Morphometric Relationship, Phylogenetic Correlation, and Character Evolution in the Species-Rich Genus *Aphis* (Hemiptera: Aphididae)

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**Abstract**

**Background:** The species-rich genus *Aphis* consists of more than 500 species, many of them host-specific on a wide range of plants, yet very similar in general appearance due to convergence toward particular morphological types. Most species have been historically clustered into four main phenotypic groups (*gossypii*, *craccivora*, *fabae*, and *spiraecola* groups). To confirm the morphological hypotheses between these groups and to examine the characteristics that determine them, multivariate morphometric analyses were performed using 28 characters measured/counted from 40 species. To infer whether the morphological relationships are correlated with the genetic relationships, we compared the morphometric dataset with a phylogeny reconstructed from the combined dataset of three mtDNA and one nuclear DNA regions.

**Principal Findings:** Based on a comparison of morphological and molecular datasets, we confirmed morphological reduction or regression in the *gossypii* group unlike in related groups. Most morphological characteristics of the *gossypii* group were less variable than for the other groups. Due to these, the *gossypii* group could be morphologically well separated from the *craccivora*, *fabae*, and *spiraecola* groups. In addition, the correlation of the rates of evolution between morphological and DNA datasets was highly significant in their diversification.

**Conclusions:** The morphological separation between the *gossypii* group and the other species-groups are congruent with their phylogenetic relationships. Analysis of trait evolution revealed that the morphological traits found to be significant based on the morphometric analyses were confidently correlated with the phylogeny. The dominant patterns of trait evolution resulting in increased rates of short branches and temporally later evolution are likely suitable for the modality of *Aphis* speciation because they have adapted species-specifically, rapidly, and more recently on many different host plants.

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**Introduction**

*Aphis* Linnaeus, 1758, which contains more than 500 species, is the largest genus within the family Aphididae [1,2]. Although attempts have been made to subdivide this species-rich genus into subgroup families [3], more than 80% of its species have not yet been assigned [1]. Historically, delimitation of *Aphis* species has been based on small differences in morphology, together with host associations, which have played a major part in identifying the species of this group [4,5]. Considering that this genus contains by far the largest number of species of any genus in the family Aphididae, it is clear that *Aphis* has diversified to exploit an extremely large number of host plants [5,6], but the morphologies of congeneric species are remarkably similar compared to those of most other genera [2,5,7,8].

*Aphis* species that morphologically resemble one another have been assigned to several groups on the basis of major morphological similarities [4,5,9]. Stroyan [4] discussed three complexes, *Aphis fabae* Scopoli, 1763, *Aphis frangulae* Kaltenbach, 1845, and *Aphis nasturtii* Kaltenbach, 1843, each grouped by morphology and host relationships. In particular, relationships within the species-group of *A. fabae* in the European region have often been discussed based on host relationships and morphological similarities [10,11,12]. Some groups composed of closely-related *Aphis* species are associated with plant families, such as the *craccivora* group that feed on plants in the family Fabaceae [3]. Similarly, Pashchenko [13] assigned *Aphis* species host-specific on *Spiraea* to the *spiraecola* group.

Recently, two phylogenetic studies using molecular characters confirmed that the associations between species-groups based on
morphological similarities are similar to those based on molecular characters [14,15]. Coeur d’acier et al. [14] examined the phylogenetic relationships among the craccivora group (black-backed species), the fabae group (black species), the gossypi group (angular-like species), and the spiraeola group (A. spiraeola), and Kim & Lee [15] corroborated the phylogenetic relationships of these groups within the tribe Aphidini. These groups roughly correspond to the Latin names that have been historically applied by Börner [9] as Cerosiphon del Guercio (gossypii group), Doralis Börner (fabae group), Medoralis Börner (spiraeola group), and Pergandeida Schouteden (craccivora group). Consistent with the morphology-based group designations, the molecular phylogenies showed that members within each species-group are monophyletic [14,15]. With respect to group relationships, the fabae group is more closely related to the craccivora and spiraeola groups than to the gossypii group, whose coloration and number and length of setae differ from those of the other three groups. Although these findings imply that in Aphis species, genetic affinities can reflect morphological similarities [15,16], the morphological hypotheses have not confirmed which characteristics are similar within a group or different between the groups.

Tests of the evolution morphological characters of Aphis species showing quite small genetic differences [14,15] may help to better understand remarkable adaptations for wide-ranging host plants during the Tertiary [6]. Except for some polyphagous aphids such as Aphis gossypii Glover, 1876 and A. fabae utilizing more than two plant families, most Aphis species are confined to several host plant species within the same genus or to a few genera within the same family [2,16]. However, Aphis has diversified promiscuously to various unrelated plant families unlike other aphids in the genera Uroleucon, Macrosiphonion, and Macrosiphum, which have evolved host specialization to one plant family [2,6,17,18]. For example, although A. taraxacicolana, A. clerodendri, A. egmoneae, and A. sumire within the gossypii group are closely related genetically and morphologically, they feed on host plants belonging to different families, namely Asteraceae, Verbenaceae, Lamiaceae, and Violaceae, respectively [15,16]. This promiscuous host-association of Aphis is unusual in aphids, and their adaptation has been predicted to be related to the tendency to morphological simplicity [19].

In this study, multivariate morphometric analyses were performed for testing relationships based on morphological similarity of Aphis, especially comprising the four species groups, namely the gossypii group, the craccivora group, the fabae group, and the spiraeola group. In particular, morphological separations or affinities were confirmed through comparisons between the species-groups. Multivariate morphometric analysis is generally used to represent complex, multidimensional patterns of variation between host-related populations in polyphagous aphids [20,21,22] and has also been proven to be a powerful and reliable method for separating closely-related aphid taxa [23,24,25]. However, in contrast to previous studies, we employed morphometric analysis to cluster species and confirm the relationships between the previously classified species-groups in Aphis. Then, we evaluated the morphometric results in light of the species-group hypotheses and phylogenetic relationships proposed in previous studies [4,5,14,15], and determined the major characteristics that differentiate the Aphis groups. In addition, we examined whether those morphological relationships are correlated with the molecular phylogeny and rates of changes [26,27], and we tested some evolutionary features of the major characteristics using Bayesian approaches [28].

