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Characterisation and cross-amplification of polymorphic microsatellite loci in ant-associated root-aphids

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Abstract Twenty-six polymorphic microsatellite loci were developed for four species of ant-associated root-aphids: Geoica utricularia, Forda marginata, Tetraneura ulmi and Anoecia corni. We found up to 9 alleles per locus, with an average of 4.8. We also report polymorphic cross-amplification of eleven of these markers between different pairs of study species. Furthermore, we tested previously published aphid microsatellites and found one locus developed for Pemphigus bursarius to be polymorphic in G. utricularia. These microsatellite markers will be useful to study the population structure of aphids associated with the ant Lasius flavus and possibly other ants. Such studies are relevant because: 1. L. flavus mounds and their associated flora and fauna are often key components in protected temperate grasslands, and 2. L. flavus and its diverse community of root-aphids provide an interesting model system for studying the long-term stability of mutualistic interactions.

Keywords Microsatellites · Root-aphids · Mutualism · Aphidoidea (Hemiptera) · Pemphigidae · Anoeciidae

Mutualistic interactions between species are widespread and play key roles in ecosystem stability and diversity (Stachowicz 2001; Bastolla et al. 2009). In Northwest Europe, the yellow meadow ant Lasius flavus keeps up to fourteen species of mutualistic root-aphids in its nests (Pontin 1978; Heie 1980; Godske 1991). The ants actively tend the aphids, which provide them with honeydew (Pontin 1978). The nest mounds are markers of high grassland biodiversity and long-term habitat stability (Dean et al. 1997; Blomqvist et al. 2000; Lenoir 2009). However, despite the decline of European temperate grasslands in recent decades and the associated losses in plant and invertebrate biodiversity (WallisDeVries et al. 2002), neither the sociobiology of the ants (but see Boomsma et al. 1993) nor the biology of the root-aphids (Pontin 1978; Godske 1991, 1992) have been extensively studied. To facilitate molecular ecological approaches in the study of this mutualism, we developed DNA microsatellite markers for the four commonest species: Forda marginata, Tetraneura ulmi, Geoica utricularia and Anoecia corni.

Samples for genomic library construction for Forda marginata, Tetraneura ulmi, and Anoecia corni were collected in 2007 from an ant-nest on the Dutch island of Schiermonnikoog (53°29′03.5″N; 6°13′46.1″E) whereas Geoica utricularia was collected near Dejret, Denmark (56°12′54.2″N; 10°24′48.2″E). All samples for molecular analysis were preserved in 96% ethanol.

Genomic DNA was extracted using the QIAGEN DNeasy Blood & Tissue kit and enriched for poly-CA and poly-CT microsatellite containing fragments using the protocol by Rütten et al. (2001). We designed PCR primers for the flanking regions of repetitive motifs using the web-based software Primer 3 (Rozen et al. 2000).

