Genetic Structure in the Seabuckthorn Carpenter Moth (Holcocerus hippophaecolus) in China: The Role of Outbreak Events, Geographical and Host Factors

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Abstract

Understanding factors responsible for structuring genetic diversity is of fundamental importance in evolutionary biology. The seabuckthorn carpenter moth (Holcocerus hippophaecolus Hua) is a native species throughout the north of China and is considered the main threat to seabuckthorn, Hippophae rhamnoides L. We assessed the influence of outbreaks, environmental factors and host species in shaping the genetic variation and structure of H. hippophaecolus by using Amplified Fragment Length Polymorphism (AFLP) markers. We rejected the hypothesis that outbreak-associated genetic divergence exist, as evidenced by genetic clusters containing a combination of populations from historical outbreak areas, as well as non-outbreak areas. Although a small number of markers (4 of 933 loci) were identified as candidates under selection in response to population densities. H. hippophaecolus also did not follow an isolation-by-distance pattern. We rejected the hypothesis that outbreak and drought events were driving the genetic structure of H. hippophaecolus. Rather, the genetic structure appears to be influenced by various confounding bio-geographical factors. There were detectable genetic differences between H. hippophaecolus occupying different host trees from within the same geographic location. Host-associated genetic divergence should be confirmed by further investigation.

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Introduction

Pests with fluctuating population size are of major concern for forest security. Knowledge of a pest’s population dynamics and associated influential factors is crucial for forest management. Habitat, weather, natural enemies and heritable traits are considered to play roles in insect population dynamics [1]. Despite many studies, the factors involved in the origin of insect outbreaks remain poorly understood. Multiple explanations have been proposed including: escape from natural enemies [2–5], favorable weather [6], changes in host quality and quantity [7–9], and genetic variation of pests [10–12].

The seabuckthorn carpenter moth, Holcocerus hippophaecolus Hua (Lepidoptera: Cossidae) is the main pest of seabuckthorn, Hippophae rhamnoides L. (Elaeagnaceae). It usually occurs on seabuckthorn, but can also occur on Ulmus pumila L. (Urticales: Ulmaceae) as well as a couple of species of Rosaceae [13]. The larvae seriously obstruct water transportation of seabuckthorn by boring into the trunk and roots. It has one generation every 3–4 years and larval stages occupy most of its life history. It is widely distributed throughout its hosts’ range, with most damage being caused to trees more than 5 years old. The adult females have limited dispersal and lay their eggs in masses on nearby plants where the larvae feed gregariously. Berryman [14] has demonstrated that pests with low dispersal properties have short, intense, restricted outbreaks whereas those with high vagility have long, extended outbreaks. Consistent with the former pattern, the seabuckthorn carpenter moth has limited dispersal ability and exhibits short but intense outbreaks that are geographically restricted [15]. Zhou reported that outbreaks of H. hippophaeocclus can lead to more than 70% mortality of seabuckthorn in plantations in the Inner Mongolia Autonomous Region [16]. Limited mobility appears to play a role in the spatial restriction of the seabuckthorn carpenter moth. The outbreaks usually continue for one or two years before pest numbers decline [15,17].

Seabuckthorn is native to western and northern China, the northern Himalayas and northwestern Europe, through to central Asia and the Altai Mountains [18]. It is a native in 11 provinces (autonomous region, municipalities) in China, with less than 500 thousand hectares of natural forest in the 1950's [19]. Because of seabuckthorn's nitrogen-fixing symbionts, this plant serves to enrich and protect soils [18]. It has been promoted widely in western and northern China to prevent soil erosion and desertification. There are now 2,900,000 ha of seabuckthorn throughout 22 provinces in China, two-thirds of which are monoculture plantations. H. hippophaeocclus was firstly reported as a pest of seabuckthorn in 1990 [20]. Today H. hippophaeocclus is considered to be the main threat to seabuckthorn in China. It infests 133,000 ha of seabuckthorn and killed 67,000 ha during the 1990’s. Most of the outbreak events occurred in Seabuckthorn monoculture plantations [16]. Prior to the spread of H. rhamnoides plantations in western and northern China, no outbreak events of
**Materials and Methods**

