“Forms” of water mites (Acari: Hydrachnidia): intraspecific variation or valid species?

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Keywords
Bayesian analysis, cryptic species, DNA barcoding, GMYC model, principal component analysis, species delimitation.

Abstract
In many groups of organisms, especially in the older literature, it has been common practice to recognize sympatrically occurring phenotypic variants of a species as “forms”. However, what these forms really represent often remains unclear, especially in poorly studied groups. With new algorithms for DNA-based species delimitation, the status of forms can be explicitly tested with molecular data. In this study, we test a number of what is now recognized as valid species of water mites (Hydrachnidia), but have in the past been treated as forms sympatrically occurring with their nominate species. We also test a form without prior taxonomical status, using DNA and morphometrics. The barcoding fragment of COI, nuclear 28S and quantitative analyses of morphological data were used to test whether these taxa merit species status, as suggested by several taxonomists. Our results confirm valid species. Genetic distances between the form and nominate species (Piona dispersa and Piona variabilis, COI 11%), as well as likelihood ratio tests under the general mixed-Yule coalescent model, supported that these are separately evolving lineages as defined by the unified species concept. In addition, they can be diagnosed with morphological characters. The study also reveals that some taxa genetically represent more than one species. We propose that P. dispersa are recognized as valid taxa at the species level. Unionicola minor (which may consist of several species), Piona stjordalensis, P. imminuta s. lat., and P. rotundoides are confirmed as species using this model. The results also imply that future studies of other water mite species complexes are likely to reveal many more genetically and morphologically distinct species.

Introduction
A sound taxonomic foundation is fundamental for all biological sciences from ecology and conservation biology to proteomics and genomics (Wheeler et al. 2004; Wilson 2004). The circumscription and naming of taxa enable the quantification of meaningful units as well as reproducibility within and between scientific studies, the very cornerstone of science. However, species show variable degrees of intraspecific variation, which may be geographically structured, and species delimitation is not always straightforward (Sites and Marshall 2003, 2004). With a vast and complex literature on different species concepts (e.g., Ruse 1969; Nixon and Wheeler 1990; Mayden 1997; Wheeler and Meier 2000), it is encouraging that a consensus view now seems to be emerging, according to which species are seen as separately evolving metapopulation lineages (de Queiroz 2007). Adhering to this “unified species concept” enables more straightforward tests of the validity of species as well as infrasubspecific taxa. Morphological variants labeled as “forms”, “varieties”, or “ecomorphs” have been described in numerous taxa, both in the past and more recently (Snyder and Hansen 1940; Askew 1970; McLean and Kanner 2005; Mateos 2008). However, what these labels really refer to often remain unclear, undefined or, untested with quantitative data. The international code of zoological nomenclature (ICZN 1999) establishes that infrasubspecific names of the type “var.” and “form” are valid as subspecific names only, if described before 1961 and the author did not explicitly intend them to be of infrasubspecific rank. Here, we leave the debate on subspecies aside, because it is only relevant...
for allopatric or parapatric distributions (circular range overlap excepted; Wilson and Brown 1953; Starrett 1958; Wilson 1994). Names that refer to sympatrically occurring phenotypic forms or varieties can be explicitly tested using recent advances in applying molecular data and statistical analyses (Sites and Marshall 2003, 2004; Pons et al. 2006; Fontaneto et al. 2007; Knowles and Carstens 2007; Rosenberg 2007; Rodrigo et al. 2008).