Primers were tested on Schiermonnikoog samples collected in 2007, 2008 and 2009 and on samples collected...
| Locus | Species               | Primer sequence (5′–3′) (F: forward, R: reverse) | Repeat motif | Size range (bp) | N | Ne | Ho | Ta (°C) | Nr. of cycles x | Primer concentration (µM) | Multiplex mix | Genbank accession number |
|-------|-----------------------|-------------------------------------------------|--------------|-----------------|---|----|----|--------|-----------------|-----------------------------|-----------------|--------------------------|
| Gu1   | Geoica utricularia    | F: ATCAAACGAACGAAACCGAAT R: GCGAAAGTTATGGCCTTTG | (GT)₈       | 113–118         | 5  | 4  | 0.740 | 1.000 | 50  | 40  | 0.35 | Gu-3 | HM582813                |
| Gu2   | Geoica utricularia    | F: CGCGATTAGATTCGGGAAAT G: GCGAAAGTTATGGCCTTTG | (GT)₁₁      | 158–177         | 227 | 5  | 0.613 | 0.361 | 50  | 40  | 0.15 | Gu-2 | HM582814                |
| Gu3   | Geoica utricularia    | F: TATCTGCGGGACACGACAT R: CGGGCTATACCCGATACACT | (TA)₉       | 192–208         | 169 | 7  | 0.665 | 1.000 | 50  | 40  | 0.15 | Gu-1 | HM582815                |
| Gu4   | Geoica utricularia    | F: CTGCTGCTGTCGAGACTTA R: GCAGATTCACGTTAGCTTGA | (TG)₆ C (AT)₁₂ | 206–222         | 8  | 4  | 0.602 | 0.125 | 50  | 35  | 0.35 | Gu-3 | HM582816                |
| Gu5   | Geoica utricularia    | F: CACAGGACGGGATACTTAATAG R: AACTTTTCGGGACATCCTTGA | (GT)₁₅      | 164–214         | 214 | 6  | 0.569 | 0.145 | 50  | 40  | 0.15 | Gu-2 | HM582817                |
| Gu6   | Geoica utricularia    | F: ATCAAACGGTGGCATGTA R: CAATATCTCATCTGCAGCAA | (TG)₁₃ CG (GT)₈ | 151–200         | 199 | 7  | 0.539 | 0.337 | 50  | 40  | 0.15 | Gu-2 | HM582818                |
| Gu7   | Geoica utricularia    | F: GITAAGGAAAATGCTTACGCTTACTGGC R: CATATAAAATGATCCGTTAGGC | (CA)₉ TA (CA)₉ | 87–103         | 13  | 4  | 0.698 | 0.000 | 50  | 40  | 0.35 | Gu-3 | HM582819                |
| Gu8   | Geoica utricularia    | F: TATAACGTCGCGACAGAT R: GTTCGTTGCTCGTGCATCTT | (AC)₁₀      | 233–237         | 199 | 3  | 0.479 | 0.060 | 50  | 40  | 0.15 | Gu-1 | HM582820                |
| Gu9   | Geoica utricularia    | F: CCGCGGATATGAAAAATGTA R: CTCGCTGTGTTGTGACACCTTT | (CA)₁₃      | 223–250         | 184 | 8  | 0.800 | 0.799 | 50  | 40  | 0.15 | Gu-1 | HM582821                |
| Gu10  | Geoica utricularia    | F: CGCGCTAAAGAAGGTTTCA R: TTACGTTAAAACGACGAGGTAA | (GT)₁₉      | 228–261         | 14  | 8  | 0.763 | 0.786 | 50  | 40  | 0.35 | Gu-1 | HM582822                |
| Gu11  | Geoica utricularia    | F: CGGTACCGGTTAAGGGCTTTA R: AATCCTGGAGTGCTCGATCGG | (CA)₁₁      | 145–153         | 223 | 6  | 0.729 | 0.677 | 50  | 40  | 0.15 | Gu-2 | HM582823                |
| Gu12  | Geoica utricularia    | F: GAGCCTAGCTGGCTTTAG R: CGGGTTTATTTAATGGCTCAGA | (GT)₁₂ GC (GT)₂₅ A (GT)₄ | 106–138 | 10 | 3  | 0.460 | 0.000 | 60  | 45  | 0.15 | HM582824                |
| Gu13  | Geoica utricularia    | F: TCGCCGGACTATTTTACA R: AGTGACGTCGCGGGAGAAAT | (CAG)₁₃ (N)₂₁ (TC)₁₀ | 202–218     | 188 | 7  | 0.754 | 1.000 | 50  | 40  | 0.15 | Gu-1 | HM582825                |
| Gu15  | Geoica utricularia    | F: TTTTTACGGGCTAACCCTATTTT R: CCACAGGATCCACCAACTTTT | (GA)₁₅ (A₁₄ (GA)₃ (A)₉ | 165–167     | 10 | 2  | 0.180 | 0.200 | 50  | 40  | 0.25 | HM582826                |
| Fm1   | Forda marginata       | F: CCTCCAATTACGTTACCAAC R: GAGAACGTTGCAACGCGGATA | (TG)₂₂ CG (TG)₈ | 182–259       | 154 | 9  | 0.458 | 0.253 | 53  | 37  | 0.15 | HM582827                |
| Fm3   | Forda marginata       | F: TCTGATTATTCGGACACCTCA R: CGGGCTGTATCTACTTTA | (AT)₁₀      | 225–349        | 138 | 6  | 0.494 | 0.246 | 50  | 40  | 0.15 | HM582828                |
| Fm4   | Forda marginata       | F: CATTACGGTGTAGTGTAAATATAGTTTT R: TGTTTTAACACGACGTTCCTT | (AC)₁₄      | 178–200       | 162 | 7  | 0.465 | 0.167 | 50  | 35  | 0.15 | HM582829                |
| Locus | Species        | Primer sequence (5'–3')                        | Repeat motif                                    | Size range (bp) | N  | N<sub>a</sub> | H<sub>E</sub> | H<sub>O</sub> | T<sub>a</sub> (°C) | Nr. of cycles | Primer concentration (μM) | Genbank accession number |
|-------|----------------|-----------------------------------------------|------------------------------------------------|----------------|----|--------------|-------------|-------------|------------------|---------------|--------------------------|------------------------|
| Fm6   | Forda marginata | F: TCACCTCGCTAGCGGTTCCTTC (T)<sub>11</sub> ATGA (T)<sub>23</sub> | (T)<sub>11</sub> ATGA (T)<sub>23</sub> | 250–280        | 125| 4            | 0.709      | 0.920      | 50               | 45            | 0.15                     | HM582830               |