**Results**

**Phylogeny**

The topologies inferred by Bayesian inference (BI) and maximum likelihood (ML) analyses revealed congruent species relationships, as shown in Figure 1, although the ML bootstrap values (BL) were lower than the Bayesian posterior probabilities (PP). Although the molecular phylogeny was almost congruent to the two previous phylogenies [14,15], we needed to combined these data to reconstruct the phylogeny for the purpose of using character reconstruction and comparison of the rate of changes (see below). Monophyly of the gossypii group (clade G) was highly supported (PP/BL=1.00/99), and the craccivora, spiraeola, and fabae groups also formed a monophyletic group (clade F) with significant nodal support (PP/BL=1.00/82). The three unclassified species, A. oenotherae, T. cirticus, and T. odinae, were closely related to clade F, and these three species and clade F formed a sister group to the gossypii group. The remaining unclassified species with morphological characteristics distinct from the species-groups were located in basal positions in the molecular phylogeny. Some species in the fabae or gossypii group (arrowed in Figure 1) might have undergone rapid diversification from the most recent common ancestor (MRCA) based on the small number of nucleotide substitutions differentiating species and the short branch lengths between the MRCA and the branch tips [29].

Multivariate morphometric analysis

In the first principal component analysis (PCA) plot based on the first two principal components (PC1, PC2), the gossypii group species aggregated on the mid-left side and were almost completely separate from species in the fabae + craccivora + spiraeola groups and the unclassified species, which were scattered and mixed on the right side (Table 1; Figure 2). Although some species, e.g., A. kurosaeci, which have a small body size and short appendages, were in close proximity to species in the gossypii group, member species of the gossypii group appeared to be closely related on the basis of morphometric characters. PC1 and PC2 accounted for 50.2% and 9.4% of the total variance, respectively. Separation by PC1 was due to the length of body parts, especially the length of the antenna and its segments, the leg parts, and the body. With regard to meristic characters, the number of setae on the GP and cauda contributed substantially to the separation. Whereas, separation by PC2 was caused by certain meristic characters, such as the number of setae on the A. longispina, A. longispina, and A. longispina species-groups. In particular, morphological separations or affinities were confirmed through comparisons between the species-groups. Multivariate morphometric analysis is generally used to represent complex, multidimensional patterns of variation between host-related populations in polyphagous aphids [20,21,22] and has also been proven to be a powerful and reliable method for separating closely-related aphid taxa [23,24,25]. However, in contrast to previous studies, we employed morphometric analysis to cluster species and confirm the relationships between the previously classified species-groups in Aphis. Then, we evaluated the morphometric results in light of the species-group hypotheses and phylogenetic relationships proposed in previous studies [4,5,14,15], and determined the major characteristics that differentiate the Aphis groups. In addition, we examined whether those morphological relationships are correlated with the molecular phylogeny and rates of changes [26,27], and we tested some evolutionary features of the major characteristics using Bayesian approaches [28].
mainly by two meristic characters: the number of setae on Ant.III and the number of setae on GP. PC3 was largely affected by two continuous characters: the length of SIPH and the length of cauda (figure not shown). In the second PCA (Figure 3), the plots of the *gossypii* group samples were arranged in a narrower band on the axis of PC2 than were those of the *fabae + craccivora* group samples, consistent with the first PCA. Similarly, the third PCA confirmed the high correlation of meristic characters between species in the *gossypii* group (Table 1; Figure S1). Although the plots of *A. craccivora* and *A. spiraecola* largely overlapped with that of the *gossypii* group, the plots of the former two species were clustered further than that of the *fabae* group, separated by contributions of 42.7% for PC1, 14.1% for PC2, and 12.2% for PC3. The low variation in meristic characters in the *gossypii* group revealed by the PCA analyses supported the use of these meristic characters to classify and separate the *gossypii* group from the other species-groups and the unclassified species.

The first canonical discriminate analysis (CDA) showed that CV1 (52.1% of the total variance), CV2 (17.5%), and CV3 (9.7%) separated the *gossypii* group from the *fabae + craccivora* groups species (Table 2; Figure S2). The lengths of the HFM and HTB contributed significantly to the separation of both CV1 and CV2, while the separation of CV3 was dependent mainly on the length of the body and Ant.I and the number of setae on the GP and cauda. The plot patterns of the first CDA were almost identical to those of the second PCA even though samples were assigned to each species cluster in the CDA. The second CDA showed that 11 meristic characters overlapped between the *gossypii* group and *A. craccivora* (Table 2; Figure S3), but the plots of these characters in the *gossypii* group were still largely concentrated on the left side in contrast to the scattered pattern of these characters seen in the other species-groups.

In the first ANOVA, seven continuous and two meristic characters separated the *gossypii* group from the other species groups (i.e., other all species) in the first PCA with high significance (P < 0.0001) (Table 3). In particular, the lengths of Ant.IV and HFM among the continuous characters and the numbers of setae on the ML and cauda among the meristic characters were highly correlated with the separation of the *gossypii* group from the other species-groups. The second ANOVA showed that the 25 characters used in the second PCA that had high scores in the first PCA were also highly significant in dividing the clustered groups, except for the number of setae on Ant.II, which showed little variation. The one-way ANOVA showed that those characters were individually significant for separating the species group clusters (*gossypii* group vs. others) revealed by PCA or CDA, even although the correlated factors in multivariate analysis, e.g., size-correlation between antenna and HFM, were excluded.

Figure 1. The phylogenetic relationships inferred from ML analysis based on the GTR + I + γ model using the combined sequences of COII (702 bp), 16S (1601 bp), CytB (737 bp), and EF1-a (802 bp). Posterior probabilities of BI analysis followed by ML bootstrap values in parentheses are given on or beneath nodes if greater than 50%. Letters enclosed in circles indicates two group partitions: *gossypii* group as ‘clade G’ and other three groups as ‘clade F’. Arrow means a short divergence point.

