Phylogenetic comparative methods reveal higher wing loading in ant-attended *Tuberculatus* aphids (Hemiptera: Aphididae)

Izumi Yao

Abstract—Aphids of the genus *Tuberculatus* Mordvilko feed on Fagaceae trees and exhibit various interactions with ants, ranging from non-attendance to intermediate or close associations. Despite possession of fully developed wings, two ant-attended species, *Tuberculatus quercicola* (Matsumura) and *Tuberculatus* sp. A, exhibited extremely low dispersal. I examined the correlation between wing loading (ratio of body volume to wing area) and ant associations in 20 species of *Tuberculatus*. Based on a 1317 bp sequence in two mitochondrial regions, cytochrome oxidase I (COI) and NADH dehydrogenase subunit 1 (ND1), phylogenetic trees were reconstructed by neighbor-joining (NJ), most parsimony (MP), maximum likelihood (ML), and Bayesian analyses. All phylogenetic trees showed that mutualistic interactions with ants have evolved in *Tuberculatus* at least five times. Comparative analyses based on the NJ, MP, and ML trees showed that increase in wing loading is correlated with ant associations, suggesting that ant-attended aphids have allocated more resources to their bodies than to their wings, resulting in lowered dispersal.

Résumé—Les pucerons du genre *Tuberculatus* Mordvilko se nourrissent d'arbres de Fagaceae et connaissent diverses interactions avec les fourmis, variant d'aucune interaction à des associations moyennes ou fortes. Malgré la possession d'ailes complètement développées, deux espèces entretenues par les fourmis, *Tuberculatus quercicola* (Matsumura) et *Tuberculatus* sp. A, ont une dispersion très restreinte. La corrélation a été déterminée entre la charge alaire (rapport du volume corporel sur la surface des ailes) et l'association avec les fourmis chez 20 espèces de *Tuberculatus*. Les arbres phylogénétiques basés sur une séquence de 1317 pb dans deux régions mitochondriales, la cytochrome oxydase I (COI) et la sous-unité 1 de la NADH déshydrogénase (ND1), ont été élaborés par les méthodes du plus proche voisin (NJ), de la parcimonie maximale (MP) et de la vraisemblance maximale (ML), ainsi que par analyse bayésienne. Tous les arbres phylogénétiques montrent que les interactions mutualistes avec les fourmis sont apparues au moins à cinq reprises chez *Tuberculatus*. Des analyses comparatives basées sur les arbres NJ, MP et ML indiquentop que l'accroissement de la charge alaire est corrélé aux associations avec les fourmis, ce qui laisse croire que les pucerons entretenus par les fourmis allouent plus de ressources à leur corps qu'à leurs ailes, ce qui entraîne une dispersion réduite.

[Traduit par la Rédaction]

Introduction

Aphids of the genus *Tuberculatus* Mordvilko (Hemiptera: Aphididae) feed on members of the beech family (Fagaceae) and exhibit various interactions with ants, ranging from non-attendance to intermediate or close associations. During the summer, regardless of colony density or ant attendance, all nymphs develop into alate viviparous females that produce offspring parthenogenetically.

A previous field study using flight intercept traps and weekly observations found that two strongly ant-attended species, *Tuberculatus quercicola* (Matsumura) and *Tuberculatus* sp. A, exhibited significantly lower dispersal levels compared with two non-attended species, *Tuberculatus japonicus* Higuchi and *Tuberculatus paiki*