|       |                 | R: GTGGCCGTAGCATGTCACTA                       |                                                |                |    |              |             |            |                  |               |                          |                        |
| Tu1   | Tetraneura ulmi | F: CGGGTGCCTGGGTACCTTAT (GT)<sub>3</sub> GAT(AG)<sub>5</sub> T- (GA)<sub>10</sub> (A)<sub>6</sub> (N)<sub>9</sub>(T)<sub>17</sub> | (GT)<sub>3</sub> GAT(AG)<sub>5</sub> T- (GA)<sub>10</sub> (A)<sub>6</sub> (N)<sub>9</sub>(T)<sub>17</sub> | 218–241        | 89 | 2            | 0.164      | 0.000      | 50               | 35            | 0.25                     | HM582831               |
|       |                 | R: ATACGTGACCTGGCTACCTACTA                    |                                                |                |    |              |             |            |                  |               |                          |                        |
| Tu2   | Tetraneura ulmi | F: TCCGACCTACCTTAAACACAAA (TA)<sub>7</sub> (TG)<sub>8</sub> | (TA)<sub>7</sub> (TG)<sub>8</sub> | 157–159        | 60 | 2            | 0.180      | 0.000      | 50               | 40            | 0.25                     | Tu-1 HM582832           |
|       |                 | R: ATGCACCCCCCTGCCACTATC                      |                                                |                |    |              |             |            |                  |               |                          |                        |
| Tu3   | Tetraneura ulmi | F: CGCCGTAAATAATAAATAACCAACA (A)<sub>11</sub> (AT)<sub>6</sub> (TA)<sub>2</sub> (C)<sub>3</sub> (GT)<sub>9</sub> | (A)<sub>11</sub> (AT)<sub>6</sub> (TA)<sub>2</sub> (C)<sub>3</sub> (GT)<sub>9</sub> | 234–264        | 89 | 5            | 0.702      | 0.921      | 50               | 35            | 0.25                     | HM582833               |
|       |                 | R: CACGAGCCTAGGATAAGGAAA                      |                                                |                |    |              |             |            |                  |               |                          |                        |
| Tu4   | Tetraneura ulmi | F: TTATTCGCAACACACCTTGG (GT)<sub>26</sub> G (GT)<sub>3</sub> | (GT)<sub>26</sub> G (GT)<sub>3</sub> | 182–203        | 94 | 6            | 0.636      | 0.904      | 50               | 40            | 0.25                     | Tu-1 HM582834           |
|       |                 | R: ACGGACACGAGAGAATACG                        |                                                |                |    |              |             |            |                  |               |                          |                        |
| Tu10  | Tetraneura ulmi | F: AGTATACGGCTCTGCAAC (TAA)<sub>3</sub> TGA (TAA)<sub>7</sub> | (TAA)<sub>3</sub> TGA (TAA)<sub>7</sub> | 233–248        | 87 | 3            | 0.226      | 0.253      | 50               | 40            | 0.25                     | HM582835               |
|       |                 | R: GGAGACATTCCCAGCTTAT                        |                                                |                |    |              |             |            |                  |               |                          |                        |
| Tu11  | Tetraneura ulmi | F: CGGAGAGGGCTATTGTTT (GT)<sub>9</sub> (TA)<sub>5</sub> | (GT)<sub>9</sub> (TA)<sub>5</sub> | 194–200        | 89 | 4            | 0.396      | 0.393      | 50               | 35            | 0.25                     | HM582836               |
|       |                 | R: CGTGGCGCGTGAATGAT                        |                                                |                |    |              |             |            |                  |               |                          |                        |
| Ac6   | Anoecia corni  | F: CGAGGCATATCTAAAGTGTTAGA (AT)<sub>3</sub> G (TA)<sub>9</sub> C (AT)<sub>2</sub> | (AT)<sub>3</sub> G (TA)<sub>9</sub> C (AT)<sub>2</sub> | 148–164        | 6  | 2            | –          | –          | 45               | 45            | 0.25                     | HM582837               |
|       |                 | R: CAGCATTTTACAGGAATGCA                 |                                                |                |    |              |             |            |                  |               |                          |                        |
| Ac8   | Anoecia corni  | F: AATTAATATTTGCGGCCGTGC (ATT)<sub>10</sub> | (ATT)<sub>10</sub> | 160 | 4  | 1            | –          | –          | 45               | 45            | 0.25                     | HM582838               |
|       |                 | R: CGCGTGAAGCAAATAATATC                  |                                                |                |    |              |             |            |                  |               |                          |                        |

