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ORIGINAL RESEARCH PAPER

Molecular identification and ecological characteristics of two cryptic lineages within a cosmopolitan aphid pest, *Brachycaudus helichrysi* (Hemiptera: Aphididae)

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Abstract The leaf-curl plum aphid, *Brachycaudus helichrysi* (Kaltenbach 1843), is a polyphagous cosmopolitan aphid. This serious pest of plum and peach orchards affects the growth of its host plants by curling infested leaves, and can transmit viruses such as the plum pox virus. Phylogenetic analyses have recently shown that *B. helichrysi* actually comprises two lineages that are morphologically similar but genetically different. In this study, we developed a PCR-RFLP test for differentiating between these two lineages, based on a mitochondrial DNA fragment. We used this test to investigate the patterns of host plant association and the geographical distribution of these lineages, with a large sample of individuals collected from numerous host plants and locations worldwide. We found that one of the lineages was never present on plum trees, and that individuals from both lineages coexisted on many secondary herbaceous hosts at almost all of the sites studied. We then carried out principal component analysis to determine whether the distribution of the two lineages could be explained by climatic features. We found that the lineage absent from plum trees was not found in regions with harsh winters. This suggests that one of the lineages within *B. helichrysi* may be an obligate parthenogenetic aphid.

Keywords Aphids · Asexuality · Climate · Host plant association · PCA · Lineage

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Introduction

Aphids (Insecta: Hemiptera: Aphididae) encompass about 4700 species (Remaudière and Remaudière 1997; Blackman and Eastop 2000), some of which are important pests on cultivated host plants worldwide. Their effectiveness as crop pests is due to both their capacity to reproduce asexually (rapidly generating very large numbers of individuals) and the broadness of their host-plant ranges. They damage host plants directly, through their feeding behaviour, and indirectly, by transmitting major plant viruses (Blackman and Eastop 2000; Emden and Harrington 2007).

Brachycaudus helichrysi (Kaltenbach 1843) is a polyphagous, cosmopolitan aphid (Blackman and Eastop 2000). It has been reported to have a heteroecious lifecycle, alternating between several *Prunus* (Rosaceae) species as primary hosts (Bennett 1955; Gupta and Thakur 1993) and numerous herbaceous secondary hosts, mostly from the Asteraceae and Boraginaceae. Holocyclic populations have also been described in mild climates and glasshouses. This

species is generally known as the leaf-curl plum aphid and is considered a major pest of sunflower (Lerin and Badenhausser 1995; Badenhausser and Lerin 1998), plum orchards (Madsen and Bailey 1958) and ornamental flowers, such as chrysanthemums (Wardlow and Gould 1981; Miller and Stoetzel 1997; Ramakers and Maaswinkel 2002). *Brachycaudus helichrysi* colonies cause damage by curling the leaves of their host plants and transmitting viruses, including the plum-pox virus, which causes a very serious viral disease of stone fruits (Bell 1983; Verma and Singh 1990; Levy et al. 2000). In a recent phylogenetic study (Piffaretti et al. 2012), we showed that *B. helichrysi* actually comprises two sibling species, hereafter called *B. helichrysi* H1 and *B. helichrysi* H2. These analyses, based on the sequencing of four DNA fragments (two mitochondrial markers, one nuclear marker and one DNA fragment from *Buchnera aphidicola*), revealed the existence of two clades separated by a genetic distance similar to that generally found between sister species within the genus *Brachycaudus* (e.g. p distance = 0.03 for the COI fragment used in aphids barcoding studies) (Coeur d'Acier et al. 2008). Conversely, intraclade distances, even between geographically distant individuals, were almost zero. Similar results were obtained for all of the DNA sequences investigated. The existence of two well-differentiated sibling species was confirmed by analyses of microsatellite DNA markers. However, *B. helichrysi* H1 could not be distinguished from *B. helichrysi* H2 on the basis of morphological features, no valid description and name could be attributed to any of the two lineages, and their taxonomic statuses have not yet been determined yet. We hope that our studies will lead to a taxonomic revision of this species complex, but in the meantime we will continue to name the two lineages *B. helichrysi* H1 and H2.

Our first study (Piffaretti et al. 2012) suggested that the two lineages might differ in terms of their biological features (e.g. host range, life cycle). However, this was based on only 65 individuals, and it was not possible to investigate ecological differences between the two lineages. A disjunction in the host-plant ranges and/or geographic ranges of the two lineages would imply that they do not represent equivalent threats to the surrounding cultivated plants. Furthermore, they might also differ in their capacity to transmit viruses (prevalence or transmission rate). It is, therefore, also important to investigate the ecological and biological differences between *B. helichrysi* H1 and H2 that are relevant to their pest status, in order to guide informed decisions regarding crop pest management. This could be achieved through the development of a cheap, easy-to-use, lineage-specific marker.