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The patristic distances calculated for each morphological dataset were significantly correlated with those of the ML tree estimated from the molecular dataset based on Pearson correlation or Mantel tests (Table 4). Pearson correlation tests revealed that the molecular dataset was correlated with each morphological dataset (0.56 < r < 0.72; P < 0.001). In particular, the 11 meristic characters were highly correlated with the molecular characters in Mantel test (r = 0.7378; P < 0.001), while the tests with the 17 continuous character set or the 28 all character set were not significant. However, the estimated K-scores indicated that the three distance-optimized trees based on the different morphological datasets were almost congruent in topology and branch lengths, implying similar changes on nodes between the trees.

Table 1. The first two principal components that contributed to the separation of the 40 species or the 247 samples for 14 species in three PCAs.

| Characters | First PCA | Second PCA | Third PCA |
|-----------|-----------|------------|-----------|
|           | PC1       | PC2        | PC3       | PC1       | PC2 | PC3 | PC1 | PC2 | PC3 |
| Length of | Body      | 0.2334     | -0.1293   | -0.0729   | 0.2517   | -0.0836 | 0.0473 |
|           | Antenna   | 0.2503     | -0.1414   | 0.0562    | 0.2545   | -0.1914 | -0.0160 |
|           | Ant.I     | 0.2388     | -0.0809   | 0.0718    | 0.2198   | -0.1059 | -0.2808 |
|           | Ant.II    | 0.2377     | -0.1010   | -0.0629   | 0.2367   | -0.0599 | -0.1761 |
|           | Ant.III   | 0.2416     | -0.1679   | -0.0149   | 0.2497   | -0.1222 | 0.0602 |
|           | Ant.IV    | 0.2431     | -0.1692   | 0.0243    | 0.2470   | -0.1878 | 0.0598 |
|           | Ant.V     | 0.2369     | -0.1385   | 0.0145    | 0.2323   | -0.2428 | 0.0791 |
|           | Ant.Vib   | 0.2151     | -0.0989   | -0.1974   | 0.2350   | -0.1122 | -0.0813 |
|           | PT        | 0.1862     | -0.0694   | 0.3282    | 0.1924   | -0.2339 | -0.1636 |
|           | URS       | 0.1784     | 0.1243    | 0.0622    | 0.1999   | 0.1621  | -0.0966 |
|           | HFM       | 0.2454     | -0.1599   | -0.0551   | 0.2544   | -0.1150 | 0.0344 |
|           | HTB       | 0.2507     | -0.1558   | 0.0305    | 0.2520   | -0.1683 | -0.0139 |
|           | 2HT       | 0.1927     | -0.0667   | -0.2923   | 0.2276   | -0.1188 | -0.0814 |
|           | SIPH      | 0.1193     | 0.0262    | 0.4202    | 0.1067   | -0.1405 | 0.6417 |
|           | Cauda     | 0.1287     | -0.1180   | 0.3007    | 0.1325   | 0.1196  | 0.4363 |
|           | Ant.III   | 0.1800     | 0.1203    | -0.3333   | 0.2133   | 0.3144  | -0.1014 |
|           | AbdT.III  | 0.0847     | 0.1338    | -0.3177   | 0.2002   | 0.2640  | -0.1568 |
| Number of | ML        | 0.1766     | 0.1763    | 0.2549    | 0.1065   | 0.0696  | 0.3325 |
|           | Ant.I     | 0.1161     | 0.2929    | 0.2846    | 0.0768   | 0.2781  | 0.0778 |
|           | Ant.II    | 0.1096     | 0.3785    | 0.0428    | 0.0293   | -0.0347 | 0.1491 |
|           | Ant.III   | 0.1950     | 0.2438    | -0.2489   | 0.1840   | 0.3549  | 0.0456 |
|           | URS*      | 0.0288     | 0.0228    | -0.1003   | 0.0637   | 0.1108  | 0.1963 |
|           | AbdT.VIII | 0.0953     | -0.0932   | -0.1649   | 0.1514   | 0.3462  | 0.0480 |
|           | GP        | 0.2152     | 0.0765    | 0.0241    | 0.1965   | 0.3141  | -0.0679 |
|           | Cauda     | 0.2029     | 0.0059    | -0.0036   | 0.2143   | 0.2011  | -0.0149 |
| Number of | Ant.III   | 0.1740     | 0.2508    | 0.0760    | -        | -       | -     |
|           | Ant.IV    | 0.1358     | 0.3570    | 0.0171    | -        | -       | -     |
|           | Ant.V     | 0.1080     | 0.4628    | -0.0936   | -        | -       | -     |
| % of total |          |            |           |           |          |        | 50.2  |
| variance  |          |            |           |           |          |        | 9.4   |
|           |          |            |           |           |          |        | 7.1   |
|           |          |            |           |           |          |        | 55.2  |
|           |          |            |           |           |          |        | 9.0   |
|           |          |            |           |           |          |        | 5.7   |
|           |          |            |           |           |          |        | 42.7  |
|           |          |            |           |           |          |        | 14.1  |
|           |          |            |           |           |          |        | 12.2  |

Abbreviations of the characters are explained in the text.

Comparison of the morphological and molecular rates of evolution

Although the branch lengths of the trees generated using the Euclidean distance matrix under the topological constraint of the molecular phylogeny were different from those of the ML tree (Figures 1, 4), they were relatively similar within each classified group (clade G and clade F in Figure 4). Comparing the morphology-based trees with the DNA-based ML tree, species in the *gossypii* group had relatively shorter branches than did species in the other groups. In the two trees generated using 28 characters and 17 continuous characters, a subclade of eight species in the *gossypii* group appeared quite divergent from the remaining members, corresponding to a short branch length in the molecular phylogeny and suggestive of rapid diversification in this clade (arrowed in Figure 4). In the tree generated using the 11 meristic characters, the branch lengths of the *gossypii* group were relatively shorter than those of the other groups.