N number of tested samples, N<sub>a</sub> number of alleles, H<sub>E</sub> expected heterozygosity, H<sub>O</sub> observed heterozygosity, T<sub>a</sub> annealing temperature
near Dejret in 2007 (Anoecia spp.), DNA for microsatellite screening was extracted using 200 µl 20%-Chelex® 100 resin (Fluka) (Walsh et al. 1991). PCR-cocktails had a total volume of 10 µl, consisting of 0.8 mM dNTPs, 2 mM MgCl₂, 1× PCR buffer, 0.25 U AmpliTaq Gold® DNA Polymerase (Applied Biosystems), 1 µl of DNA template and a varying concentration of primers (Table 1). Several primer pairs were multiplexed in PCR (Table 1). The amplification conditions were 95°C for 5 min, x number of cycles of 95°C for 30 s, Tₐ for 30 s and 72°C for 30 s (1 min for Gu3, Gu8, Gu9, Gu10 and Gu13) and a final extension of 15 min at 72°C. The respective x and Tₐ for each primer are listed in Tables 1 and 2.

Amplified fluorescent labeled PCR-products were run on an ABI-PRISM 3130XL (Applied Biosystems) sequencer and chromatograms were analyzed in Genemapper an ABI-PRISM 3130XL (Applied Biosystems). The respective x and Tₐ for each primer are listed in Tables 1 and 2.