In several cases, molecular tools have proved useful for species identification and delimitation in aphids (Raboudi et al. 2005; Carletto et al. 2009; Footitt et al. 2009; Peccoud and Simon 2010). As the DNA sequences of *B. helichrysi* H1 and *B. helichrysi* H2 differ for several markers, it seemed appropriate to use these differences to develop a test distinguishing between H1 and H2. The principal objective of this study was to develop such a discrimination test, based on RFLP (restriction fragment length polymorphism). We chose to work on a well-conserved coding region of mitochondrial DNA to avoid the problems associated with the presence of too many mutations or insertion–deletion events that might modify the restriction sites. The second objective of this study was to analyse the geographical distributions of the two lineages in terms of climate characteristics and to investigate their host-plant ranges. We collected a large sample of individuals from numerous host plants (primary woody hosts and secondary herbaceous hosts) from regions with contrasting climatic conditions in all continents.

Materials and methods

Aphid sampling and DNA extraction

Brachycaudus helichrysi is a truly cosmopolitan and polyphagous aphid species, having been reported on all continents and on more than 120 plant species, with a notable preference for Asteraceae and Boraginaceae. We collected 271 aphid colonies between 1999 and 2011, from numerous host plants (9 plant families, 32 genera) and different parts of the world (10 countries on 5 continents), focusing in particular on three zones: (1) France, where we had easy access to samples; (2) southern Kazakhstan, in Central Asia, where a high species diversity has been reported for both *Prunus* (Bortiri et al. 2001) and *Brachycaudus* (Coeur d'Acier et al. 2008), and where *B. helichrysi* has been reported to cause major damage (Berim 2008); and (3) the western coast of the USA, where *B. helichrysi* occurs on a regular basis, from California to Washington State, according to information compiled in the Essig Museum database (http://essigdb.berkeley.edu/: 622 records for *B. helichrysi* specimens with locations, host-plant association, and collection date). We tried to maximise the diversity of the host plants and climatic conditions in which the samples were collected (Table 1; Table S1 of the Electronic supplementary material, ESM). Each sampled colony was georeferenced and the host plant was identified down to the genus level at least and—where possible—to the species level with a local flora. All colonies were immediately preserved in 90 % ethanol for storage. Each colony corresponded to specimens collected from a single host plant or from two neighbouring host plants of the same species if only a very small number of individuals were present on a single plant. A voucher

Table 1 Colony classes (with different compositions) and their associated host plants (categorised at the family and genus levels)

Host plant	*B. helichrysi* H1	*B. helichrysi* H2	*B. helichrysi* H1 and *B. helichrysi* H2
Apocyinaceae	2	0	0
Vinca	2	0	0
Asteraceae	106	80	31
Achillea	18	18	5
Anaphalis	1	2	0
Antennaria	0	1	0
Anthemis	5	2	1
Artemisia	2	2	0
Aster	0	2	1
Centaurea	0	2	0
Chrysanthemum	0	0	1
Conyza	56	29	19
Coreopsis	1	0	0
Erechtites	0	1	0
Eupatorium	0	1	0
Euryops	2	1	0
Helianthus	7	1	0
Helichrysum	2	1	0
Inula	0	1	0
Koelpinia	0	1	0
Leucanthemum	0	1	0
Matricaria	6	0	0
Osterospermum	0	1	0
Pallenis	0	2	0
Santolina	0	1	0
Saussurea	1	0	0
Senecio	2	9	3
Boraginaceae	1	1	2
Myosotis	1	0	2
Symphytum	0	1	0
Ericaceae	1	0	0
Arctostaphylos	1	0	0
Goodeniaceae	0	1	0
Lamiaceae	2	1	0
Stachys	2	1	0
Oenotheraceae	0	1	0
Oenothera	0	1	0
Ranunculaceae	1	0	0
Anemone	1	0	0
Rosaceae	35	0	0
Prunus	35	0	0

The number of colonies of each "class" (i.e. colonies comprising H1 individuals only, H2 individuals only, or mixed colonies) as indicated by the PCR-RFLP results is given for each plant family and genus

number was attributed to each colony, which was then transferred to the INRA collection (CBGP, Montpellier), from which they are available upon request.

We first examined the colonies under a binocular microscope in order to perform rapid initial sorting to remove species other than *B. helichrysi* from the plants. We then took 1–10 individuals from each sampled colony for DNA extraction and identification by RFLP.

Total genomic DNA was extracted from a single individual (frequently a larva) with the BioBAsics extraction kit (Bio Basic Inc., Markham, ON, Canada) in a 96-well plate containing 50 µl of extraction buffer, or in 10 % (w/v) Chelex® 100 resin solution (Bio-Rad Laboratories, Hercules, CA, USA) in a 96-well plate (see Piffaretti et al. 2012 for details). All DNA samples were stored at −20 °C.