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Hille Ris Lambers (Yao 2010). The total numbers of winged individuals trapped and observed in trees across all seasons were 8 and 1342 for *T. quercicola*; 2 and 194 for *T.* sp. A; 52 and 200 for *T. japonicus*; and 137 and 1315 for *T. paiki*, respectively. As a consequence of low dispersal, the two ant-attended species also showed low genetic diversity and high genetic differentiation between subpopulations compared with those of the two non-attended species. The migratory behavior of the non-attended species allows them to escape from predators or move to host plants with a higher nutritional value. Fully developed wings of ant-attended aphids, however, seems to be a less adaptive trait. Given that developing a flight apparatus prolongs development and results in a 20% reduction in gonad size (Dixon *et al.* 1993), selection should favor the loss of the flight apparatus. Studies focusing on insects with a dispersal polymorphism found trade-offs between wing development and body size or between wing development and fecundity (Roff and Fairbairn 1991; Zera and Denno 1997). When reared in a homogenous environment in the field, *T. quercicola* had larger body volumes associated with higher embryo numbers than *T. paiki*; the larger body volume resulted in larger wing loading (the ratio of body volume to wing area; Ellington 1984) (I. Yao, personal observation). Moreover, morphological traits such as the longer mouthparts of poplar-feeding *Chaitophorus* Koch aphids (Shingleton *et al.* 2005) and the shorter cornicle length of Macrosiphini aphids (Mondor *et al.* 2002) are likely to be associated with ant attendance. It is expected that ant-attended *Tuberculatus* aphids are less likely to fly and that they have developed a proportionally larger body size to increase embryo numbers or to produce more honeydew for attending ants. This study used phylogenetically independent contrasts to determine if the relationship between wing loading and ant association is found among 20 species of *Tuberculatus*.

Material and methods

Sampling

From 2005–2009, specimens of 20 *Tuberculatus* species were collected from leaves on 2–14 trees at various sites in Japan (Table 1,Fig. 1*a*). A species was regarded as ant-attended if aphids offered honeydew directly from their anus to attending ants (Table 1). To avoid multiple collections of an aphid clone, a single aphid was collected from each sampled leaf. All aphids were preserved in 99.5% ethanol. Specimens of two other aphid species, *Tinocallis zelkowae* (Takahashi) and a species of *Takecallis* Matsumura, were included as outgroups.

Measurements of wing loading

Wing loading is defined as the ratio of an insect's mass to the surface area of its wings (Ellington 1984). Here, body volume was used as an index of weight because it was difficult to weigh the aphids. To calculate wing loading, the following characteristics were measured for each aphid: (1) the body volume of alate adults

$$V = V1 + V2$$

where $V1$ is the volume of a sphere, $4\pi(tl/2)^3/3$, $V2$ is the volume of an ellipsoid, $4\pi(bl)(bw)^2/3$, and tl is thorax length, bl is body length, and bw is body width; and (2) the total area of fore- and hindwings of alate adults using the formula for calculating the area of a rhombus,

$$A = (fl)(fs)/2 + (hl)(hs)/2,$$

where fl and hl are fore- and hindwing long axes, respectively, and fs and hs are fore- and hindwing short axes, respectively (Fig. 1*b*). Finally, measurements of wing loading were calculated as V/A.

Body part dimensions were measured using an eyepiece micrometer installed in a binocular microscope. On average, 38.7 aphids per species (range: 10–64, Table 1) were measured. Measurements of each sampling tree were averaged, and the averages were used for analyses.

DNA extraction, polymerase chain reaction (PCR), and sequencing

Total DNA was extracted using a DNeasy Tissue kit (Qiagen, Tokyo, Japan). Two mitochondrial regions, COI (940 bp) and ND1 (377 bp), were used for a total of 1317 bp of DNA sequences. Primer sets, C1-J-1718 (5′-GGAGGATTTGGAAATTGATTAGTTCC-3′)