Cross-amplification was tested for all markers except Gu12 and Fm5 (Table 2), yielding eleven markers that amplified in one or more additional species. Moreover, most markers used (species specific and cross-amplified) for Forda marginata were also suitable for the sibling species Forda formicaria. The loci Fm3, Fm4, Fm6 and Gu13 proved to be diagnostic for distinguishing between Forda marginata and Forda formicaria (Table 2). Three markers from Pemphigus bursarius (Pb02 (Miller et al. 2000)) and P. sypothecae (97PS12 and 98PS8 (Johnson et al. 2000)) were tested for cross-amplification in our focal species, but only Pb02 reliably cross-amplified in Geoica utricularia (Table 2).

Although we enriched specifically for (CA)ₙ and (CT)ₙ repeats, the aphid DNA appeared to be especially AT-rich, including repeats that were suitable for microsatellite

### Table 2 Cross-amplifications of microsatellite markers in different species of ant-associated root-aphids

| Locus | Cross-amplified species | Size range (bp) | N | Nₐ | Hₑ | Hₒ | Tₐ (°C) | Nr. of cycles | Primer concentration (µM) | Genbank accession number |
|-------|-------------------------|----------------|---|----|----|----|--------|---------------|---------------------------|--------------------------|
| Gu6   | Forda marginata         | 151–176        | 159 | 5  | 0 681 | 0 672 | 49   | 40           | 0 15                      | HM582818                 |
| Gu11  | Forda marginata         | 135–147        | 162 | 6  | 0 489 | 0 234 | 49   | 40           | 0 15                      | HM582823                 |
| Gu13  | Forda marginata         | 143–178        | 159 | 5  | 0 430 | 0 000 | 45   | 45           | 0 15                      | HM582825                 |
| Tu11  | Forda marginata         | –              | 2  | –  | –   | –   | 49   | 40           | 0 15                      | HM582836                 |
| Fm3   | Forda formicaria        | 121            | 18  | 1  | 0 000 | 0 000 | 50   | 40           | 0 15                      | HM582828                 |
| Fm4   | Forda formicaria        | 174–178        | 18  | 3  | 0 495 | 0 777 | 50   | 35           | 0 15                      | HM582829                 |
| Fm6   | Forda formicaria        | 206–291        | 18  | 2  | 0 500 | 1 000 | 50   | 45           | 0 15                      | HM582830                 |
| Gu6   | Forda formicaria        | 151–152        | 17  | 2  | 0 110 | 0 000 | 49   | 40           | 0 15                      | HM582818                 |
| Gu11  | Forda formicaria        | 142–146        | 18  | 3  | 0 439 | 0 277 | 49   | 40           | 0 15                      | HM582823                 |
| Gu13  | Forda formicaria        | 156            | 19  | 1  | 0 000 | 0 000 | 45   | 45           | 0 15                      | HM582825                 |
| Fm1   | Anoecia corni, A. zirnitsi | 110–134    | 7   | 3  | –   | –   | 45   | 45           | 0 25                      | HM582827                 |
| Tu2   | Anoecia corni, A. zirnitsi | 137–148      | 3   | 2  | –   | –   | 45   | 45           | 0 25                      | HM582832                 |
| Tu11  | Anoecia corni, A. zirnitsi | 69–126      | 7   | 5  | –   | –   | 45   | 45           | 0 25                      | HM582836                 |
| Ac 8  | Anoecia zirnitsi, A. major | 130–146      | 2   | 2  | –   | –   | 45   | 45           | 0 25                      | HM582838                 |
| Pb02a | Geoica utricularia      | 118–124        | 8   | 2  | –   | –   | 50   | 40           | 0 20                      | AF267192                 |

N number of tested samples, Nₐ number of alleles, Hₑ expected heterozygosity, Hₒ observed heterozygosity, Tₐ annealing temperature

*a Developed by Miller et al. 2000 for the lettuce root-aphid Pemphigus bursarius
design. This observation is in accordance with earlier findings (Weng et al. 2007).

In conclusion, the 26 newly developed microsatellite markers presented here cover a large proportion of the known root-aphid fauna associated with L. flavus and other ant species (Heie 1980), and will be useful for detailed studies of the ecology and evolution of this mutualistic association.

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