Based on the ideas of the unified species concept, there are multiple relevant lines of evidence of speciation, all of which are found in previous species concepts, but as part of the definition (de Queiroz 2007). Examples include the cessation of gene flow, phenetical distinctiveness, diagnosability, ecological niche differentiation, and reciprocal monophyly and several recent methods have been developed to quantitatively test the evidence in favour of, or against, speciation. The general mixed Yule coalescence method (GMYC) (Pons et al. 2006; Fontaneto et al. 2007) provides a quantitative way of circumscribing species without any prior knowledge using single-locus DNA. The method only delimits reciprocally monophyletic species, hence all recognized species under the GMYC model satisfy at least that nonabsolute, but indicative criterion. Specifically, GMYC combines the coalescent process model for populations with the Yule speciation model for species to find the maximum likelihood threshold solution of an ultrametric gene tree. It separates branches that likely represent separate species from branches that are better modeled as within-species coalescents. Rosenberg (2007) and Rodrigo et al. (2008) developed different tests, but aimed at testing the same null hypothesis: could the observed pattern be derived by chance from a single-panmictic population? In Rosenberg’s (2007) test, the pattern observed is two reciprocally monophyletic cladesth and the sample size of each clade determines the probability of observing the pattern under a single-panmictic population. Rodrigo et al.’s (2008) test instead focus on the branch length ratio of the assumed species ingroup node to the tips and the ingroup node to the immediate ancestral node. This is basically a quantitative measure of the “distinctiveness of clusters” often referred to visually on NJ-trees in DNA barcoding studies (Hogg and Hebert 2004; Koch 2010), but is here tested against the probability of seeing the observed ratio under a single-panmictic population. Rejecting the null under both tests imply reduced or absent gene flow between populations and if sympatically occurring, evidence of species.

Preferably, the circumscription of separately evolving metapopulation lineages should be based on multiple lines of evidence (de Queiroz 2007), why we use quantitative morphological, nuclear, and mitochondrial data for species delimitation. This integrative taxonomic methodology is a powerful tool in resolving taxonomical problems and will in this study on water mites (Hydrachnidia) be applied to already known species (Unionicol a minor (Soar, 1900), Piona stjordalensis (Thor 1897) [=curvipes stjordalensis], P. imminuta s. lat. (Piersig 1897), P. rotundoides (Thor 1897)) (Biesiada 1977; Davids and Kouwets 1987; Gerecke 2011), which have in the past been regarded as intraspecific forms to a sympatrically occurring nominate species (U. minor in relation to U. crassipes (Müller, 1776), P. stjordalensis and P. imminuta s. lat. both in relation to Piona coccinea (Koch, 1836), P. rotundoides in relation to P. pusilla (Neuman, 1875)) (Viets 1982, 1987). We also test a form presently without accepted species status, synonymous to the nominate species (P. dispersa Sokolow 1926 in relation to P. variabilis (Koch, 1836)) (Böttger and Ullrich 1974; Gerecke 2011). They can all be found in freshwater habitats in Europe and have a chaotic taxonomical history (Lundblad 1962; Viets 1987; European Water Mite Research 2009). For example, the following taxon names are also involved in the same species complexes, but of debated taxonomic status: U. crassipes f. octopora Maglio, 1924, U. crassipes f. reducta Lundblad 1924; U. laurantiana Crowell and Davids 1979; U. nearticca Crowell and Davids 1979; P. coccinea f. confertipora Walter, 1927, P. coccinea f. hankensis Sokolow, 1931, Piona coccinea f. recurva Lundblad 1920; P. coccinea f. gracidipalpis Lundblad 1924; the colour variant P. coccinea f. caesia Thor, 1925; P. pusilla f. disjuncta Viets, 1930, the smaller variant P. pusilla f. tenera Lundblad 1925, P. pusilla f. disparilis (Koenike, 1895), P. pusilla f. acutipes Viets, 1954, P. pusilla f. rotundiformes Lundblad, 1938, P. africana Viets, 1940, and P. sudamericana Viets, 1910) (Lundblad 1920, 1924, 1962; Viets 1982, 1987). Within the species-rich Hydrachnidia, variable sympatrically occurring intraspecific populations have in the past frequently been called forms (Lundblad 1962; Viets 1982, 1987). Despite the large extent of water mite forms currently still unsolved, for example, the problematic P. nodata group, there are few molecular studies on cryptic water mite species (but see Edwards and Dimock 1997; Bohonak 1999; Edwards et al. 1999; Bohonak et al. 2004; Ernsting et al. 2006, 2008). This is the first time the status of Unionicol a minor, Piona stjordalensis, P. imminuta s. lat., P. rotundoides, and P. dispersa are tested using molecular data. We apply statistical phylogenetic, species delimitation, and population genetic methods to explicitly test diagnosability, gene flow, monophyly, and phenetic distinctiveness.