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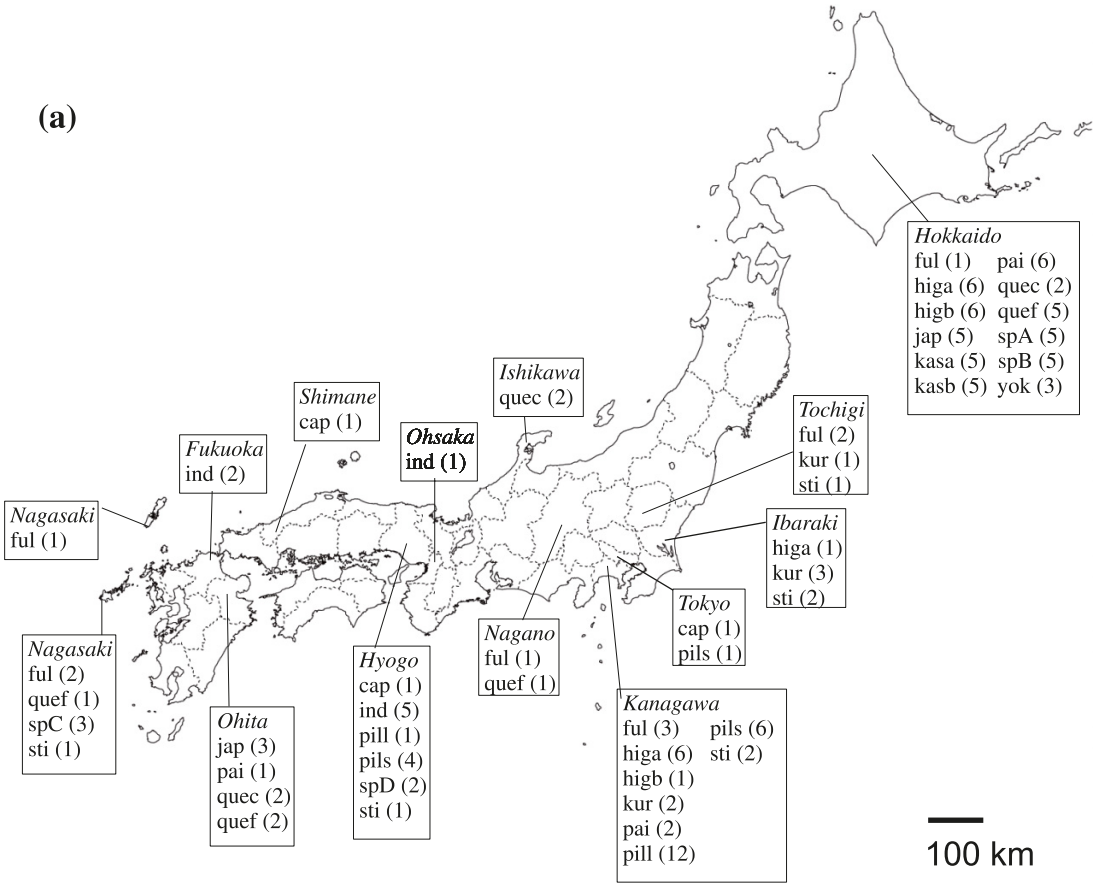
Table 1. Collection sites, host plants, wing-loading data, and DNA Data Bank of Japan accession numbers for *Tuberculatus* aphid species used in sequencing and measurements of wing loading.

Species	Abbreviation	Collection sites[a]	Ants[b]	Host plant	n[c]	Wing loading[d]	COI[e]	ND1[e]
T. capitatus Essig & Kuwana	cap	Shirahama	1	*Quercus acutissima* Carruthers	51	0.73±0.09	AB592750	AB592772
T. fulviabdominalis (Shinji)	ful	Toyama	1	*Quercus dentata* Thunberg	30	0.57±0.19	AB592751	AB592773
T. higuchii-a	higa	Nishinomiya	0	*Quercus aliena* Blume	64	0.40±0.10	AB592752	AB592774
T. higuchii-b	higb	Tomakomai	0	*Quercus crispula* Blume	21	0.35±0.07	AB592753	AB592775
T. indicus L.K. Ghosh	ind	Kobe	1	*Quercus serrata* Thunberg	37	0.64±0.10	AB592754	AB592776
T. japonicus Higuchi	jap	Ishikari	0	*Quercus dentata* Thunberg	50	0.46±0.12	AB592755	AB592777
T. kashiwae-a	kasa	Shimizu-cho	0	*Quercus dentata* Thunberg	18	0.38±0.10	AB592756	AB592778
T. kashiwae-b	kasb	Ebetsu	0	*Quercus crispula* Blume	23	0.36±0.14	AB592757	AB592779
T. kuricola (Matsumura)	kur	Fukuoka	1	*Castanea crenata* Siebold & Zucc.	30	0.54±0.11	AB592758	AB592780
T. paiki Hille Ris Lambers	pai	Shikaoi-cho	0	*Quercus dentata* Thunberg	51	0.39±0.09	AB592759	AB592781
T. pilosulus Quednau	pill	Hakone-machi	1	*Quercus serrata* Thunberg	50	0.61±0.12	AB592760	AB592782
T. pilosus (Takahashi)	pils	Kawasaki	0	*Lithocarpus edulis* (Makino) Nakai	50	0.43±0.11	AB592761	AB592783
T. quercicola (Matsumura)	quec	Ishikari	1	*Quercus dentata* Thunberg	54	0.66±0.08	AB592762	AB592784
T. querciformosanus (Takahashi)	quef	Kashiwa	0	*Quercus dentata* Thunberg	50	0.38±0.06	AB592763	AB592785
Tuberculatus sp. A	spA	Syari-cho	1	*Quercus crispula* Blume	50	0.76±0.12	AB592764	AB592786
Tuberculatus sp. B	spB	Shikaoi-cho	1	*Quercus dentata* Thunberg	43	0.84±0.16	AB592765	AB592787
Tuberculatus sp. C	spC	Gotoh	0	*Quercus dentata* Thunberg	21	0.56±0.10	AB592766	AB592788
Tuberculatus sp. D	spD	Kobe	0	*Quercus aliena* Blume	15	0.49±0.01	AB592767	AB592789
T. stigmatus (Matsumura)	sti	Shimane-Pref.	1	*Quercus serrata* Thunberg	56	0.55±0.14	AB592768	AB592790
T. yokoyamai (Takahashi)	yok	Tomamae-cho	0	*Quercus dentata* Thunberg	10	0.40±0.17	AB592769	AB592791

