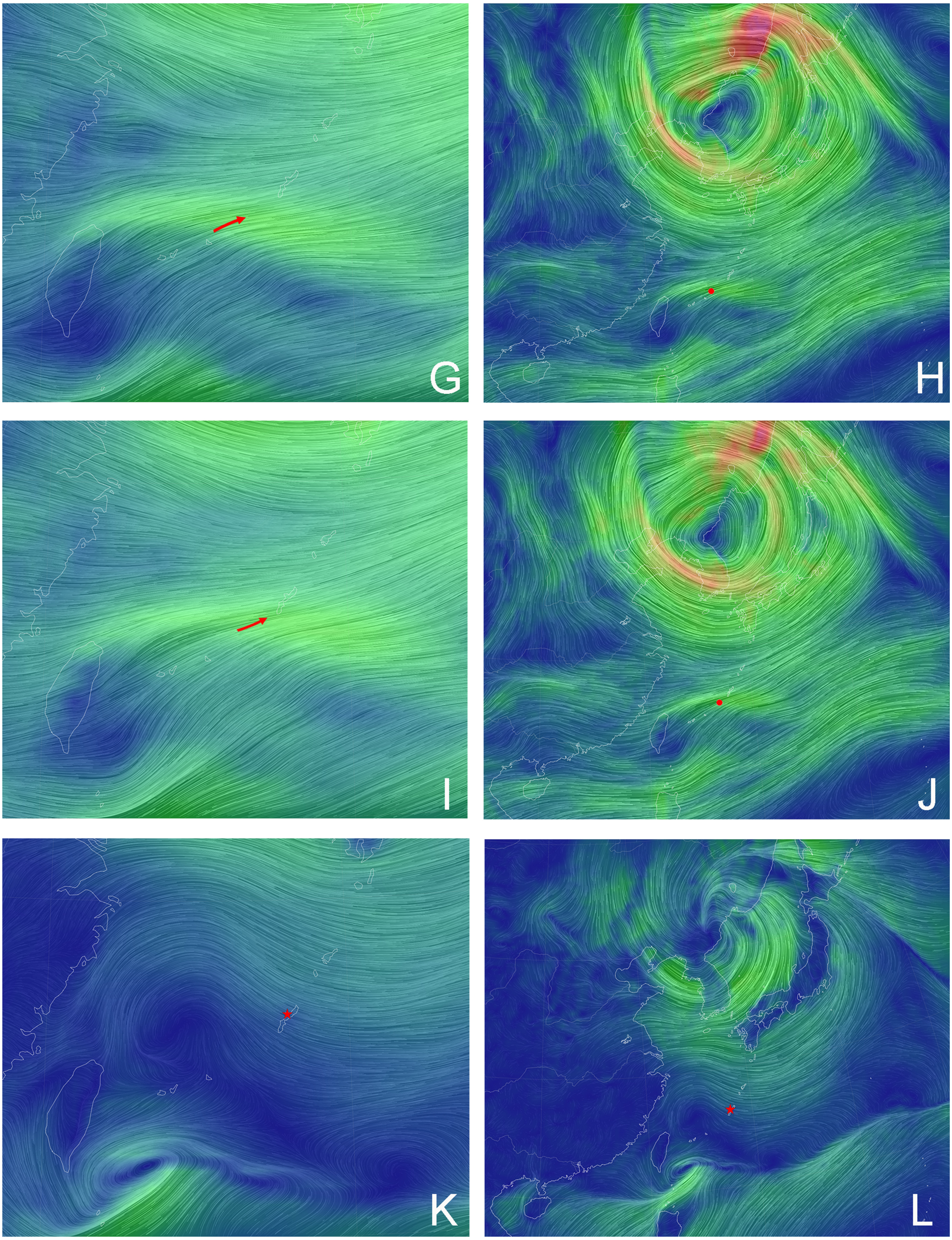

FIGURE 8 (Continued). (G, H) At 700 hPa at 3 am on August 31. (I, J) At 700 hPa at 6 am on August 31. (K, L) On the surface at 9 am on August 31. (G, I, K) Around Taiwan and Okinawa Main Island. (H, J, L) Around the Japanese archipelago. © 2024 Cameron Beccario. Red arrows and dots indicate the dispersal pathways and locations of the Sesoko *Achillides* for each period, respectively.