Restriction site identification

We chose ten sequences from individuals from each of the two lineages, *B. helichrysi* H1 and *B. helichrysi* H2, as identified in our previous study on the basis of several molecular markers (see Piffaretti et al. 2012). Potential restriction sites were then identified in these sequences with the NEBcutter online software package (Vincze et al. 2003). This program searches each submitted sequence for unique restriction sites for all type II and commercially available type III restriction enzymes. We selected restriction enzymes that met the following criteria: (1) the enzyme had a restriction site exclusive to *B. helichrysi* H1 or *B. helichrysi* H2, and (2) the size of the digestion fragment had to be sufficiently different (by at least 100 bp) from that of the undigested fragment to allow easy interpretation of electrophoresis results. We used a 750 bp fragment of the mitochondrial cytochrome *b* gene [*Cytb*, amplified with CP1 (5′-GATGATGAAATTTTGGATC-3′) (Harry et al. 1998) and CB2 (5′-ATTACACCTCCTAAT TTATTAGGAAT-3′) (Jermiin and Crozier 1994)] and a 658 bp fragment of the mitochondrial cytochrome oxidase subunit I gene [COI, amplified with the LepF (5′-ATTC AACCAATCATAAAGATATTGG-3′) and LepR (5′-TAA ACTTCTGGATGTCCAAAAAATCA-3′) primers (Sheffield et al. 2009)]. The restriction enzymes retrieved for the COI fragment did not match our criteria, so we selected two restriction enzymes that digested the *Cytb* fragment.

*Rsa*I was selected because its restriction site is present in the *Cytb* sequences of *B. helichrysi* H1 but not in those of *B. helichrysi* H2. Digestion generates fragments about 300 and 400 bp in length (Fig. 1a; Fig. S1 of the ESM). We then checked that this restriction site was conserved in a batch of 50 *B. helichrysi* H1 sequences and absent from a batch of 50 *B. helichrysi* H2 sequences.

We also used *Bst*XI, as its restriction site is present in the *Cytb* sequences of *B. helichrysi* H2 but not in those of *B. helichrysi* H1. The DNA fragments resulting from digestion are approximately 550 and 150 bp long (Fig. 1b; Fig. S1 of the ESM). We checked that this restriction site was conserved in a batch of 50 *B. helichrysi* H1 sequences and absent from a batch of 50 *B. helichrysi* H2 sequences.

PCR-RFLP test

Sixteen individuals (eight individuals from each lineage identified in our previous study on the basis of six DNA fragments; see also Fig. S1 and Table S2 of the ESM) were subjected to the RFLP test as a control and for the estimation of discrimination error.

A DNA fragment of about 750 bp from the mitochondrial cytochrome *b* gene (*Cytb*) was amplified with the CP1/CB2 primers. All PCRs were performed in a final volume of 25 µl containing 1× reaction buffer (CoralLoad PCR Buffer, Qiagen®, Hilden, Germany), 0.1 mM of each dNTP, 0.7 µM of each primer, 1 U of *Taq* DNA polymerase and 2 µl of DNA extract in an Eppendorf Mastercycler® ep thermal cycler as follows: initial denaturation at 94 °C for 4 min, followed by 30 cycles of 30 s at 94 °C, 1 min at 48 °C and 1 min at 72 °C, and a final elongation phase at 72 °C for 5 min.

We then digested the PCR products with the restriction enzymes *Rsa*I and *Bst*XI separately: we digested 2 µl of the PCR products with 0.2 µl of *Rsa*I enzyme (10 U/µl, Promega, Madison, WI, USA) in 1 µl of 10× buffer C and 6.8 µl of H_2O. The preparation was incubated at 37 °C for 1 h. We then digested 2 µl of the PCR products with 0.2 µl of *Bst*XI (10 U/µl, Fermentas GmbH, St. Leon-Rot, Germany) in 1 µl of 10× buffer 0 and 6.8 µl of H_2O. The preparation was incubated at 55 °C for 1 h.

We added 5 µl of 10× molecular weight ladder to the digestion products in each well, and the mixtures were loaded onto 2 % agarose gels containing 5 % GelGreen™ nucleic acid gel stain (Biotium Inc., Hayward, CA, USA). DNA fragments were separated by electrophoresis in TAE buffer for 20 min at 100 V. They were then examined under blue light (470 nm). Fragment sizes were compared with a 10000 bp size ladder with bands at 100 bp intervals (GeneRuler™ DNA Ladder Mix, Fermentas GmbH, St. Leon-Rot, Germany) (Fig. 1).

The test was then performed on 755 individuals from 271 colonies.

Analyses of ecological data

Each colony was associated with a "class" on the basis of RFLP identification. Class was set to (1) if only H1 individuals were identified in the colony, to (2) if only H2 individuals were identified in the colony, and to (3) for mixed colonies.