[a]Collection sites for the individuals used in sequencing.
[b]0, non-attended species; 1, faculative or obligate ant-attended species.
[c]Total number of aphids used for measurements of wing loading.
[d]Average and standard deviation.
[e]Accession numbers in DDBJ.

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Fig. 1. (*a*) Distribution of *Tuberculatus* aphid species collection sites in Japan. Italics indicate prefecture names. Species abbreviations are defined in Table 1. Numbers in parentheses indicate the number of trees from which aphid samples were collected. (*b*) A typical *Tuberculatus* species after Quednau (1999) with the following body and wing measurements indicated: thorax length (*tl*), body length (*bl*), body width (*bw*), fore- and hindwing long axes (*fl* and *hl*, respectively), and fore- and hindwing short axes (*fs* and *hs*, respectively).



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(Simon *et al.* 1994) + R2711 (5′-GGGAAA-AAAGTTAGGTTTACACC-3′) and N1-N-12595 (5′-GTAGCATTTTTAACTTTATTA-GAACG-3′) (Simon *et al.* 1994) + ND1BR (5′-AATCATATTCAACCAGAATG-3′), were used to amplify COI and ND1 regions, respectively. All PCRs were performed in 20 μL volumes with 2 μL of 10× PCR buffer (Takara, Shiga, Japan), 1.6 μL of dNTP mixture (each of 2.5 mM), 1 μL of 2 pM of each primer, 20 ng/μL of genomic DNA, and 0.5 units of Ex-*Taq* DNA polymerase (Takara). The reaction cycle was 94 °C for 3 min followed by 30 cycles of 94 °C for 30 s, 45 °C for 20 s, and 65 °C for 90 s. For each fragment, an entire PCR product was purified using the QIAquick PCR purification kit (Qiagen). The volume of the sequencing reaction was 5 μL and consisted of 2 μL of Quick Start Mix (Bechman Coulter, Tokyo, Japan), 0.5 μL of 10 pM forward or reverse primers, and 2.5 μL of 10 ng/μL template DNA. The reaction cycle was 33 cycles of 94 °C for 30 s, 50 °C for 15 s, and 65 °C for 90 s. DNA sequencing was performed using CEQ2000XL DNA Analysis System (Bechman Coulter). Sequences of COI and ND1 were deposited in the DNA Data Bank of Japan under accession numbers AB592750–AB592769 and AB592772–AB592791 for *Tuberculatus* species (Table 1), AB592748 and AB592770 for *Tinocallis zelkowae*, AB592749 and AB592771 for *Takecallis* sp.