Material and methods

Biological material sampled

All included taxa were collected in the years 2007–2008 in Sweden. Specimens identified and extracted were
Unionicola crassipes (14♀), U. minor (12♀), Piona cocci-nea (10♂), P. stjordalensis (6♂, 4♀), P. imminuta s. lat. (3♂1♀), P. pusilla (9♂, 1♀), P. rotundoides (3♂, 1♀), P. variabilis (9♀), and P. dispersa (10♀). Piona longipalpis (Krendowskij, 1878) (Pionidae) (10♀) was included as a reference species for comparison since it neither in the past or present contains described forms (Viets 1987) and Arrenurus suecicus Lundblad, 1917 (Arrenuridae) (1♂) was used as an outgroup.

Six localities were chosen on the basis of earlier findings in the provinces of Uppland and Småland (Lundblad 1962, 1968) (Fig. 1, Table 1). The localities included both running and standing water. The examined species were sampled together with its former nominate species in at least one of the sampled localities. Piona dispersa coexisted with P. variabilis in Lake Mälaren (Fig. 1, Table 1, Appendix 1). Water mites were sampled with a hand net (mesh size 0.5 mm) and sorted in the laboratory. The material was preserved in frozen water (−20°C) until identification and then in ethanol (80%, −20°C). Species were identified with the help of Viets (1936) and Lundblad (1962, 1968). Vouchers and DNA extractions are deposited at the Entomology Department, Swedish Museum of Natural History (NHRS), Stockholm, under the catalogue numbers NHRS-ACAR000000001-94. Images of all vouchers are available on Morphbank (2013; see Appendix 1 for Morphbank accession numbers).

**Molecular analysis**

The molecular work was carried out at the Molecular Systematics Laboratory (MSL), Swedish Museum of Natural History. DNA was extracted from the tissue of four legs of each individual. In some cases the whole mite was included as a reference species for comparison since it neither in the past or present contains described forms (Viets 1987) and Arrenurus suecicus Lundblad, 1917 (Arrenuridae) (1♂) was used as an outgroup.

**Figure 1.** The localities were chosen on the basis of earlier records of targeted nominal species and forms in Sweden (Lundblad 1962, 1968). Material was collected from Lake Mälaren (A; site 1–3), the stream Helgöan (site 4), Lake Anebysjön (B; site 5), and Lake Flisbysjön (B; site 6).

**Table 1.** Localities in the province of Uppland and Småland, Sweden, with coordinates, temperature (air), water depth, and bottom substrate. The habitat of Lilla Ullfjärden and Helgöan (site 3 and 4) lacked dominating plants.

| Site (Province Parish lake/stream) | Latitude   | Longitude   | Temp. (°C) | Depth (m) | Bottom substrate          |
|-----------------------------------|------------|-------------|------------|-----------|---------------------------|
| 1 Upl Vasslunda Lake Mälaren, Kyrkviken/Ekhamnsviken | 59°43’26.66”N | 17°40’49.16”E | 8, 15, 20 | 0–1 | Phragmites, sand          |
| 2 Upl Vasslunda Lake Mälaren, Skofjarden | 59°42’46.17”N | 17°38’34.13”E | 8, 15, 20 | 0–1 | Phragmites, detritus      |
| 3 Upl Yttergrans Lake Mälaren, Lilla Ullfjärden | 59°35’25.93”N | 17°31’17.46”E | 17 | 0–0.5 | Detritus, gravel          |
| 4 Upl Osseby Stream Helgöan | 59°36’03.66”N | 18°12’45.67”E | 18 | 0–1 | Detritus, fine sediment    |
| 5 Sm Norra Solberga-Flisby Lake Anebysjön | 57°47’28.84”N | 14°48’52.18”E | 20 | 0–1 | Schoenoplectus, Carex, sand |
| 6 Sm Norra Solberga-Flisby Lake Flisbysjön | 57°44’39.57”N | 14°50’51.64”E | 20 | 0–0.5 | Carex, Typha, sand        |
was used, with body fluids removed to avoid contamination. The extraction method followed the tissue protocol of Blood and Tissue Genomic Mini kit (Viogene, Taipei, Taiwan). A few individuals were extracted using GeneMole (Mole Genetics, Lysaker, Norway), QIAamp Mini Kit (Qiagen), or QIAamp DNA Micro Kit (Qiagen, Hilden, Germany). The protocols were modified by increasing the time of lysis with 4 °C (30 sec at 96 °C). Each sequencing reaction ran for 1 min at 96 °C, 10 min at 37 °C, 15 sec at 55 °C, 30 sec at 72 °C) × 40, 10 min at 72 °C. PCR products showing low amplification by gel electrophoresis were reamplified with 30 cycles. A fragment of the D2 region of 28S rDNA (28S) was amplified using primers D2F-forward 5′-AGTCGTGTTGCTTGATA-3′ and D2R-reverse 5′-TTGGTCCGTGTTTCAA-GACGGG-3′ (Campbell et al. 1993; Goolsby et al. 2006). Amplification of 28S was accomplished by 3 min at 94 °C, (30 sec at 94 °C, 15 sec at 55 °C, 30 sec at 72 °C) × 42, 10 min at 72 °C. PCR products showing low amplification by gel electrophoresis were reamplified with 30 cycles.