**Sample collection and DNA extraction**

Individuals (n = 217) were collected from 10 locations across the carpenter moth range during the summer of 2008 (Table 1) by directly sampling under the bark of infested trees and by using light and pheromone traps. Sampling locations represented two contrasting patterns of historical outbreak events, based on a literature survey and unpublished data (J. Zong, personal communication) (Figure 1). Populations from some areas have experienced outbreaks while in other populations densities have been consistently low. In Jianping, a further 24 insects were collected from different hosts (U. pumila [JPI, n = 7], Prunus armeniaca [JPX, n = 8], Pyrus pyrifolia [JPL, n = 9]). Individuals were transported alive to the laboratory, and then kept at −80°C. Prior to DNA extraction, insects were washed in 80% ethanol. Total genomic DNA was isolated using the SDS-method of Zhang and Hewitt [30]. After extraction, DNA was dissolved in TE buffer and stored at −20°C until further use.

**AFLP protocol**

Amplified fragment length polymorphism (AFLP) analysis was used to assess genetic diversity among sampled populations of *H. hippophaecolus*. The AFLP procedure followed Vos et al. [23] with minor modifications. Genomic DNA was digested with EcoRI and *Msal* restriction enzymes (New England Biolabs) and double stranded adapters were ligated to the sticky ends of the fragments. After 4 h incubation at 37°C, each sample was diluted 1:9 with H2O and a two-step amplification strategy was used. Pre-selective amplification was performed for 3 min at 94°C, then 30 cycles of 30 s at 94°C, 30 s at 56°C and 1 min 72°C. A 20 µL Pre-selective amplification PCR mixture consisted of 30 mM MgCl2, 4.5 mM dNTP, 0.6 U Taq DNA polymerase, 30 ng EcoRI-C and *Msal*-A primer. In the selective amplification, we used the following nine primer combinations selected from 100 tested combinations [31]: EcoRI-AA/C/Msal-CAA, EcoRI-AA/C/Msal-CAC, EcoRI-AA/C/Msal- CCT, EcoRI-AA/C/Msal-CTT, EcoRI-AAG/Msal-CCA, EcoRI-AAG/Msal-CTG, EcoRI-CA/Msal-CAA, EcoRI-CA/Msal-CAC, EcoRI-CA/Msal-CCT. The EcoRI primers were labeled with IRD-700. Selective amplification was performed with the following touchdown thermal profile: 3 min at 94°C; 12 touchdown cycles at 94°C for 30 s, 65°C for 30 s (decreasing the temperature by 0.7°C per cycle), and 72°C for 60 s; 30 cycles at 94°C for 30 s, 56°C for 30 s, 72°C for 1 min; 5 min at 72°C. The 10 µL PCR mixture contained 15 MgCl2, 1.3 ng *Msal* and EcoRI primer, 2 mM dNTP), 2 µl diluted (1:9) pre-amplified
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Table 1. Geographical location, average annual rainfall, and hosts for populations of *H. hippophaecolus*.

| No. | Location             | Coordinates       | Average annual rainfall/mm | Population identifier | Host plant     | Sample Size |
|-----|----------------------|-------------------|----------------------------|-----------------------|----------------|-------------|
| 1   | Liaoning, Jianping   | 119.71E/41.84N    | 478.37                     | JPS                   | *H. rhamnoides* | 26          |
| 2   | Inner mongolia, Linxi| 118.23E/43.61N    | 378.98                     | LX                    | *H. rhamnoides* | 16          |
| 3   | Hebei, Fengning      | 116.61E/41.21N    | 535.16                     | FN                    | *H. rhamnoides* | 25          |
| 4   | Shanxi, Youyu        | 112.39E/39.96N    | 377.24                     | YY                    | *H. rhamnoides* | 26          |
| 5   | Inner mongolia, Dongsheng | 111.25E/39.87N | 410.52                     | DS                    | *H. rhamnoides* | 15          |
| 6   | Shanxi, Wuzhai       | 111.87E/38.88N    | 431.54                     | WZ                    | *H. rhamnoides* | 18          |
| 7   | Shanxi, Yulin        | 109.76E/38.28N    | 397.43                     | YL                    | *H. rhamnoides* | 19          |
| 8   | Shanxi, Wuqi         | 108.26E/36.90N    | 534.83                     | WQ                    | *H. rhamnoides* | 29          |
| 9   | Nixia, Yanchi        | 107.48E/37.89N    | 290.24                     | YC                    | *H. rhamnoides* | 22          |
| 10  | Nixia, Pengyang      | 106.50E/35.82N    | 498.67                     | PY                    | *H. rhamnoides* | 21          |

DNA. All PCRs were conducted on a GeneAmp PCR System 9700 (USA Applied Biosystems).