Phylogenetic analysis

Neighbor-joining (NJ), most parsimony (MP), and maximum likelihood (ML) analyses were performed using PAUP* 4.0b10 PPC (Swofford 2002). For MP analysis, all characters were equally weighted. MP trees were searched heuristically with 1000 random addition replication using tree bisection- reconnection (TBR) branch swapping. To assess confidence in clades, non-parametric bootstrap tests were performed for NJ and MP trees using a full heuristic search and 10 000 replicates with TBR branch swapping.

Parameters for ML analysis were chosen based on the Akaike Information Criterion as implemented in Modeltest ver 3.7 (Posada and Crandall 1998). The GTR + I + G model was selected for the combined mitochondrial sequences. The ML trees were searched heuristically with TBR branch swapping using a stepwise addition as a starting option. For the ML tree, a branch-and-bound search and 1000 replicates with the NNI branch swapping were used to save time.

For Bayesian analysis, MrBayes ver. 3.1.2 (Ronquist and Huelsenbeck 2003) was performed with the GTR + I + G model selected by MrModeltest ver. 2 (Nylander 2004). The number of generations, sample frequency, and burn-in were set to 10^6, 100, and 500, respectively.

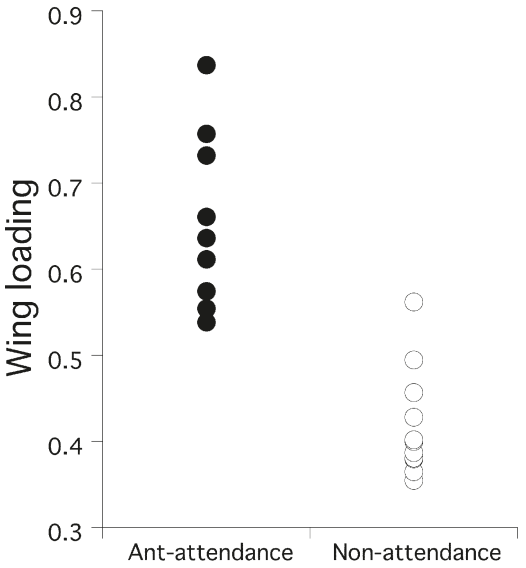
Comparative analysis by independent contrasts

As a consequence of their common ancestry, closely related species share many characteristics, and similarity between lineages is often influenced by relatedness rather than by independent evolution. Most statistical tests assume independence of data points and, therefore, data that are phylogenetically non-independent will tend to inflate the degrees of freedom (Felsenstein 1985; Harvey and Pagel 1991). Comparative analysis by independent contrasts uses independent comparisons of components of a phylogeny, with each comparison being made at a different node in the phylogeny (Purvis and Rambaut 1995). To examine the correlation between ant associations and wing loading in each of the *Tuberculatus* species , the phylogenetically independent contrasts that were calculated at bifurcating nodes were used. Calculation of contrasts in phylogenetic trees was performed using Comparative Analysis by Independent Contrasts (CAIC) ver. 2.6.9, which allows a categorical x-variable to be included in the analysis (Purvis and Rambaut 1995). Although the extent of ant association ranges from facultative to obligate mutualism, it is difficult to quantify the degree of intimacy with ants. The extent of ant association, thus, was categorized as either 0 (non attendance) or 1 (facultative and obligate). Wing loading was not log-transformed because all comparative analyses with the logarithm-transformed data resulted in the assumptions in the CAIC being violated. After calculation using the CAIC, the correlation between independent standardized linear contrasts of wing loading and ant

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Fig. 2. Average wing loading for 20 *Tuberculatus* aphid species classified by ant-attendance or non-attendance.



association was analyzed with a Wilcoxon's signed-ranks test.

Results

Wing loading ranged from 0.35 ± 0.07 (mean ± SD) (*T. higuchii*-b) to 0.84 ± 0.16 (*Tuberculatus* sp. B) (Table 1). Averages of wing loading classified by ant-attendance or non-attendance were 0.66 ± 0.10 (mean ± SD) and 0.42 ± 0.06 (Fig. 2), respectively.