PCR products were purified using ExoSAP (Fermentas, Vilnius, Lithuania) for 30 min at 37 °C and 15 min at 80 °C. Gene regions were sequenced with the same primers as in the PCR using the ABI BigDye™ Terminator ver. 3.1 Cycle Sequencing Kit (Applied Biosystems, Foster city, CA). Each sequencing reaction ran for 1 min at 96 °C, (30 sec at 96 °C, 15 sec at 50 °C, 4 min at 60 °C) × 25. Sequencing reactions were purified using the DyeEx 96 kit (Qiagen) and cycle sequencing reactions were run on an ABI 3130xl Genetic Analyzer (Applied Biosystems). Raw data and contigs were viewed and assembled using the Pregap4 and Gap4 modules of the Staden package 1.6.0 (Staden et al. 1998). Primer sequences were removed from the beginning and end of each sequence. The 28S sequences were aligned using the FFT-INS-I strategy in MAFFT v. 6 (Katoh et al. 2005), which resulted in an alignment length of 734 bp. The alignment of COI was straightforward as sequences were length invariant. Only sequences with <15% missing data were used, expect for one 28S sequence with 55% missing values (Piona pusilla specimen 7). Sequence data of COI and 28S were available for all examined species including P. dispersa. However, some specimens were successfully sequenced for only COI (P. longipalpis (5 of 10), P. variabilis (3 of 9), and P. dispersa (5 of 10)) or 28S (U. sp D nr minor (1 of 1), P. coccinea (3 of 10), P. stjordalensis (1 of 10), P. rotundoides (3 of 4)) (Appendix 1). Nucleotide composition statistics, genetic distances, and parsimony informative characters were obtained using MEGA v.4.1 (Tamura et al. 2007). All sequences, trace files, primer sequences, voucher catalogue numbers, and collection data are submitted to BOLD (Ratnasingham and Hebert 2007). In addition, sequences are deposited in Genbank under the accession codes JN034731-JN034895 (Appendix 1). Phylogenetic trees were reconstructed with Bayesian methods using MrBayes v.3.2.1 (Ronquist and Huelsenbeck 2003). Substitution models (GTR + I + F) for COI (with 1st + 2nd and 3rd codon position partitioned separately) and 28S were selected with MrModeltest v. 2.3 (Nylander 2004). All parameters except topology and branch lengths were unlinked across partitions. Markov chain Monte Carlo (MCMC) settings were 10 million generations sampled every 500 generations, with the first 25% of samples discarded as burn-in. We ran both COI and 28S separately. COI and 28S analyses had a standard deviation of split frequencies below 0.01 (0.006, 0.004, respectively). A combined COI and 28S analysis was made with the same models as specified above and had an average standard deviation of split frequencies below 0.02 (0.018) after 15 million generations (not shown). Genetic distances within and between species were calculated with a K2P model (Kimura 1980).

To test if Piona dispersa and the examined species are likely to be separately evolving lineages and species under the unified species concept, we used GMYC analysis (Pons et al. 2006; Fujisawa and Barracough 2013), Rosenberg’s (2007) test of reciprocal monophyly and Rodrigo et al.’s (2008) test of branch length ratios. These are all tests for single-locus gene trees. We ran three different GMYC analyses: one separate analysis for each genus (Piona and Unionicola), and a combined analysis with data from both genera (not shown), as the influence of taxon sampling is a concern (Fujisawa and Barracough 2013). The GMYC analysis requires an ultrametric tree which was inferred with a clock model in MrBayes after identical COI haplotypes had been removed (Fujisawa and Barracough 2013). As a strict molecular clock could not be rejected over a nonclock model (likelihood ratio test; P < 0.05 for all three datasets), we used a strict-clock model to infer the ultrametric gene tree. The MCMC settings were the same as above, with the run length being 10 million generations. The GMYC analysis was performed in R version 3.0.1 with the ‘splits’ package (Ezard et al. 2013; R Core Team 2013). Rosenberg’s (2007) and Rodrigo et al.’s (2008) tests were conducted using the COI trees from
MrBayes, with the software Genious and the species delimitation plugin described by Masters et al. (2011).