Analysis of phylogenetic correlation and trait evolution

The estimates of the three scaling parameters and their LR against the log-likelihood of null hypothesis’ test fixing parameter...
The results of LR tests for directional trends showed that the log-likelihood values of Model A (random walk) mostly tended to be larger than those of Model B (directional), but length and number of setae on Ant.III similarly showed a directional trend.

Discussion
Relative stasis of morphological characters in the *gossypii* group more than in other species-groups

Our morphometric analyses revealed that species in the *gossypii* group diverged more morphologically from species assigned to the *craccivora* + *spiraecola* + *fabae* groups [2,4,5]. This result, based on morphological characters, agrees well with the reciprocally monophyletic separation between the *gossypii* group and the cluster of the other three groups in the molecular phylogeny (Figure 1; [14,15]). With respect to coloration and pigmentation, although these were not included in the morphometric analysis, the apterae of species in the *gossypii* group have a pale body color, and little or no sclerotisation or reticulation on the thoracic and abdominal dorsum [5,16,30].

In the morphometric analyses, species in the *gossypii* group all showed a relative reduction in the length and number of measured characters, primarily the lengths of the body, antennal segments,
HFM, HTB, and the numbers of rhinaria on Ant.III and Ant.IV (Tables 1, 2; Figures 2, 3). The quantitative morphological characteristics of species in the *gossypii* group were less variable than those of species in the *craccivora* + *spiraecola* + *fabae* groups. The *gossypii* group generally showed reduced quantitative values for characters used in morphometric analysis, and many characters were simplified. Although the numbers of specimens for each species were insufficient to fully gauge the extent of intraspecific variation, the lengths of almost all parts of the *gossypii* group e.g., lengths of the body, antennal segments, leg parts, and setae, were relatively shorter than those of the *craccivora* + *spiraecola* + *fabae* groups, except for *A. kurosawai*. In addition, species in the *gossypii* group showed relatively lower within-group variation than the other species groups for most of the meristic characters used for morphometric analyses (data not shown). Consequently, relatively reductive or regressive stasis of morphological characters in the *gossypii* group made them more morphologically similar to one another than to other species-group.

**Correlation between morphological and molecular characters**

Correlation between phenotypic and molecular changes is controversial [31,32], but in *Aphis*, morphological traits appeared to be significantly correlated to molecular characters based on comparisons of rates of evolution [Figure 4; Table 4]. The test of trait evolution (parameter λ in Table 5) revealed that phylogenetic signals existed in the major characters separating the *gossypii* group from the other groups, which also suggested a significant correlation between morphological and molecular evolution [28]. The sizes and numbers of characters in the *gossypii* group were significantly reduced compared to those in the other groups, and this correlation was congruent with the molecular phylogeny. Despite the fact that many species in the *gossypii* group feed on unrelated host plants, most taxonomists have assumed that these species are monophyletic and have attempted to assign species to subgeneric partitions or species-groups based on the strong affinities of morphological characters [3,4,5,9]. The phylogenetic correlation between two heterogeneous characters and the correlation of their rates of evolution suggest that these characters played a significant role in the diversification of the *Aphis* group.

Furthermore, the rapid divergence of the genus *Aphis* likely affected the morphological convergence that more characterizes each species-group. The eight species in the *gossypii* group that formed a sub-cluster (arrowed in Figures 1, 4) more closely resembled one another than they did any other *Aphis* species. Similarly, *Aphis fabae*, *A. fukii*, *A. hederae*, and *A. nevoni* in the *fabae*
suitable for the modality of *Aphis* and temporally later evolution (Table 5; [35,36]). These are likely species-specifically adapted for many different host plants [2,5,19].

_A. fabae_ length leading to the two polyphagous species, sampled only a few species in the _fabae_ group (arrowed in Figure 1) were also morphologically more similar to one another than to other _Aphis_ species. Although we sampled only a few species in the _fabae_ group, the short branch length leading to the two polyphagous species, _A. fabae_ and _A. gossypii_, may be seen as evidence of a common pattern of diversification for _Aphis_ species. In addition, the six subspecies [1] recognized biologically or morphologically to belong to _A. fabae_ likely originated as a result of rapid diversification [33,34]. It is hypothesized that although the _A. fabae_ and _A. gossypii_ related to the rapid divergence points have both host alternation and polyphagous ability [2,5]. For this reason, many other species morphologically similar to the _fabae_ or _gossypii_ group, even though they feed on the unrelated host plants, are therefore highly predicted to belong to those unique lineages.

**Evolution of the morphological characters in _Aphis_**

The dominant patterns of character evolution revealed by the two parameters, , were increased rates in short branches and temporally later evolution (Table 5; [35,36]). These are likely suitable for the modality of _Aphis_ speciation because they have species-specifically adapted for many different host plants [2,5,19].

The phylogenetic tree is suggestive that the _Aphis_ species radiated rapidly (Figures 1, 4). For example, among the _gossypii_ group, closely-related species (e.g., *A. sumare* and *A. taraxacii*) have been become isolated in short divergence times, and this pattern of speciation also similarly appeared in the _fabae_ group (arrowed in Figure 1; [14,15]). Their relatively simple and unspecialized structures might be rather advantageous for their rapid adaptation to various hosts [19]. It is hypothesized that although the morphological adaptation seems to be less important than the physiological constraint requiring the adaptation for some new leaf, stem, and trichome) on the new host even though the change is microscopic [21]. On the other hand, it is suggested that the physiological constraint requiring the adaptation for some new host affected the variation of structures in _Aphis_, thereby their characters could be altered in a relatively short divergence time [40,41]. It is clear that the long-term or incipient speciation pattern of _Aphis_, especially in the _gossypii_ group, by the transition that they have drastically shifted their hosts to many unrelated plants is quite different from most other species in _Acyrthosiphon_,

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**Table 2.** The first two canonical variables (total-sample standardized) that contributed to the separation of the 247 samples for 14 species in two CDAs.