The phylogenetic trees from NJ, MP, and ML were in broad agreement with each other (Fig. 3). A Bayesian consensus tree has a polytomy. All but NJ trees showed a basal position for *Tuberculatus querciformosanus* (Takahashi) followed by a clade consisting of *Tuberculatus kuricola* (Matsumura) and *Tuberculatus pilosus* (Takahashi). All phylogenetic trees showed that mutualistic interactions with ants have evolved at least five times. In MP analysis, 13 trees resulted: for all trees, length = 957, consistency index = 0.508, retention index = 0.514, and rescaled index = 0.261. Of 1317 total characters, 258 characters were informative and 123 variable characters were parsimony uninformative.

Five contrasts were calculated at the nodes where an ant association evolved or was lost (Fig. 3). Positive direction was found in ant-attended species in all contrasts. The Wilcoxon's signed-ranks test showed a significant correlation between wing loading and ant associations, irrespective of any data from comparative analysis using the NJ tree, each of the 13 MP trees, and the ML tree (for the NJ tree, $n = 5$ contrasts, $z = -2.023$, $P = 0.043$; for each of 13 MP trees, $n = 5$ contrasts, $z = -2.023$, $P = 0.043$; and for the ML tree, $n = 5$ contrasts, $z = -2.023$, $P = 0.043$). These results indicate that higher wing loading is correlated with ant associations. For comparative analysis using a consensus tree obtained from Bayesian analysis, independent contrasts failed because of a violated assumption probably due to a polytomy.

Discussion

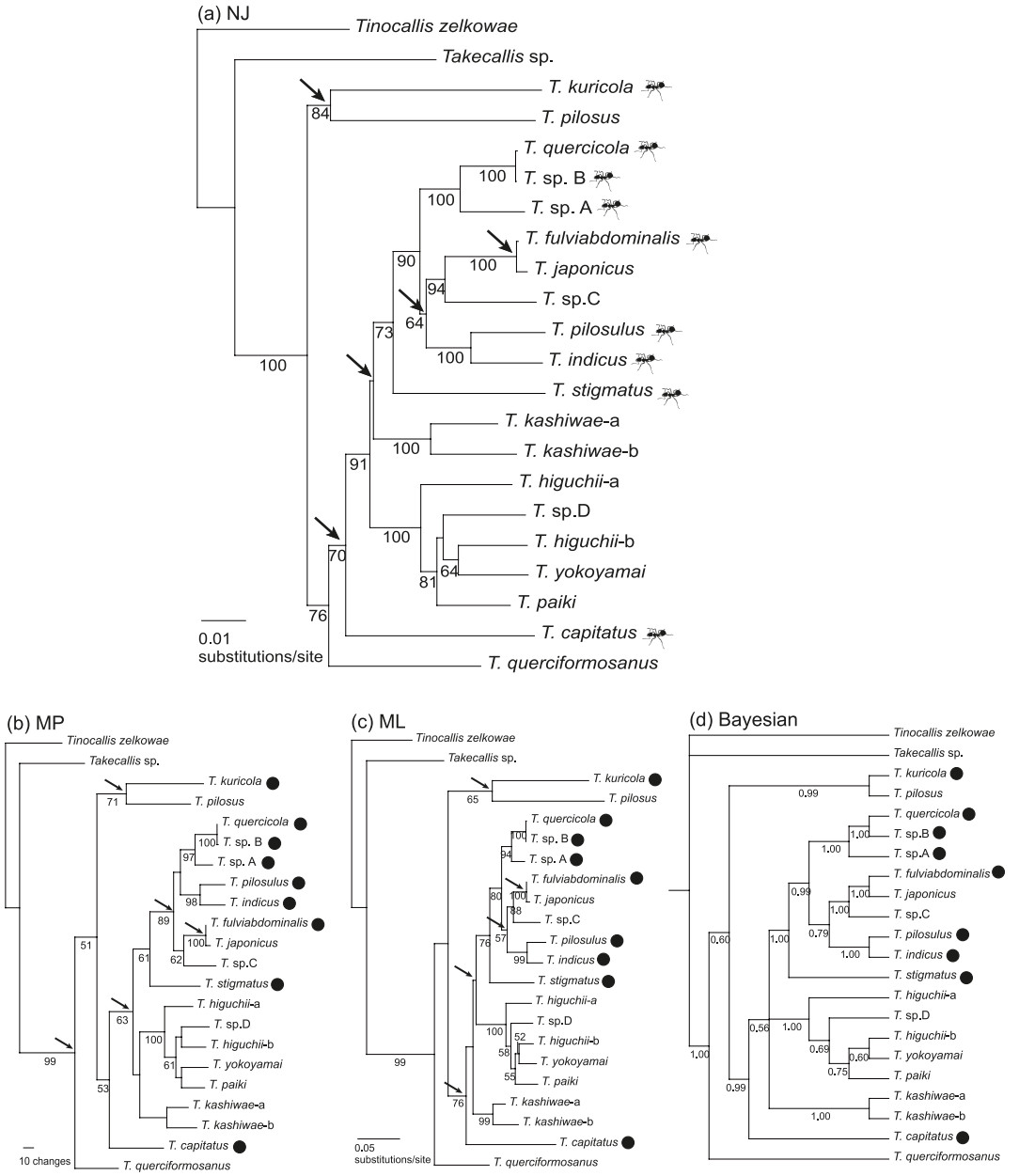
Tuberculatus phylogenetic trees indicated that mutualistic interactions with ants have evolved at least five times in the genus. Multiple gains or losses of ant mutualism suggest that aphid–ant mutualisms are labile, which is consistent with Shingleton and Stern (2003) who demonstrated that the evolution of the interaction between *Chaitophorus* aphids and ants has occurred at least five times. A variety of factors in aphid–ant mutualisms, *e.g.*, costs of ant attendance through honeydew production (Stadler and Dixon 1998; Yao *et al.* 2000; Katayama and Suzuki 2002; Yao and Akimoto 2001, 2002), host plant quality (Stadler *et al.* 2002), and the availability or predictability of suitable ants (Bristow 1991), have been reported as critical for promoting or hindering the formation of association with ants and they could be responsible for unstable mutualisms.