**Morphometric analysis**

Prior to examination, body fluids were removed from the specimen by gently puncturing the body with an insect pin. Measurements of the width and length of the body, fourth coxa (in *Unionicola* specimens the fused coxa III and IV), palpal femur (P-II), and palpal tibia (P-IV), were taken with a Leitz Wetzlar Laborlux S microscope and an ocular micrometer. We also measured the dorsal length of the remaining segments of the palp (Table 2). In addition, we counted the sclerotized and unsclerotized genital aceta-

**Results**

**Molecular analysis**

Cytochrome c oxidase subunit I yielded a 658 bp sequence with 305 variable characters, of which 281 were parsimony informative, the vast majority in third codon positions (Table 3). Region D2 of nuclear ribosomal 28S gave a sequence length of 517–609 bp with 395 variable characters, of which 374 were parsimony informative (Table 3).

Bayesian phylogenetic analyses of COI and 28S gave similar topologies and strong support for most clades, including the two genera *Piona* and *Unionicola* (Figs 2, 3). The main area of disagreement is the relationship between the three complexes of *P. coccinea*, *P. variabilis*, and *P. pusilla*, where the two genes indicated different solutions, but with weak support. Also within the *P. coccinea*-complex, the phylogenetic signals from the two genes conflicted with respect to the relationships among the four specimen clusters, but again without convincing support. The combined Bayesian analysis of COI and 28S lent stronger support to some internal nodes and suggested that *P. longipalpis* is the sister species to the *P. coccinea*-complex (not shown).

All the examined taxa were separated by large genetic distances from their respective nominate species, presented as COI distances below if not otherwise stated. In addition, some species consisted of more than one distinct genetic cluster. Both genes indicated that *Unionicola crassipes* and *U. minor* together represent a complex of four to five genetic clusters. The 28S tree divided *U. minor* into four clusters (A–D), three of which were matched by COI (COI data were missing for representatives of the fourth cluster, cluster D). A genetic distance

### Table 2. Length and width (mean values in μm) of body size, coxal plate IV (length of III + IV for *Unionicola*), and palp, as well as the number of genital acetalba (right and left side) for each species. Length and width (μm) on claw and tarsus (leg segment) of the males' third leg are shown for *Piona coccinea* and *P. pusilla*-complex. All values were rounded to integers.