| Characters | First CDA | | | | Second CDA | | | |
|------------|-----------|-----------|-----------|-------------|-----------|-----------|-----------|
|            | CV1       | CV2       | CV3       | CV1         | CV2       | CV3       |
| Length of  |           |           |           |             |           |           |
| Body       | 0.0453    | −0.0514   | −1.0166   | −            | −          | −          |
| Antenna    | 0.8638    | 1.3202    | −0.6988   | −            | −          | −          |
| Ant.I      | 0.4670    | −0.8511   | 0.9101    | −            | −          | −          |
| Ant.II     | 0.2452    | −0.7713   | 0.6338    | −            | −          | −          |
| Ant.III    | −0.2235   | −0.0639   | 0.6785    | −            | −          | −          |
| Ant.IV     | −0.5507   | −0.0705   | 0.2843    | −            | −          | −          |
| Ant.V      | −0.6651   | 0.4376    | −0.7908   | −            | −          | −          |
| Ant.Vib    | 0.3510    | −0.6680   | 0.5632    | −            | −          | −          |
| PT         | −0.1437   | −0.9334   | 0.3267    | −            | −          | −          |
| URS        | −0.1738   | 0.4179    | −0.1816   | −            | −          | −          |
| HFM        | −1.7508   | 1.6107    | −0.4296   | −            | −          | −          |
| HTB        | 2.0143    | −2.3330   | −0.6675   | −            | −          | −          |
| ZHT        | 0.6434    | −1.4677   | 0.8028    | −            | −          | −          |
| SIPH       | −0.6537   | 1.3879    | −0.8786   | −            | −          | −          |
| Cauda      | −0.2051   | 0.1500    | −0.1576   | −            | −          | −          |
| Length of setae on | 1.6154 | 1.0154 | 0.6649 | - | - | - |
| Ant.II     | 0.7400    | 0.4446    | 0.2514    | −            | −          | −          |
| Number of setae on | 0.1288 | 0.0017 | −0.2218 | 0.0782 | 0.1852 | −0.4744 |
| Ant.I      | 0.1462    | 0.2353    | 0.0127    | 0.2903       | −0.3454   | 0.3121    |
| Ant.II     | −0.0689   | 0.0183    | 0.1061    | −0.0041      | −0.1621   | −0.0477   |
| Ant.III    | 0.4437    | 0.9276    | 0.3863    | 0.7454       | −0.8408   | −1.6160   |
| URS*       | 0.0635    | 0.1258    | 0.0415    | 0.0550       | 0.1586    | −0.6803   |
| Abd.T.VIII | 0.4734    | 0.3713    | −0.1030   | 0.8375       | 0.0696    | 0.3883    |
| GP         | 0.4548    | −0.1828   | 0.8792    | 0.6261       | −1.5202   | 1.1344    |
| Cauda      | 1.0266    | 0.3259    | −1.0410   | 1.6037       | 2.2498    | 0.2873    |
| Eigenvalue | 29.4107   | 9.8950    | 3.4880    | 11.7011      | 2.7816    | 0.8378    |
| % of total variance | 52.1 | 17.5 | 9.7 | 71.8 | 17.1 | 5.1 |

*subsidiary setae on URS. 
\[10.1371/journal.pone.0011608.t002\]
Hyalopterus association to unrelated hosts occurring in Myzus persicae nae, which have diverged to the plant species closely associated tempo (morphological differentiation, it will be intriguing to reveal the or anholocycly [2], so they may have similar speciation variations in life cycle, i.e., host alternation, monoecious holocycly, have a lot of similarity in regard to polyphagy, and with displaying specialized host-adapted populations [20,47]. These two species morphological characters in morphometric analysis between Aphis gossypii possibly correlated with evolutionary rapid radiation.

In the analysis of trait evolution comparing Model A and Model B (Table 5), directional trends of length and number of setae on Ant.III appeared weakly even though the low LR and confidence value \( P = 0.179 \) and \( P = 0.093 \), respectively. If a directional trend exists, species that have diverged more from the root in the phylogeny will also tend to have changed more morphologically in a given direction [20]. In fact, those characters showing directional trends contribute to identify the Aphis species or divide species-groups, and also have been considered as important diagnostic keys in the taxonomic studies of Aphis [4,5]. Compared with that the species (e.g., A. ervinia, A. horii) in the basal position of the phylogeny have longer and much more setae on Ant.III than the others, the directional walk is likely toward regression in Aphis. In particular, these two characters are largely different and tend to diverge between in the gossypii and fabae groups. The former species is characterized by short and sparse setae on Ant.III, while the latter is by relatively long and dense ones. Although we cannot determine what factors historically affected these characteristics to change toward regression in this study, different convergence of these morphological characters between the species-groups need to be considered for their potential adaptive or functional ways (e.g., mechanical sensory, protective) in further study [48,49].

Materials and Methods

Sampling

A total of 40 species were included in the multivariate morphometric analysis (Table S1). Only thirty-three of the above species were available from the two previous molecular phylogenies [14,15]. Most species were classified into the species groups as suggested by previous studies [2,4,5,14,15], while the group assignments of the new species included in this study were established based on phylogenetic relationships or morphological similarities with representative species of the existing Aphis group. Four Toxoptera and Aleurophenia species were also included in the study due to their close phylogenetic relationship to Aphis [15]. Despite the stridulatory organs that characterize Toxoptera [50], species in this genus are morphologically very similar to Aphis.

The specimens examined for this study were obtained from the Insect Museum of the College of Agriculture and Life Sciences in Seoul National University, Seoul, Rep. of Korea; the National Academy of Agricultural Science, Suwon, Rep. of Korea; and the Institute of Czech Academy of Science (Jaroslav Holman & Jan Havelka), Ceske Budejovice, Czech Republic.