The Wilcoxon's signed-ranks test with the CAIC revealed that increases and decreases in wing loading are linked to ant associations in *Tuberculatus* aphids. Ant-attended or non-attended species were characterized by a higher or lower wing loading, suggesting that dispersal ability is closely associated with the physical difficulty of flight. Given that a trade-off between dispersal polymorphism and fecundity

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Fig. 3. Phylogenetic trees of 20 *Tuberculatus* aphid species obtained from the methods of (*a*) neighbor-joining (NJ), (*b*) most parsimony (MP), (*c*) maximum likelihood (ML), and (*d*) Bayesian analyses. Of the 13 MP trees, a single tree that scored the highest *P* value in comparative analysis is shown. Bootstrap percentages greater than 50% are shown below or near the branches in (*a*), (*b*), and (*c*). Probabilities greater than 0.5 are shown below or near the branches in (*d*). Ant-attended species are indicated with an ant image (*a*) or closed circles (*b*, *c*, and *d*). Arrows indicate nodes where contrasts were calculated.



has been reported in many insects, higher wing loading in *Tuberculatus* aphids could be attributed to allocation of resources to allow increased body size and thus increased fecundity or the production of larger volumes of honeydew. In preliminary experiments with

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the ant-attended species *T. quercicola*, the removal of ants always resulted in the extinction of *T. quercicola* colonies (Yao *et al.* 2000). Because *T. quercicola* benefited from ant attendance, low dispersal arising from increased wing loading could be adaptive. It is unclear, however, why full or partial wing-loss has not evolved in ant-attended *Tuberculatus* aphids. Ontogenetic constraints or hidden adaptive significance may be possible explanations. If morphological constraints render wing-loss difficult, changes in the allometric relationship between body size and wing area could precede loss of wings. Alternatively, wing-loss may not have evolved because some dispersal may still be required to maintain some level of outcrossing. To determine if retention of wings arises from morphological or phylogenetic constraints, anatomical and physiological studies on the formation and maintenance of wings are needed.

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