| Species                  | N  | Body       | Cx-II + IV | Palp (P-1, P-II, P-III, P-IV, P-V) | Genital aceta-
|--------------------------|----|------------|------------|-----------------------------------|----------------
| *Unionicola crassipes*   | 14 | 1175 × 950 | 408 × 330  | 25–210 × 125–90–210 × 62–172      | 6 (0) + 6 (0)  |
| *U. sp. A nr minor*      | 5  | 777 × 681  | 276 × 207  | 14–132 × 91–53–123 × 40–90        | 6 (0) + 6 (0)  |
| *U. sp. B nr minor*      | 3  | 943 × 815  | 324 × 252  | 17–154 × 94–57–138 × 49–92        | 6 (0) + 6 (0)  |
| *U. sp. C nr minor*      | 3  | 827 × 645  | 284 × 216  | 20–157 × 88–52–131 × 44–97        | 6 (0) + 6 (0)  |
| *U. sp. D nr minor*      | 1  | 1071 × 714 | 324 × 216  | 20–155 × 80–55–153 × 40–105       | 6 (0) + 6 (0)  |
| *Piona coccinea*         | 10 | 1345 × 1120| 322 × 421  | 66–287 × 142–125–316 × 74–159     | 23 (3) + 23 (2) |
| *P. stjordalensis*       | 6  | 907 × 856  | 214 × 319  | 51–252 × 160–114–261 × 79–126     | 27 (4) + 27 (3) |
| *P. stjordalensis*       | 4  | 1575 × 1391| 230 × 429  | 63–318 × 208–124–317 × 92–160     | 29 (2) + 30 (3) |
| *P. imminuta* s. lat.    | 3  | 975 × 867  | 223 × 338  | 55–260 × 162–23–272 × 83–138     | 24 (2) + 24 (2) |
| *P. imminuta* s. lat.    | 1  | 1000 × 900 | 180 × 410  | 55–241 × 163–113–280 × 70–148     | 23 + 24        |
| *P. longipalpis*         | 10 | 2982 × 2870| 400 × 717  | 93–501 × 269–195–587 × 140–253    | 96 (18) + 97 (14)|
| *P. pusilla*             | 7  | 805 × 702  | 194 × 261  | 28–148 × 108–74–148 × 36–74       | 17 (3) + 17 (2) |
| *P. sp. A nr pusilla*    | 1  | 730 × 620  | 175 × 230  | 28–150 × 100–75–145 × 40–70       | 22, 23         |
| *P. sp. A nr pusilla*    | 1  | 625 × 550  | 190 × 175  | 25–110 × 87–55–110 × 30–55        | 16, 22         |
| *P. sp. B nr pusilla*    | 1  | 790 × 690  | 220 × 260  | 20–155 × 125–90–175 × 40–80       | 23, 26         |
| *P. rotundoides*         | 3  | 887 × 787  | 320 × 248  | 32–223 × 157–109–237 × 54–110     | 35 (1) + 33 (3) |
| *P. rotundoides*         | 1  | 770 × 680  | 320 × 255  | 35–205 × 150–103–225 × 50–93      | 38, 36         |
| *P. variabilis*          | 9  | 1047 × 789 | 135 × 225  | 30–125 × 83–65–122 × 35–59        | 9 (1) + 8 (1)  |
| *P. dispersa*            | 10 | 1095 × 921 | 152 × 258  | 32–150 × 107–75–141 × 46–69       | 10 (2) + 11 (1) |
of 18% separated U. crassipes from U. minor and clusters A–C were separated with a similar distance from each other (17–23%). The intraspecific distance of U. crassipes was 6.9% (4.2% without specimen number 14), whereas the average distance within clusters A–C were 0.5% (SD = 0.9). Piona stjordalensis and P. imminuta s. lat. each formed a distinct cluster with a genetic distance of 19% from each other and separated from P. coccinea by 21%. One specimen of P. variabilis (number 9) differed by 8% in COI from other P. variabilis specimens, but this divergence was not reflected in 28S. Piona variabilis was separated from P. dispersa with a genetic distance of 11%. The intraspecific distance of P. dispersa was 1.6%. Piona pusilla was composed of three entities ranging from 18% to 27% in genetic distance from each other, in addition to P. rotundoides at a similar distance (15–20%). Average differences between nominate species and former forms of Piona were 19% (SD = 3.8) compared to 2.6% (SD = 3.0) within clusters. Excluding P. variabilis specimen 9, the average intraspecific distance reduced to 1.5% (SD = 1.3). In comparison, the distance of the reference species Piona longipalpis (without any described forms) to the congenic Piona species in this study showed a similar range (18–29%), while the 10 sequenced specimens showed no, or very little, intraspecific variation (0% COI, 0–1% 28S).

The favoured GMYC model had a significantly better fit than the null model for the Piona species (logL = 196.29 vs. 180.15, df = 2, P-value = 9.8 × 10⁻⁶) and for the Unionicola crassipes-complex (logL = 63.36 vs. 60.20, df = 2, P-value = 0.04). Two to six clusters were included in the confidence interval for Unionicola, but the maximum likelihood solution was four separate units (U. crassipes and the U. minor clusters A–C: Fig. 4). For Piona, the maximum likelihood model identified 11 separately coalescing mtDNA entities with only one alternative solution included in the confidence interval (marked with * in Fig. 4). In the P. coccinea-complex, both P. stjordalensis and P. imminuta s. lat. were confirmed as separately evolving species. Piona dispersa was also identified by the model as a distinct species, separate from P. variabilis.