Morphometric measurements

A morphometric dataset was constructed by measuring/counting 28 characters in 40 species; 17 characters were continuous, and 11 characters were meristic (Table 1; Figure S4). To obtain the mean value of each character, we examined a total of 1,264 slide mounted specimens from 40 species (Table S2). The continuous characters measured in this study have been shown to be useful in other morphometric studies of aphids [22,23,25,51], while the meristic characters were new characters adopted from morphometric and biometric analyses of Aphis [52,53,54]. Length measurements were taken using Image Lab ver. 2.2.4.0 software (MCM Design, Hillerød), and digital images were captured with a CCD, SPOT Insight TM Color Mosaic 14.2 (Diagnostic Instruments, Inc. Sterling Heights, MI) attached to a Leica DM 4000B microscope (Leica Microsystems GmbH, Wetzlar). The size and location of each character follows that described by Blackman & Eastop [2]. Morphological terminology follows that used by Heie [5] and Blackman & Eastop [2].

| Table 3. Statistical values by ANOVA tests for gossypii group versus all other species. |
|-----------------------------------------------|--------|--------|--------|--------|--------|
| Characters                      | F value | P value | F value | P value |
|-----------------------------------------------|--------|--------|--------|--------|
| Length of Body                    | 32.59  | <.0001 | 410.02 | <.0001 |
| Antenna                         | 30.77  | <.0001 | 307.88 | <.0001 |
| Ant.I                           | 20.12  | 0.0021 | 123.08 | <.0001 |
| Ant.II                          | 22.33  | 0.0001 | 189.34 | <.0001 |
| Ant.III                         | 36.36  | <.0001 | 277.90 | <.0001 |
| Ant.IV                          | 40.04  | <.0001 | 319.64 | <.0001 |
| Ant.V                           | 28.75  | <.0001 | 268.54 | <.0001 |
| Ant.Vlb                         | 21.80  | 0.0002 | 207.31 | <.0001 |
| PT                              | 6.03   | 0.0188 | 92.63  | <.0001 |
| URS                             | 8.93   | 0.0049 | 260.18 | <.0001 |
| HFM                             | 42.12  | <.0001 | 410.25 | <.0001 |
| HTB                             | 35.49  | <.0001 | 350.32 | <.0001 |
| 2HT                             | 23.81  | <.0001 | 333.32 | <.0001 |
| SIPH                            | 2.47   | 0.1242 | 42.34  | <.0001 |
| Cauda                           | 7.81   | 0.0081 | 78.69  | <.0001 |
| Ant.III                         | 16.49  | 0.0002 | 296.96 | <.0001 |
| AbdT.III                        | 4.41   | 0.0425 | 223.53 | <.0001 |
| Number of setae on ML           | 31.98  | <.0001 | 67.72  | <.0001 |
| Ant.I                           | 1.02   | 0.3181 | 10.42  | <.0001 |
| Ant.II                          | 1.46   | 0.2349 | 0.01   | 0.9085 |
| Ant.III                         | 15.09  | 0.0004 | 89.65  | <.0001 |
| URS*                            | 2.86   | 0.0992 | 20.93  | <.0001 |
| AbdT.VIII                       | 2.14   | 0.1518 | 69.68  | <.0001 |
| GP                              | 14.17  | 0.0006 | 90.92  | <.0001 |
| Cauda                           | 27.15  | <.0001 | 236.05 | <.0001 |
| Number of rhinaria on (alate)   | 13.51  | 0.0007 | -      | -      |
| Ant.IV                          | 7.79   | 0.0082 | -      | -      |
| Ant.V                           | 2.68   | 0.1101 | -      | -      |

The specimens examined for this study were obtained from the Insect Museum of the College of Agriculture and Life Sciences in Seoul National University, Seoul, Rep. of Korea; the National Academy of Agricultural Science, Suwon, Rep. of Korea; and the Institute of Czech Academy of Science (Jaroslav Holman & Jan Havelka), Ceske Budejovice, Czech Republic.

Hyalopterus, Macrosiphoniella, Megoura, and Uroleucon within Aphidinae, which have diverged to the plant species closely associated within a genus or within a family [17,18,42,43]. However, similar association to unrelated hosts occurring in Myzus persicae [24,44,45] must be associated with that in Aphis gossypii [38,39,46], which seems to similarly affect the noticeable changes of these morphological characters in morphometric analysis between specialized host-adapted populations [20,47]. These two species have a lot of similarity in regard to polyphagy, and with displaying variations in life cycle, i.e., host alternation, monoeocious holocycly, or anholocycly [2], so they may have similar speciation mechanism. Due to these, if correlated between the genetic and morphological differentiation, it will be intriguing to reveal the tempo (k, δ) of trait evolution [23] for closely-related species (e.g., M. nicotiana) with M. persicae [47]. Based on the results, it is important to investigate more whether these patterns of trait evolution appear in other aphid group, and whether these are possibly correlated with evolutionary rapid radiation.
Abbreviations for the characters used in the text are as follows: Ant.I, Ant.II, Ant.III, Ant.IV, Ant.V, Ant.VI, antennal segments I, II, III, IV, V, VI and the base of Ant.VI, respectively; PT, processus terminalis; ML, mandibular lamina; URS, ultimate rostral segment; HFM, hind femur; HTB, hind tibia; 2HT, second segment of hind tarsus; AbdT.I, AbdT.II, AbdT.III, AbdT.IV, AbdT.V, AbdT.VI, AbdT.VII, AbdT.VIII, abdominal tergite I, II, III, IV, V, VI, VII, VIII, respectively; SIPH, siphunculus; GP, genital plate.

Phylogeny

To reconstruct a comprehensive phylogenetic background against which to evaluate morphological relationships between the species groups and to estimate the evolution of characters, we

Figure 4. Comparison of branch lengths between the trees of combined DNA and three morphological datasets optimized with the topological constraint based on the ML tree (Figure 1). Arrow means a short divergence point in the gossypii group. Green background - gossypii group (typical morphology from A. glycines), yellow - craccivora + fabae + spiraecola groups (typical morphology from A. craccae), red - unclassified species.