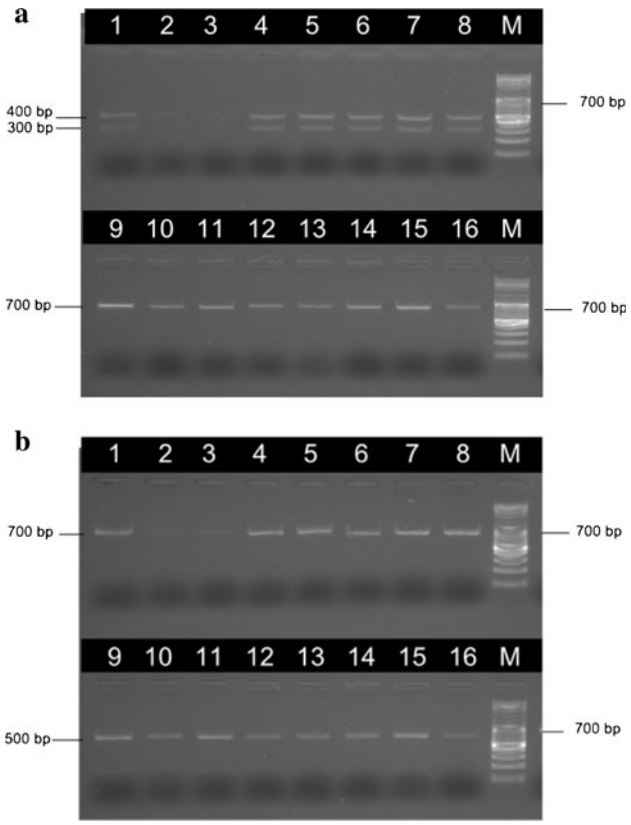


Fig. 1 PCR-RFLP profile on an agarose gel of the *Cytb* amplicon (750 bp) with the CP1/CB2 primers. **a** Digestion with *Rsa*I. *Lanes 1–8*: *B. helichrysi* H1; *lanes 9–16*: *B. helichrysi* H2; *M*: the 10000 bp DNA ladder. **b** Digestion with *Bst*XI. *Lanes 1–8*: *B. helichrysi* H1; *lanes 9–16*: *B. helichrysi* H2; *M*: the 10000 bp DNA ladder

We first tested whether *B. helichrysi* H1 and H2 displayed a disjunction in their herbaceous host-plant ranges. We organised our data into a contingency table with two factors: lineage (two categories: 1 or 2), with the reallocation of mixed colonies to H1 and H2 colony counts; and herbaceous host-plant genus (seven genera). We checked whether the mixed colonies were found on particular host plants by determining whether colony class was dependent on herbaceous host plant. We also organised the data into a second contingency table with two factors: colony class (three categories) and herbaceous host-plant genus (six genera). For each contingency table, we only retained the host-plant categories from which more than five colonies had been collected.

As the data consisted of many small counts, we applied Fisher's exact test to each contingency table to assess the association between lineage or colony class and herbaceous host-plant genus. The matrices used were larger than 2 × 2, so we calculated *p*-values for a Monte Carlo test (Hope 1968) with 2000 replicates. Tests were performed with R software (R Development Core Team 2010).

The geographical distribution of the sampled colonies and their compositions were mapped on worldwide maps with the programs "maptools" (Lewin-Koh et al. 2012) and "raster" (Hijmans and van Etten 2012) found in the R package (Figs. 2, 3; for the collection details for each colony, see Table S1 of the ESM).

Climate descriptors for each sampling site were retrieved from the Worldclim database (Hijmans et al. 2005). These data included variables such as minimum, maximum and mean monthly temperatures and precipitation averaged over the period 1950–2000. We also used 19 global bioclimatic variables derived from monthly temperatures and rainfall values to generate more biologically meaningful variables (see Hijmans et al. 2005 for details). In total, we collected 67 variables. As we were working with variables describing monthly values, we removed the data points corresponding to sampling locations in the Southern Hemisphere (4 of 267) from subsequent analyses to prevent confusion due to seasonal effects (Fig. 3).

We carried out a standard principal component analysis (PCA) (Manly 1994), together with a between-class test, with the R package "ade4" (Dray and Dufour 2007). The between-class test was used to determine whether samples grouped as a function of colony class (i.e. 1, 2 or 3; see above) differed. We simulated 999 between-class analyses (BCA, i.e. PCA constrained to maximise the divergence between the centres of gravity of the classes) on randomised data. For each sample, the values of climatic variable combinations were preserved for each data point, but class attribution was randomised. The real and randomised data were then compared.

We compared the mean values of each climate variable for each class in a one-way ANOVA followed by Tukey's HSD (honestly significant difference) test. These tests were performed with R software.

Results

PCR-RFLP discrimination between *B. helichrysi* H1 and H2

*Rsa*I digestion of the *Cytb* gene fragment amplified from individuals belonging to *B. helichrysi* H1 resulted in two DNA fragments of the expected sizes (8/8 of the PCR products tested), whereas a single fragment the size of the PCR product was obtained for individuals belonging to *B. helichrysi* H2 (8/8 of the PCR products tested) (Fig. 1a). *Bst*XI digestion of the *Cytb* gene fragment yielded a single DNA fragment the size of the PCR product for individuals belonging to the H1 lineage (8/8 PCR products tested), and a slightly shorter fragment (550 bp) for individuals belonging to H2 (8/8 of the PCR products tested; the 150 bp fragments are masked by the primer dimers) (Fig. 1b). *Rsa*I and *Bst*XI gave consistent results (Fig. 1).

Aphid identification

We identified 755 individuals from the 271 colonies: 151 colonies consisted of H1 individuals only, 87 colonies consisted of H2 individuals only, and 33 colonies consisted of a mixture of H1 and H2 individuals. Colony composition and host-plant associations are shown in Table 1, and geographical distributions are shown in Figs. 2 and 3.

Do *B. helichrysi* H1 and H2 have different host-plant ranges?