Finally, P. pusilla consisted of three distinct lineages in addition to P. rotundoides which was also resolved as a separate coalescing unit. Also specimen number 9 of P. variabilis constituted a distinct unit from remaining P. variabilis specimens, but, as mentioned above, this deep divergence was not reflected by 28S (Fig. 3). The combined GMYC analysis with both Unionicola and Piona in a common ultrametric tree gave the same results for Piona, but the U. crassipes-complex had a narrower confidence interval, ranging from 4 to 6 separately coalescing units (not shown).

Rosenberg’s test is based on sampling individuals from predefined groups and testing the probability of reciprocal monophyly given the sample sizes and assuming a single-panmictic population. Two taxa, Unionicola minor and Piona pusilla, were not monophyletic in one or both of the gene trees in the sense of the a priori defined hypothesis as additional genetic clusters were discovered. Likewise, Rodrigo et al. confessed that their branch-length ratio test is too liberal if the hypothesis is defined a posteriori. Therefore, we only applied these tests to the hypotheses concerning the remaining a priori defined taxa. Rodrigo et al.’s test rejected the null hypothesis for all taxon nodes in the coccinea - complex; P. coccinea, P. stjordalensis and P. imminuta s. lat. (P < 0.05 for all three). Rosenberg’s test of reciprocal monophyly applies to pairs and the null hypothesis could be rejected for both P. coccinea versus P. stjordalensis + P. imminuta s. lat. (P = 6.4 × 10⁻⁶), and for the latter two only (P = 2.3 × 10⁻⁴). The null hypothesis for the reciprocal monophyly of P. variabilis and P. dispersa was also rejected by Rosenberg’s test (P = 5.1 × 10⁻⁶). With Rodrigo et al.’s test, the null hypothesis could be rejected for the P. dispersa defining node (P < 0.05), but because of specimen no. 9 it could not be rejected for the P. variabilis defining node (P = 0.3).

**Morphometric analysis**

In the morphometric analysis, the first principal component (PC1) represented an isometric size component, whereas the second principal component (PC2) represented shape changes not related to size. For Unionicola, only the isometric size component separated U. crassipes and U. minor; U. crassipes specimens differ mostly from
Figure 2. Majority-rule consensus from the Bayesian phylogenetic analysis of COI. Numbers above branches are posterior probability values. Note the large genetic distances between the species (scale bar). Outgroup taxon is Arrenurus suecicus.
Figure 3. Majority-rule consensus from the Bayesian phylogenetic analysis of 28S. Numbers above branches are posterior probability values. Note the large genetic distances between the species (scale bar). Outgroup taxon is Arrenurus suecicus.
U. sp. A-D nr minor due to their larger size (Fig. 5, Table S1). *Unionicola* sp. A–B near minor versus C–D near minor seems to be partly separable by the second principal component (Fig. 5). High values on the second principle component represent longer and more slender palpal segments relative to the body (Fig. 6, Table S1).

*Piona pusilla* and *P. rotundoides* aggregated into distinct clusters mainly along the size axis of PC1 (Fig. 7, Table S1). Along the PC2 axis, *P. sp. B nr pusilla* was most similar to *P. rotundoides* due to the higher number of genital acetabula (23+26) (Fig. 7, Table S1). The female of *P. sp. A nr pusilla* had 22+23 genital acetabula in contrast to *P. pusilla*, which had an average of 17 acetabula (Table 2). Both the first size component and the second component, reflecting length and width of terminal leg claw, palpal segment width, and the number of genital acetabula, distinguished males of *P. stjordalensis* and *P. imminuta* s. lat. from *Piona coccinea* (Figs 7, 8, Table S1).

In the analysis based on females, *P. variabilis* and *P. dispersa* were separated into two distinct clusters based on PC2 representing the number and sclerotization percentage of genital acetabula, the relative length of coxa and the width of palpal segment IV (Fig. 7, Table S1). In general, *P. variabilis* have sclerotized genital plates, whereas *P. dispersa* have no sclerotization, but there is some morphological variation among specimens in this character. Specifically, the examined specimens of *P. dispersa* had between zero and eight sclerotized acetabula, whereas specimens of *P. variabilis* could have incomplete sclerotization displayed as divided plates or one unsclerotized acetabulum (Fig. 9). The reference species, *Piona longipalpis*, had a comparatively large variation in the number of genital acetabula, in contrast to the low genetic variation (Table 2).