Table 4. Correlation of patristic distance matrices by Pearson correlation coefficient, Mantel test, and K-tree score between the molecular dataset and the different morphological dataset assessed under the ML tree topology.

| Compared datasets                      | Pearson r | Mantel test | K-tree score |
|----------------------------------------|-----------|-------------|--------------|
|                                         | r         | P value     | r            | P value     | Scale factor | K-score      |
| DNA vs morphology - 28 all characters  | 0.6584    | <0.001      | -0.0318      | 0.2468      | 0.0159       | 0.1965       |
| DNA vs morphology - 17 continuous characters | 0.5667    | <0.001      | 0.0055       | 0.4599      | 0.0186       | 0.2376       |
| DNA vs morphology - 11 meristic characters | 0.7245    | <0.001      | 0.7378       | 0.0001      | 0.0239       | 0.1956       |

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used the phylogeny based on molecular markers which were used in the previous studies [14,15]. Of the 40 species used in the morphological analyses, data from 33 species were available for phylogenetic reconstruction. We retrieved the sequences of two mitochondrial genes (partial tRNA-leucine + cytochrome c oxidase II [COI; 702 bp]; partial 12S+ tRNA-valine + 16S [16S; 1601 bp]) and one nuclear gene (nuclear elongation factor 1 alpha [EF1-α; 802 bp excluding introns]) for the species including an outgroup species

\( \text{A. argrimoniae} \) corresponding sequences for two species,

\( \text{A. craccivora} \) and

\( \text{A. ulmariae} \) obtained from GenBank. We generated cytochrome oxidase II (CytB; 1,501 bp) previously used by Coeur d’acier et al. [14] were also obtained from GenBank. We generated cytochrome b (CytB; 757 bp) sequences for 31 species in this study, as described previously [18]. For the phylogenetic analysis, the sequences of 33 ingroup species plus one outgroup were concatenated and aligned using MEGA4.0 [55] to yield a combined dataset containing \( \sim 3,842 \) bp of sequence. The GenBank accession numbers of all 40 species used in the phylogenetic reconstruction are listed in Table S3.

**Table 5.** Evolutionary analysis of the 16 characters appeared to be significant in ANOVA, using BayesContinous implemented in BayesTraits, showing model parameters and likelihood ratio (LR).

| Characters | Scaling parameter estimates | LR [R]andom walk vs [D]irectional |
|------------|-----------------------------|----------------------------------|
| Length of |                             |                                  |
| Body       | 0.53 7.39** 0.30 31.31*** 2.03 5.61* 2.19 [R] |
| Antenna    | 0.67 11.66*** 0.41 21.15*** 1.69 3.71 <0.01 [R] |
| Ant.II     | 0.54 9.00** 0.26 37.92*** 2.10 7.35** 1.85 [R] |
| Ant.IV     | 0.64 11.64*** 0.36 22.20*** 1.46 4.08* 2.52 [R] |
| Ant.V      | 0.69 13.15*** 0.33 20.98*** 1.22 1.04 1.46 [R] |
| HFM        | 0.60 15.01*** 0.32 28.43*** 1.64 4.04* 0.99 [R] |
| HTB        | 0.61 13.81*** 0.32 29.42*** 1.69 4.61* 2.98 [R] |
| ZHT        | 0.81 14.38*** 0.51 5.19** 0.63 –1.00 2.63 [R] |
| Cauda      | 0.50 2.71 0.21 24.15*** 0.85 –1.12 4.71* [R] |
| Number of setae on |                             |                                  |
| Ant.II     | 0.61 3.76 0.88 3.45 2.47 4.54* 1.81 [D] |
| Number of setae on |                             |                                  |
| ML         | 0.68 11.86*** 0.36 17.75*** 1.19 0.48 3.98* [R] |
| Ant.II     | 0.81 12.60*** 0.90 –5.81 1.89 1.92 2.821 [D] |
| GP         | 0.82 9.24** 0.98 –0.35 2.00 2.03 0.31 [R] |
| Cauda      | 0.72 9.73** 0.54 11.43*** 1.60 3.11 0.37 [R] |
| Number of rhinaria on (alate) |                             |                                  |
| Ant.II     | 0.61 5.27* 0.37 12.88*** 1.06 –4.86 0.46 [R] |
| Ant.IV     | 0.61 4.00* 0.45 11.73*** 1.13 0.15 0.13 [R] |

### Multivariate morphometric analysis

Principal component analysis (PCA; SAS Procedure PRINCOMP; SAS version 9.1.3; SAS Institute, Inc., Cary, NC) was carried out on the morphometric dataset of 40 species using the 28 measured/counted characters to confirm the correlations of species-groups and to determine the main components of variation in the morphological data.

Principal component analysis was performed again on the morphometric dataset of 247 apterous samples from 14 representative species; seven (\( \text{A. gossypii} \), \( \text{A. glycines} \), \( \text{A. ichigus} \), \( \text{A. angustioria} \), \( \text{A. sumire} \), \( \text{A. egomae} \), \( \text{A. hederae} \), \( \text{A. rumicis} \), \( \text{A. sudzuki} \), \( \text{A. craccivora} \), \( \text{A. fabae} \), \( \text{A. fukii} \), \( \text{A. neopata} \), \( \text{A. rumics} \), \( \text{A. spiracola} \)) from the \( \text{gossypii} \) group and seven (\( \text{A. craccivora} \), \( \text{A. fabae} \), \( \text{A. fukii} \), \( \text{A. hederae} \), \( \text{A. neopata} \), \( \text{A. rumics} \), \( \text{A. spiracola} \)) from the \( \text{fabae} \) + \( \text{craccivora} \) + \( \text{spiracola} \) groups, using the 25 morphological characters except for three alate characters (number of rhinaria). Canonical discriminant analysis (CDA; SAS Procedure CANDISC; SAS version 9.1.3) was then performed to determine which character(s) contributed most to the separation of each species cluster. We also performed PCA and CDA using 11 meristic characters only, to exclude the effects of highly size-correlated variables between groups.