No *B. helichrysi* H2 individuals were found on plum trees (*Prunus domestica*, *Prunus spinosa*). However, these individuals were found to coexist with H1 individuals on many herbaceous plants (Table 1). Two plant genera from

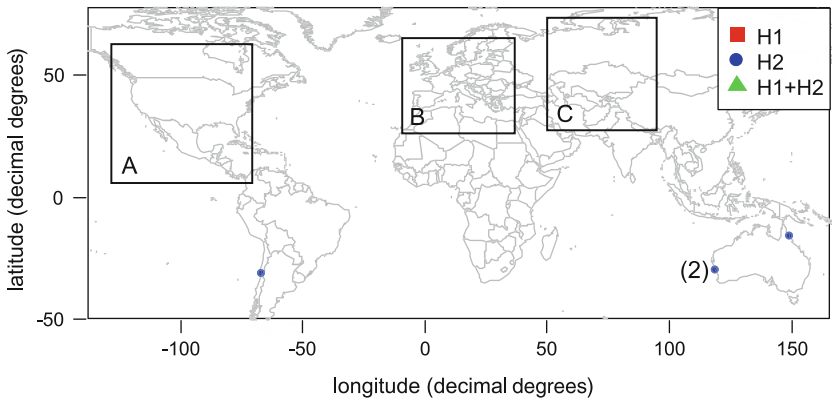
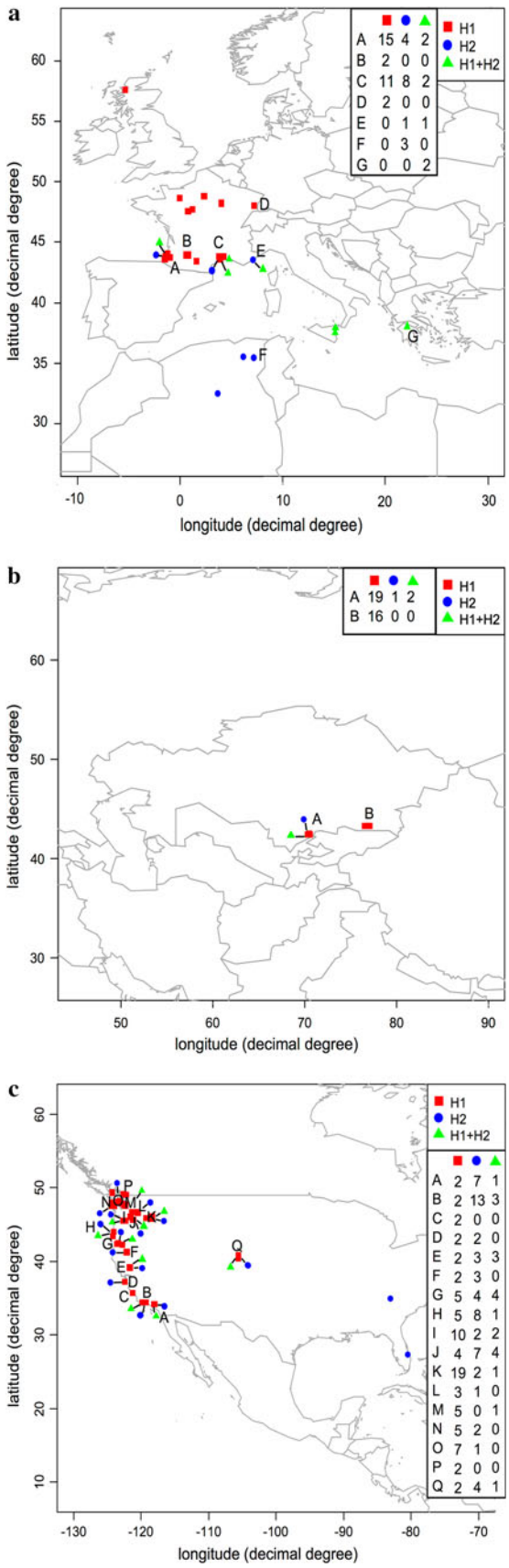


Fig. 2 World map showing the regions sampled. *Large squares* indicate the regions used for the climatic survey in the Northern Hemisphere. *A* Europe, *B* Kazakhstan, *C* USA. In the Southern Hemisphere, colonies in Australia and Chile were sampled. *Squares* indicate colonies of *B. helichrysi* H1; *circles* show colonies of *B. helichrysi* H2; *triangles* correspond to mixed colonies of *B. helichrysi* H1 and H2. *The number in brackets* indicates the number of colonies sampled (when there were several)



◀ **Fig. 3** Map of *B. helichrysi* colonies in the Northern Hemisphere. **a** *B. helichrysi* colonies in Europe. **b** *B. helichrysi* colonies in Kazakhstan. **c** *B. helichrysi* colonies in North America. *Squares* show colonies of *B. helichrysi* H1; *circles* indicate colonies of *B. helichrysi* H2; *triangles* depict mixed colonies of *B. helichrysi* H1 and H2. Sites at which more than one colony were sampled are indicated by a *letter*, and colony composition data are given in the *box on the right*

the Asteraceae, *Achillea* and *Conyza*, were repeatedly identified as host plants for both H1 and H2, in pure and mixed colonies. However, Fisher's exact tests suggested that the presence of the two lineages, H1 and H2, and colony class were dependent on the associated host-plant genera (p-values of 0.01749 and 0.01649, respectively). The significance of this test resulted principally from the pattern of occurrence of *B. helichrysi* H1 and H2 on two genera (*Helianthus* and *Matricaria*). *Brachycaudus helichrysi* H2 was never found on *Matricaria* (five colonies in Kazakhstan and one colony in France). When we analysed the occurrence of the two lineages on one of the principal cultivated host plants of *B. helichrysi*, sunflower (*Helianthus annus*), we found that, for the samples ($n = 7$ colonies) included in this study, no H2 colonies were present on cultivated sunflower fields in France. One H2 colony was found on sunflowers in Australia.