Amplification products were separated on 6% polyacrylamide gels for 2.5 h on a LI-COR 4300 DNA Analyzer (LI-COR Biosciences, USA), using LI-COR 50–700 bp (labeled with IRD-700) as a size standard. Fragments from 100–700 bp in size were scored as present (1) or absent (0) using SAGA MX (LI-COR Biosciences, USA), and exported for data analysis. A blank control was carried out along with each set of DNA extractions and PCR amplifications to monitor any possible cross contamination. Poor-quality DNA samples that did not amplify were excluded from further analysis.

Data analysis

Genetic variation and structure of *H. hippophaecolus* populations. The diversity of geographic populations was assessed by estimating the percentage of polymorphic loci (%P) and Nei’s heterozygosity. Percentage of polymorphic loci estimates were based on 99% criteria and heterozygosity estimates were made using the software TFGPA [32]

The genetic structure was examined by an analysis of molecular variance (AMOVA) performed by the software ARLEQUIN 3.1 [33]. This method was used to partition the genotypic variance among and within populations. Two separate analyses were performed to test the hypotheses of genetic structure attributable to variation: among individuals across the different localities feeding on *H. rhamnoides* and among individuals across different host plants in Jianping. An additional analysis of individuals feeding on *H. rhamnoides* compared to the group combining three other host plants in Jianping was also performed. Genetic differentiation coefficients between populations (both geographic and host-associated) were calculated as $F_{ST}$ with 95% confidence intervals (CI) obtained by bootstrapping 1000 replicates over loci. The TFGPA software was also applied to calculate Nei’s genetic distance ($D$) [34]. Neighbor-joining (NJ) trees were constructed based on $D$ using MEGA4 [35]. Outlier loci were identified using the Dfdist approach [36,37] in Mcheza program [38] (available at http://popgen.eu/soft/mcheza/). Allele frequencies are estimated in Dfdist based on Zhivotovsky’s [39] Bayesian approach. Because of our particular interest in outbreak-associated divergence, the Dfdist was run for two groups of populations (outbreaking population vs non-outbreaking population). A total of 50000 realizations were performed and maximum allowable allele frequency was 0.99. We chose the 0.995 confidence interval and set the significance level at 99%. The Benjamini and Hochberg false discovery rate (FDR) correction method was used to correct for the occurrence of false positives in loci identified as under selection [40]. Loci with significant $P$-values at FDR threshold of 50% were identified using the Benjamini and Hochberg method.

Testing outbreaks and environmental factors driving genetic structure. The following analysis tested outbreaks and environmental factors that potentially influenced genetic population structure. The effect of geographical distance was assessed using linear map distances between *H. hippophaecolus* populations. Secondly, outbreak patterns were scored with 1 indicating populations from areas where outbreaks had occurred and 0 representing populations in non-outbreaking areas. Finally, an index for the “degree of drought,” represented by the average annual rainfall collected over 50 years was obtained (1955–2007, China meteorological data sharing service system http://cdc.cma.gov.cn/). Mantel tests were conducted with the software TFGPA to test the correlation between Euclidean distances for all the factors and genetic distances.
The general linear models (GLM) method was also used to test the effect of outbreak and drought on the genetic differentiation between populations. In this analysis the factor “drought” was defined as locations with less than 400 mm average annual rainfall. Values of 1 were used for drought locations (YY, YL, YC, LX) and 0 for other locations (PY, JP, WQ, DS, WZ, FN). The outbreak factor was standardized, as previously, for an outbreak area of 1 and a non-outbreak area of 0. We performed a GLM analysis of the heterozygosity with outbreak and drought as fixed factors. A \( P \)-value of \(< 0.05\) was used to indicate statistical significance. GLM was implemented using SPSS 16.0.