documented that the larvae of this subspecies feed on *Citrus* spp., *Euodia lepta*, *E. glauca*, *Tetradium glabrifolium*, *T. ruticarpum*, *Toddalia asiatica, Zanthoxylum ailanthoides, Z. nitidum*, *Z. schinifolium* and *Z. simulans* (Igarashi & Fukuda 2000; Hsu *et al.* 2018). Among these, *T. glabrifolium* and *Z. ailanthoides* are native to Okinawa Main Island (Murata & Yonekura 2013). Therefore, while there is a possibility that *P. b. thrasymedes* temporarily occurred on Sesoko Island, it seems unlikely due to few host plants and only a small number of *P. ryukyuensis* flying on the island (Arata *et al.* 2021). There is a slight possibility that *P. b. thrasymedes* temporarily occurred on Okinawa Main Island and then flew to Sesoko Island. However, although the second author has conducted almost daily surveys on Okinawa Main Island side, opposite Sesoko Island, no similar individuals have been observed. Furthermore, while this individual potentially be is a hybrid of *P. bianor* and *P. ryukyuensis*, we have determined that this specimen is not a hybrid, since it does not exhibit any characteristics of *P. ryukyuensis.* It has been reported that hybrids between *P. b. polyctor*, which lacks reproductive isolation from *P. b. thrasymedes*, and *P. ryukyuensis* did not produce females (Ae 1990). In addition, hybrids between *P. b. okinawensis*, a close relative of *P. b. thrasymedes*, and *P. ryukyuensis* have almost never produced females, or their offspring resulting from backcrossing with F1 hybrids failed to grow normally (Ae 1990). Therefore, it is considered highly unlikely that this individual is the descendant of a hybrid from several generations ago, beyond the previous generation.

Despite having been carried from Taiwan, it is noteworthy that the wings of this individual are almost undamaged. The lack of damage may suggest that this individual should be the descendant of a female that flew from Taiwan earlier, as mentioned above. On the other hand, there are examples of butterflies, such as *Ixias pyrene* (Linnaeus) and *Talbotia naganum* (Moore), that flew from Taiwan to the Ryukyu Islands with little or no wing damage during transportation (Takagi 1996; Sugawara & Takahashi 2014). The host plants of these species are not distributed in Japan, and it is rational to assume they arrived via atmospheric phenomena. Additionally, other species in the same family as the Sesoko *Achillides*, such as *Papilio castor* Westwood and *Papilio alphenor* Cramer, which have been recorded as occasional vagrants (Saijo 1999; Arata 2000), also show a little or no wing damage. Similarly, many danaine species with almost no wing damage have been recorded frequently as vagrants (Sugawara & Takahashi 2014). Therefore, it is not surprising to consider that the Sesoko *Achillides*, with minimal wing damage, was able to fly directly from Taiwan.

The ND5 gene was used for genetic analyses to identify the Sesoko *Achillides*, but future studies may need to include analyses with other genes. While migrated butterflies are frequently observed in the Ryukyu Islands, conducting genetic and morphological analyses, as demonstrated in this study, will likely make it possible to infer their origins.

Conclusion

Based on morphological and genetic analyses, the Sesoko *Achillides* was assigned to *P. b. thrasymedes,* which is native to Taiwan. Regarding its dispersal pathway, it was inferred that it migrated from Taiwan to Sesoko Island from the night of August 30 to the morning of August 31, 2016, via southwestern winds generated by Typhoon No. 10.

Papilio* (*Achillides*) *bianor thrasymedes **Fruhstorfer, 1909**

Specimen data: One female (Figs 1–2), 31. viii. 2016 (8:29 am), Ishika [Sesoko Island], Motobu-cho, Okinawa Prefecture, Japan, Satoshi Arata leg. (S. Arata's private collection), forewing length 66 mm.

Notes: This individual was collected while sucking the nectar of *Hibiscus rosa* in the garden of a house along the farm road on the south coast of Sesoko Island. As the butterfly was female, the second author attempted to extract eggs from its abdomen but did not obtain any. Therefore, it is possible that it laid eggs around Sesoko Island. However, it should also be noted that, instead of eggs, a larger amount of body fat was stored.

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