Both lineages appeared to be highly polyphagous: *B. helichrysi* H1 occurred on at least three *Prunus* species and 21 herbaceous plant genera, and *B. helichrysi* H2 occurred on 32 herbaceous plant genera.

Do *B. helichrysi* H1 and H2 occur in locations with different climatic characteristics?

Both H1 and H2 were found on all continents and at many sites. However, H2 seemed to be largely absent from regions with a continental climate, with the exception of three individuals from southern Kazakhstan, Central Asia and five individuals from Colorado, USA (Figs. 2, 3).

PCA of the climate variables yielded a first principal component accounting for 41 % of the total inertia, which accounted principally for temperature patterns, as revealed by the large contributions of mean annual temperature and autumn and spring monthly temperatures (maximum, minimum and mean) (Fig. 4; Table S3 of the ESM). Interestingly, this principal component corresponded to a clear segregation between samples from class 1 (i.e. featuring H1 only) and those from classes 2 and 3 (showing only H2 and mixed colonies, respectively) (Fig. 5). These differences were statistically significant ($p < 0.001$) in the between-class test performed with $n = 1000$ randomizations. The first axis of the PCA revealed that *B. helichrysi* H2 was absent from the coldest regions, particularly those with cold autumns and springs.

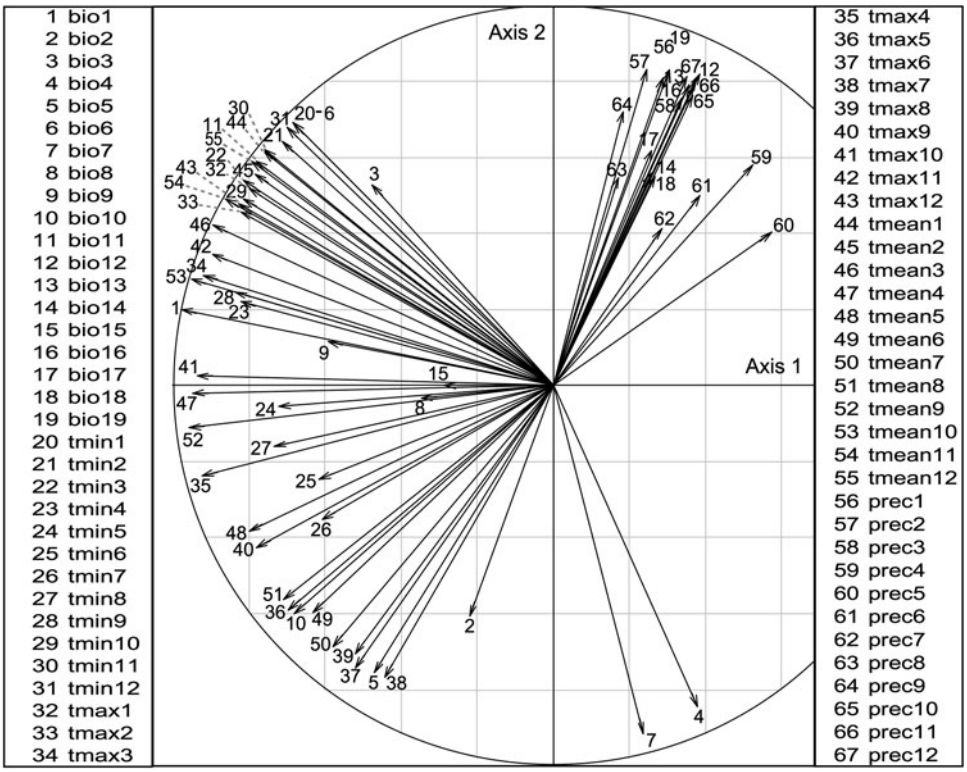


Fig. 4 Correlation circle showing the contributions of the climatic variables to the first two axes of the PCA. Each climatic variable corresponds to a number according to the correspondence table on either side of the circle. *bio1* annual mean temperature, *bio2* mean diurnal range [mean monthly temperature (max temp − min temp)], *bio3* isothermality (bio2/bio7) (×100), *bio4* temperature seasonality (SD×100), *bio5* maximum temperature of warmest month, *bio6* minimum temperature of coldest month, *bio7* temperature annual range (bio5–bio6), *bio8* mean temperature of wettest quarter, *bio9* mean temperature of driest quarter, *bio10* mean temperature of warmest quarter, *bio11* mean temperature of coldest quarter, *bio12* annual precipitation, *bio13* precipitation of wettest month, *bio14* precipitation in driest month, *bio15* precipitation seasonality (coefficient of variation), *bio16* precipitation in wettest quarter, *bio17* precipitation in driest quarter, *bio18* precipitation in warmest quarter, *bio19* precipitation in coldest quarter, *tmin1 to tmin12* minimum temperature in January to minimum temperature in December in degrees Celsius × 10, *tmax1 to tmax12* maximum temperature in January to maximum temperature in December in degrees Celsius × 10, *tmean1 to tmean12* mean temperature in January to mean temperature in December in degrees Celsius × 10, *prec1 to prec12* mean precipitation in January to mean precipitation in December in millimetres