### Results

#### Genetic variation and structure of *H. hippophaecolus* populations

The nine primer combinations produced a total of 933 bands. The global \( G \) among the 10 sites was 0.2106 (95% CI 0.1981–0.2230). Nei’s heterozygosity for each geographical population was moderate and ranged from 0.1505–0.2042 (Table 2).

AMOVA conducted on AFLP markers confirmed the presence of moderate genetic differentiation showing that 22.54% of total variability was due to the variation among geographic populations.

#### Table 2. Percentage of polymorphic loci (%P) and Nei’s heterozygosity of *H. hippophaecolus* populations.

| Population identifier | JP | LX | FN | YY | DS | WZ | YL | WQ | YC | PY |
|-----------------------|----|----|----|----|----|----|----|----|----|----|
| %Polymorphic loci (p) | 72.56 | 51.23 | 77.60 | 58.41 | 74.49 | 68.27 | 70.85 | 66.34 | 56.38 | 61.74 |
| Heterozygosity (H)    | 0.1854 | 0.1529 | 0.1702 | 0.1495 | 0.2042 | 0.1505 | 0.1872 | 0.1749 | 0.1604 | 0.1679 |

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The pair-wise comparisons between populations were characterized by values of $F_{ST}$ ranging from 0.0424–0.3663 (Table 3). Most of the populations showed highly significant differences ($P<0.0001$) with the exception of the YY and LX populations ($P=0.0182$). This result indicates that most of the 10 sampled populations represent differentiated populations.

The Neighbor Joining phenogram (Figure 2) indicates that the clusters comprised populations with a mixture of outbreak patterns. For instance, populations from Dongsheng and Youyu were in two distinct NJ genetic clusters, although they have the same intensity of outbreak events.

Examination of the AFLP data using Dfist in Mcheza sought to determine whether there was evidence of any highly differentiated loci. $F_{ST}$ is plotted against heterozygosity in Figure 3. The outbreak and non-outbreak population comparison performed with Dfist resulted in four markers out of 993 (loci 93, 188, 223, 390) showing more differentiation than expected at the 99.5% confidence level. All these loci were detected as potential positive outliers at the 50% FDR threshold (Figure 3).

Testing outbreaks and drought as factors driving $H. \text{hippophaecolus}$ genetic structure

The Mantel test based on the 10 localities gave an $r$ value of 0.0554 ($P=0.3460$ for 10000 randomizations), indicating no correlation between geographic and genetic differences. The Nei's genetic distances between populations were not significantly correlated to outbreak differences in the Mantel test ($r=0.2516$, $P=0.0740$). The interaction between Euclidean distances for average annual rainfall and genetic distances was also not significant (Mantel test $r=0.1271$, $P=0.2070$). GLM analysis showed that the factors of outbreak and drought, and their interaction, did not have a significant effect on heterozygosity ($F_{1,10}=0.053$, $P=0.826$, $F_{1,10}=1.329$, $P=0.293$ and $F_{1,10}=2.904$, $P=0.139$ respectively).

Host-associated diversity

The host plant was found to have a larger effect on the genetic structure among populations than geographic location. The global $O$ value among different hosts was 0.2785 (95% CI 0.2548–0.3024), higher than the value among 10 sites (0.2106). AMOVA

**Table 3.** Analysis of molecular variance (AMOVA) of $H. \text{hippophaecolus}$ populations.

| Source of variation               | d.f. | SS       | Percentage of variation (%) | $P$     |
|----------------------------------|------|----------|----------------------------|---------|
| Geographical grouping            |      |          |                            |         |
| Among localities                 | 9    | 5831.247 | 22.54                      | $<0.0001$|
| Individuals within localities     | 206  | 18395.396| 77.46                      | $<0.0001$|
| Host-plant grouping              |      |          |                            |         |
| Among host plants in Jianping    | 3    | 1537.343 | 31.73                      | $<0.0001$|
| Individuals within host plants in Jianping | 30 | 3895.897 | 68.27                      | $<0.0001$|
| Among two host groups in Jianping| 1    | 1249.317 | 34.82                      | $<0.0001$|
| Individuals within groups in Jianping | 48  | 4183.923 | 65.18                      | $<0.0001$|

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($F_{ST}=0.2254$, $P<0.0001$) (Table 3).
with ARLEQUIN found greater variation among populations in host-plant groupings (31.73%) than populations in geographical groupings (22.54%) (Table 3). Pairwise F<sub>ST</sub> statistics between JPS and each other location population ranged from 0.0856 to 0.2978 (Table 4), while the genetic divergences were all highly significant 0.3510, 0.3773 in the host-associated analysis (Table 5).