One-way analysis of variance (ANOVA; SAS Procedure ANOVA; SAS version 9.1.3) was performed for each character to evaluate the significances of differences between the \( \text{gossypii} \) group and all other species in the first and second PCAs.
Comparison of the evolutionary rates between morphological and molecular characters

Using the ML tree obtained from the combined molecular dataset as a topological constraint, we compared the branch lengths estimated from the three different morphological datasets (17 continuous, 11 meristic, and 28 continuous+meristic characters) used in the first PCA. The lengths of the branches of the morphological datasets were calculated using a Euclidean distance matrix (SAS Procedure DISTANCE; SAS version 9.1.3). Comparison of the evolutionary rates between the molecular and morphological traits was conducted using PAUP*4.0b10 [60] to optimize the distance matrix and to describe the length-estimated tree on the basis of two recent studies [26,27]. We computed patristic distances using PATRISTIC v1.0 [61] for the morphological and molecular datasets in order to test the significance of correlations by Pearson correlation coefficient (r) and Mantel test. Mantel test’s significance was assessed by comparing the morphological and molecular matrices obtained in the above analysis using ZT v1.1 [62] with 1,000,000 permutations. In addition, topologies and relative branch lengths of the length-estimated trees based on each morphological dataset were compared using Ktreedist v1.0 [63].

Hypothesis testing for the evolution of the major characters

We tested hypotheses for the evolution of the 16 major characters, which were estimated as significant according to ANOVA (Table 3), using Pagel’s BayesContinuous in BayesTraits version 1.0 [28,35]. This approach has been useful to test whether some continuous characters are correlated with the phylogeny by applying a Generalized Least Squares (GLS) method [28,64,65]. GLS approach provides a natural framework in which to represent some evolutionary features that arise from phylogenetic association [28]. Models of trait evolution under GLS can be compared using a likelihood-ratio (LR) test in which $LR = 2[\log{\text{likelihood of the better-fitting model}}] - \log{\text{likelihood of the worse-fitting model}}$. We used the harmonic mean instead of the log-likelihood as recommended by Pagel and Meade [35]. The LR statistic is asymptotically distributed as a chi-square variate with one degree of freedom [35].

We first estimated the three scaling parameters, $\lambda$ (lambda), $\kappa$ (kappa), and $\delta$ (delta), modeled in BayesContinuous, which can allow tests of the tempo, mode, and phylogenetic associations of trait evolution for a given phylogeny [28,36]. The parameter $\lambda$ estimates whether the phylogeny correctly predicts the patterns of covariance among species on a given trait, where $\lambda = 0$ indicates phylogenetic independence like a big-bang phylogeny, and $\lambda = 1$ denotes evolution in accordance with the topology of the phylogeny. Intermediate values of $\lambda$ arise when the topology overestimates the covariance among species. The parameter $\kappa$ can be used to test for a punctuational versus gradual mode of trait evolution in branches of different lengths. Values of $\kappa < 1$ indicate increased rates of trait evolution in short branches, while values of $\kappa > 1$ indicate that longer branches contribute more to trait evolution. A value of $\kappa = 0$ is consistent with a punctuational mode of evolution over very short time scales. The parameter $\delta$ estimates whether the rate of trait evolution has accelerated or slowed over time as one moves from the root to the tips of the tree. Values of $\delta < 1$ can indicate temporally early evolution of a trait (i.e., adaptive radiation), while values of $\delta > 1$ indicate temporally later evolution of a trait (i.e., species-specific adaptation). Values of $\kappa = 1$ or $\delta = 1$ are interpreted as a default gradualism in each case. For determining the confidence of each estimated parameter, the LR were obtained from tests for the estimated value against little phylogenetic correlation ($\lambda = 0$), or a gradual pace ($\delta = 1$, $\kappa = 1$). Second, we estimated directional trends by comparing Model A (random walk) with Model B (directional walk) via their log-likelihoods [28,35]. The random walk of Model A is sometimes called as a Brownian motion. If Model B fits the data better (larger log-likelihood closer to zero) than Model A, it says that a directional trend exists.

For each test, log-likelihood and rate parameter values were explored to find acceptance rates when running the Markov chains of between 20 and 40% as recommended by Pagel and Meade [35,66]. The Markov chain was run for 10 million generations after a burnin of a million generation. Convergence of the model was investigated by finding a stationary phase of the harmonic means.

Supporting Information

Figure S1 Plot of the mean scores on the first two principle components for 14 species representing the gossypii group (red-type symbols) and the craccivora + fabae + spiraecola groups (blue-and purple-type symbols) based on 11 meristic characters. Found at: doi:10.1371/journal.pone.0011608.s001 (3.15 MB TIF)

Figure S2 Plot of the mean scores on the (A) CV1 vs CV2 and (B) CV1 vs CV3 for 14 species representing the gossypii (open circle), the craccivora (blue closed triangle), the spiraeola (red closed square), and the fabae (black closed diamond) groups based on 25 characters. Found at: doi:10.1371/journal.pone.0011608.s002 (2.35 MB TIF)

Figure S3 Plot of the mean scores on the (A) CV1 vs CV2 and (B) CV1 vs CV3 for 14 species representing the gossypii (open circle), the craccivora (blue closed triangle), the spiraeola (red closed square), and the fabae (black closed diamond) groups based on 11 meristic characters. Found at: doi:10.1371/journal.pone.0011608.s003 (2.25 MB TIF)

Figure S4 Picture of the structures and reference parts employed in the morphometric analyses (represented by A. craccivora; aptera (A-G) and alata (H): A, body; B, antennal segment III; C, second hind tarsal segment; D, ultimate rostral segment; E, seta on abdominal tergite III; F, cauda; G, genital plate; H, antennal segment III–V. Abbreviations are explained in the text. Found at: doi:10.1371/journal.pone.0011608.s004 (5.16 MB TIF)

Table S1 Taxonomic, biological, and species-group information of species used for morphometric analysis. Found at: doi:10.1371/journal.pone.0011608.s005 (0.10 MB DOC)

Table S2 Samples of apterous (Ap.) and alate (Al.) viviparae of species measured/counted for morphometric analysis. Found at: doi:10.1371/journal.pone.0011608.s006 (0.07 MB DOC)

Table S3 GenBank accession numbers of the sequences used in this study. Found at: doi:10.1371/journal.pone.0011608.s007 (0.07 MB DOC)

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Author Contributions

Conceived and designed the experiments: HK, SL. Performed the experiments: HK. Analyzed the data: HK. Contributed reagents/materials/analysis tools: HK, WI, SL. Wrote the paper: HK.
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