Axis 2 accounted for 32 % of the total inertia and revealed differences in precipitation-related variables such as mean annual precipitation, winter precipitation, temperature seasonality and the annual temperature range (Fig. 4; Table S3 of the ESM). Axis 2 mostly expressed differences between samples from class 2 and those from classes 1 and 3 (Fig. 5). The second axis of the PCA revealed that *B. helichrysi* H1 was underrepresented in regions with low annual temperature variation and high levels of precipitation.

The one-way ANOVA results were consistent with the PCA results. The factor "class" had a significant effect on the mean values of each climate variable, particularly for variables related to temperature. All of the tests for which significant results were obtained involved comparisons with pure H1 colonies. Differences between H2 colonies and mixed colonies were not significant, regardless of the variable considered (the details are given in Table S4 of the ESM). These findings suggest that H1 colonies may occur in climatic conditions incompatible with the occurrence of *B. helichrysi* H2 (in pure or mixed colonies).

Discussion

This study demonstrates the efficacy of a mtDNA PCR-RFLP test for distinguishing between the two lineages found within *B. helichrysi*. In a worldwide sample, we identified 433 individuals belonging to the taxon that we named *B. helichrysi* H1, and 286 individuals belonging to the taxon that we named *B. helichrysi* H2. We carried out control digestions for 16 individuals: no discordance with the results of the *Rsa*I and *Bst*XI identification method was observed. Our PCR-RFLP identification test is therefore an effective tool for rapid discrimination between *B. helichrysi* H1 and H2. This test is based on four easy steps:

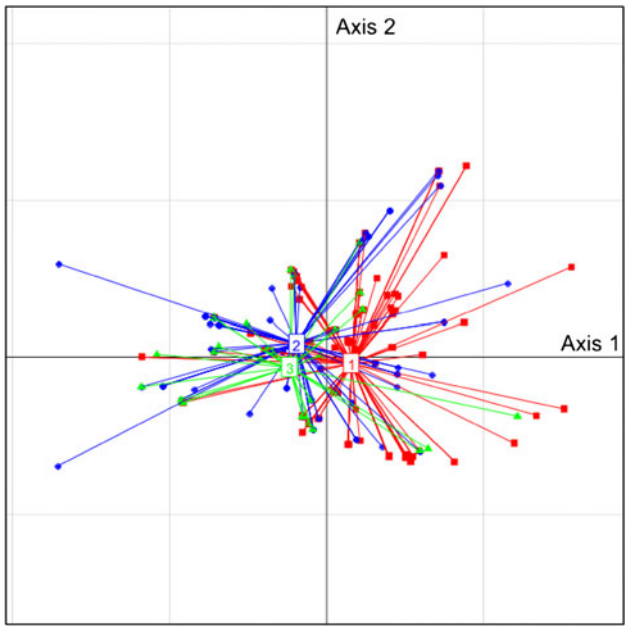


Fig. 5 Climatic spaces for each colony class on the first two axes. *1 B. helichrysi* H1 colonies, *2 B. helichrysi* H2 colonies, *3* mixed colonies of *B. helichrysi* H1 and H2

DNA extraction, partial *Cytb* amplification, digestion and electrophoresis. All these processes are routinely carried out in molecular biology laboratories.

Our survey of a worldwide sample demonstrated that the two lineages occurred together at almost all the sites studied (Figs. 2, 3). Furthermore, they were frequently found together on the same herbaceous plants, in mixed colonies of indistinguishable *B. helichrysi* H1 and H2 individuals.

We confirm here that, as suggested by Piffaretti et al. (2012), one of the lineages within *B. helichrysi*—*B. helichrysi* H2—is never found on plum trees. In addition, some H2 colonies were found late in autumn and early in spring on herbaceous plants. These observations suggest that *B. helichrysi* H2 never occurs on woody hosts. This lack of occurrence of H2 on woody host plants, which generally serve as primary hosts in the life cycle of *B. helichrysi*, suggests that H2 is either monoecious on herbaceous hosts (i.e. *B. helichrysi* H2 does not shift onto a woody host plant for the sexual phase of its life cycle) or anholocyclic (i.e. there has been a transition to obligate parthenogenesis in this lineage, with loss of the sexual generation).