In Jianping, individuals feeding on <i>H. rhamnoides</i> had a great separation from individuals feeding on other host plants. When combined individuals feeding on <i>U. pumila</i>, <i>P. amenaca</i> and <i>P. pyrifolia</i> as a group, compared to individuals feeding on <i>H. rhamnoides</i>, the variation among two groups rose up to 34.82% by AMOVA with ARLEQUIN. Pairwise comparisons of F<sub>ST</sub> values between all host plant combinations further supported the pattern of genetic structure. F<sub>ST</sub> values were much greater in comparisons between the <i>H. rhamnoides</i> feeders (0.3510–0.3773) and each other host-plant feeders (0.0527–0.1180) (Table 5).<sup>31</sup> <i>H. rhamnoides</i> feeders showed strongly significant differences (<i>P</i>, 0.0001) with the moth on other host plants (Table 5).

### Discussion

Genetic patterns associated with outbreak events of <i>H. hippophaecolus</i>

Genetic clustering did not support distinct outbreak-associated genetic clades in <i>H. hippophaecolus</i>. NJ genetic population clusters contained a combination of populations from historical outbreak areas as well as non-outbreak areas (Figure 2). The outbreak effect may have been difficult to detect among different geographical populations due to various confounding biogeographical factors that also shape genetic structure in <i>H. hippophaecolus</i>. In addition, one cannot exclude the possibility that the outbreak and non-outbreak patterns are associated with a single genotype, but depend on the expression of different phenotypes in different environments.

Indeed, our results support the notion that outbreak events were likely to be endemic population changes from latent to epidemic rather than being due to insects with an outbreak-associated

### Table 4. Nei’s genetic distance and F<sub>ST</sub> value between all geographic combinations.

|   | PY | JP | YY | WQ | DS | YL | WZ | YC | LX | FN |
|---|---|---|---|---|---|---|---|---|---|---|
| PY | — | 0.0902 | 0.2409 | 0.0683 | 0.1589 | 0.2179 | 0.2929 | 0.2779 | 0.2601 | 0.2298 |
| JP | 0.0191 | — | 0.2682 | 0.0856 | 0.1969 | 0.2204 | 0.2978 | 0.2754 | 0.2737 | 0.2467 |
| YY | 0.0465 | 0.0587 | — | 0.2654 | 0.1115 | 0.2938 | 0.3663 | 0.3477 | 0.1047 | 0.2715 |
| WQ | 0.0170 | 0.0196 | 0.0583 | — | 0.1813 | 0.2048 | 0.2914 | 0.2404 | 0.2856 | 0.2454 |
| DS | 0.0401 | 0.0442 | 0.0423 | — | 0.1550 | 0.1828 | 0.2511 | 0.1089 | 0.0986 |
| YL | 0.0530 | 0.0559 | 0.0888 | 0.0527 | 0.0362 | — | 0.1129 | 0.1642 | 0.2520 | 0.1298 |
| WZ | 0.0816 | 0.0780 | 0.1247 | 0.0869 | 0.0375 | 0.0254 | — | 0.2998 | 0.3131 | 0.0424 |
| YC | 0.0538 | 0.0541 | 0.0774 | 0.0447 | 0.0617 | 0.0437 | 0.0871 | — | 0.3387 | 0.2819* |
| LX | 0.0456 | 0.0578 | 0.0209 | 0.0606 | 0.0343 | 0.0678 | 0.0944 | 0.0731 | — | 0.2126 |
| FN | 0.0669 | 0.0652 | 0.0969 | 0.0740 | 0.0207 | 0.0298 | 0.0062 | 0.0827 | 0.0705 | — |

Nei's genetic distances are below the diagonal. F<sub>ST</sub> value and their significance level are above the diagonal. Significance level of associated F<sub>ST</sub> value are shown as: *P<0.01, unmarked mean P<0.0001.

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Table 5. Pairwise comparisons of genetic divergence estimates ($F_{ST}$) between all host plants combinations.

|     | JPY | JPL | JPX | JPS |
|-----|-----|-----|-----|-----|
| JPY | 0.00000 |     |     |     |
| JPL | 0.07590* | 0.00000 |     |     |
| JPX | 0.05271* | 0.11808** | 0.00000 |     |
| JPS | 0.36080*** | 0.37700*** | 0.35109*** | 0.00000 |

Significance level of associated $F_{ST}$ value are shown as:
* $0.01 < P < 0.05$
** $0.001 < P < 0.01$
*** $P < 0.001$

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The role of the plant

Host races are genetically differentiated sympatric populations of parasites that use different hosts and between which there is limited gene flow [54]. Our analyses uncovered very high $F_{ST}$ values (0.3510–0.3773) between JPS and other non-seabuckthorn populations. It is indicated that *H. rhamnoides* constitutes a barrier to gene flow between *H. hippophaeacolus* populations from other host plants in Jianping. *H. hippophaeacolus* feeding on *H. rhamnoides* in Jianping are more genetically differentiated than those from other hosts in sympatric rather than other geographically distant populations of seabuckthorn in Liaoqing. Host races might therefore exist in seabuckthorn and other host plant used by *H. hippophaeacolus*. Factors favoring host race formation include correlations between host choice and mate choice. Although host fidelity and assortative mating has been fully explored in *H. hippophaeacolus*, tests using both artificial and natural methods suggest female host preferences may exist. Adult emergence from the seabuckthorn roots confirmed oviposition preference on *H. rhamnoides*, rather than on *U. pumila* and *P. armeniaca* [55].

Seabuckthorn was an endemic perennial, sporadically growing in Inner Mongolia, Shanxi and areas of Liaoning province before
it was widely promoted. The timing of host shifting of *H. hippophaecolus* in Jiaxing is likely due to the introduction of *H. rhamnoides*. However, how did host shifting occur in *H. hippophaecolus* in Jiaxing? When did host-associated genetic divergence initially occur in *H. hippophaecolus*? Data from many host utilization systems gave rise to a possible scenario that host shifts occur as a result of host plant’s increased abundance and availability as a potential resource following human-mediated plant community changes [56,57]. If this is the case, our data suggests a local host shift and genetic differentiation of *H. hippophaecolus* following the introduction of seabuckthorn in Jiaxing. Though a rapid range expansion of *H. hippophaecolus* following human-mediated changes is possible, it does seem unlikely given the wide extent of genetic divergence observed during such a brief time. This scenario was also rejected by Sword et al in the *Hesperotettix viridis* host utilization system [58]. Another possibility is a genetic divergence of moth between *H. rhamnoides* and other hosts, prior to the host shift. Feder et al. [59] found genetic divergence between apple and hawthorne host races of *Rhopalitis pannoniella* L. pre-dating the introduction of the apple to North America. Given the long life history of *H. hippophaecolus* and brief plant history of *H. rhamnoides* in Jiaxing, we suppose the latter scenario is the case. Seabuckthorn is native to parts of western and northern China although records for the historical host plant use by *H. hippophaecolus* are lacking. Our results indicate that an *H. hippophaecolus* lineage might have adapted to utilize *H. rhamnoides* in China prior its spread. The possibilities of an ancestral host shift and stable host-associated genetic divergence in seabuckthorn carpenter moth are suggested.

We found no fixed diagnostic differences in AFLP data between the different host-associated forms. Host-associated genetic divergence should also be further demonstrated by sampling additional populations feeding on different host plants in more locations. In future studies, more different genetic markers are recommended in this system. They should include co-dominant markers such as microsatellites (not currently available for this species) and incorporation of variable regions of the mitochondrial genome. Microsatellites are highly polymorphic, locus-specific and can show co-dominant inheritance. They may recover higher levels of variability than other markers, particularly if following a population bottleneck associated with host shift. Mitochondrial sequences can be analyzed to determine patterns of evolutionary relationships between different haplotypes. This may provide information on the historical evolution of host-associated forms in the seabuckthorn moth.

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**Author Contributions**

Conceived and designed the experiments: JT MC S-XZ Y-QL. Performed the experiments: JT MC. Analyzed the data: JT MC S-XZ Y-QL. Contributed reagents/materials/analysis tools: JT MC S-XZ Y-QL. Wrote the paper: JT. Collected samples: JT S-XZ.

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