Principal component analyses of the climatic variables associated with the sites from which samples were taken showed that (1) *B. helichrysi* H2 was absent from the coldest regions sampled, particularly those with cold autumns and springs, and that (2) *B. helichrysi* H1 was underrepresented in regions with low annual temperature variations and high levels of precipitation. These results are consistent with the hypothesis that *B. helichrysi* H2 is an asexual aphid lineage (Rispe et al. 1998; Vorburger et al. 2003). Indeed, aphid colonies cannot withstand freezing conditions. Only eggs (the product of sexual reproduction) can survive harsh winter conditions (Strathdee et al. 1995), and anholocyclic lineages cannot persist from year to year in regions with harsh winters. The regions from which H2 was absent were those with harsh dry winters. We sampled many H2 individuals from the northern part of the western coast of the USA, where winter temperatures fall sufficiently for snow to occur, but this snow does not preclude the occurrence of parthenogenetic lineages, as it may protect the aphid colonies. No such protection can occur in harsh, dry winter conditions. Nevertheless, we found a small number of H2 individuals in Kazakhstan and Colorado (USA), where the climatic conditions preclude the maintenance of a purely anholocyclic aphid lineage. These colonies may have settled on their host plants after a dispersion event (by wind or by human activities) from an area in which climatic conditions were more favourable to a parthenogenetic *B. helichrysi* H2. Indeed, four of the five colonies from Colorado containing H2 were found in cities, on ornamental flowers (botanical gardens, house fronts). The presence of anholocyclic H2 in Colorado may easily have resulted from the human transport of ornamental flowers produced in greenhouses. Alternatively, the H2 populations found in Kazakhstan and Colorado may be holocyclic. If this is the case, we can draw no firm conclusions about the type of life cycle they display (heteroecious or monoecious on herbaceous host plants). However, if they are heteroecious, the primary host of these populations has yet to be identified, as we have sampled *Brachycaudus* species extensively from various *Prunus* species in previous studies (Jousselin et al. 2010) and we have never found *B. helichrysi* H2.

There may be several explanations for the apparent underrepresentation of pure H1 colonies in regions with low levels of temperature variation and high levels of precipitation. Firstly, *Prunus* may be less well adapted in some of these regions, providing *B. helichrysi* H1 with fewer opportunities to find its primary host plant, resulting in a lower abundance of this aphid (Vorburger et al. 2003). Alternatively, the two lineages may be in competition in these regions, and H2 may outcompete H1 due to higher levels of fecundity linked to its asexuality (Rispe et al. 1998; Nespolo et al. 2009).

Hypotheses about the life cycle of H2 should now be tested explicitly in population genetic studies using microsatellite markers, for example, and aiming to identify the clonal lineages that form the signature of an anholocyclic species (Delmotte et al. 2002; Wilson et al. 2003; Figueroa et al. 2005; Halkett et al. 2005). Attempts could also be made to induce sexual phases experimentally in

B. helichrysi H2, as a means of determining its life cycle (Lees 1959; Voegtlin and Halbert 1998).

In addition, experiments could be carried out to assess the competition between the two lineages (Rochat et al. 1999; Brevault et al. 2011). The discrimination test that we have developed would provide a useful tool for such studies.

Our sampling scheme was designed to maximise the diversity of the host-plant genera sampled, in order to provide a general overview of the feeding behaviour of *B. helichrysi* H1 and H2. We did not conduct intensive surveys of a few host-plant taxa to investigate the host-plant preferences of H1 and H2. Many plant genera were thus sampled only once, and we cannot draw any firm conclusions as to whether there are real disjunctions in the host-plant ranges of H1 and H2 (i.e. whether there are some plants on which H1 can feed but H2 cannot and vice versa). However, this initial survey revealed that *B. helichrysi* H1 and H2 were both highly polyphagous and that they were frequently found together on diverse herbaceous host plants, including ornamental plants such as *Chrysanthemum* and *Achilleae* that are commercially important. Nevertheless, our data revealed some differences in host-plant distribution between H1 and H2: only *B. helichrysi* H1 was found on *Matricaria*. However, five of the six samples included in our study were collected in Kazakhstan, where *B. helichrysi* H2 was found to be very rare. The observed distribution of the two aphid lineages on this host-plant genus may therefore be due to geographical biases in sampling. We did not focus specifically on cultivated plants, but we noticed that H2 seemed to be absent from sunflower fields in France (in the north or south). Furthermore, our findings confirmed that only H1 was a potential pest of plum orchards. We have extensively sampled diverse *Brachycaudus* species on other cultivated *Prunus* species that were previously reported to serve as occasional hosts for *B. helichrysi* (Blackman and Eastop 2000) (i.e. *Prunus dulcis*, *P. armeniaca*, *P. cerasus*), and on *P. tianshanica*, a wild *Prunus* species native to Central Asia (Jousselin et al. 2010). In none of these samplings have we found either H1 or H2, suggesting that H1 is a threat only to plums in the sampled regions. It seems to occur more rarely on other *Prunus* species. In any case, additional studies of host-plant preference (empirical and experimental) are required to improve our understanding of the host-plant association patterns of these two lineages, and their associations with cultivated host plants in particular.

In conclusion, in a worldwide sample, the DNA-based discrimination of *B. helichrysi* H1 and H2 showed that individuals of both clades frequently coexist on secondary herbaceous host plants. This highlights the need for methods of discriminating between lineages before conducting ecological, epidemiologic or population genetic studies of the cosmopolitan pest *B. helichrysi*. The PCR-RFLP test developed here provides such a tool, and it can be carried out rapidly and easily by almost all molecular laboratories.

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