**Discussion**

**Species delimitation**

The Bayesian phylogenetic analyses and the species delimitation with a single locus (e.g., the GMYC model, Rosenberg’s and Rodrigo’s test) revealed large and consistent genetic distances between all forms with or without already known species status (*U. minor* in relation to *U. crassipes*, *Piona stjordalensis* and *P. imminuta* s. lat. both in relation to *Piona coccinea*, *P. rotundoides* in relation to *P. pusilla*, *P. dispersa* in relation to *P. variabilis*). The molecular patterns observed cannot be due to random
coalescence processes, but in fact, as they occur sympatri-
cally even in the same locality, support species status with
no or limited geneflow between them. Therefore, these
taxa cannot be treated as intraspecific variation. The
genetic distance between *P. variabilis* and *P. dispera*
were lower, but still comparable to the interspecific distances
of the examined species, including the reference species of
*P. longipalpis*. In fact, the genetic distances in the barcode
region were larger than the distances among the majority of recognized, closely related species of other animal groups tested to date (Grant and Bowen 1998 [fish]; Hebert et al. 2003 [moths]; Hebert et al. 2004 [birds]; Hogg and Hebert 2004 [springtails]; Kumar et al. 2007 [mosquitoes]; Koch 2010 [bees]). The differentiation

Figure 7. Multivariate analysis on measured morphological characters from Piona (A) females and (B) males. Parameters included: dorsal length of palp segments (P-I, P-II, P-III, P-IV, P-V), width of second and fourth segment (P-II, P-IV), coxa of IV (length and width), number of genital acetabula and in females sclerotization (percentage of genital acetabula) and in males length of tarsus and claw of the third leg.

Figure 8. Palp morphology drawn to scale of (A) Piona coccinea (specimen 6), (B) P. stjordalensis (specimen 9) and (C) P. imminuta s. lat. (specimen 1) of the Lake Mälaren population (site 1 and 2). Differences occur in the ventral side of the second palpal femur (arrow); (A) concave, (B) convex, and (C) straight.
(11–27% including examined species) is, however, similar to closely related species in other groups of Acari (Navajas et al. 1998; Dabert et al. 2008; Skoracka and Dabert 2010; Lv et al. 2013) including water mites (Martin et al. 2010 [18–31%]; Pécsi et al. 2012 [11%]).

To use a threshold of genetic distance to identify samples or even to delimit species, for example 2%, has been proposed and used widely, especially in the DNA barcoding literature (Hebert et al. 2003; Hebert et al. 2004; Kumar et al. 2007). However, such a threshold is artificial and not justified by known biological processes. The GMYC is also a method that is based on a simplified threshold and assumes species monophyly, but the value of the threshold is not artificially constructed, but optimized in a maximum likelihood framework based on realistic and established models of intraspecific coalescence and interspecific speciation. Originally developed for species delimitation of community samples in poorly studied groups (Pons et al. 2006), the GMYC model optimizes the transition between a slow interspecific branching rate compared to a relatively faster intraspecific coalescence rate in an ultrametric tree. The combined GMYC likelihood is tested against the likelihood of modeling the entire ultrametric tree as a single coalescence. This test is moderately informative when multiple species in a tree are tested at the same time. In the case of Piona, for example, rejecting the null only means that at least one of all jointly tested species should be regarded as a separately evolving unit. On the other hand, the ability, or statistical power, to identify the transition in branching rate is reduced if the tested ultrametric tree was to be subdivided into pairs of taxa. Instead, the strength with the GMYC method lies in not requiring an a priori species hypothesis and by using a proximate confidence interval of 2 log likelihood units from the maximum likelihood solution (Pons et al. 2006), initial species hypotheses can be erected for further testing beyond the single locus (see also Powell (2012) for an alternative confidence measure). The tests by Rosenberg (2007) and Rodrigo et al. (2008) are better suited to the testing of specific questions as oppose to large-scale biodiversity assessments, and require a priori defined hypotheses to be stringent tests (Rodrigo et al. 2008). As putative cryptic species are often discovered as a result of genetic analyses (not a priori), for example, in DNA barcoding studies, a careless usage of the one-click plug-in tool applying these tests (Masters et al. 2011) risk unjustified taxonomic inflation. Species delimitation method development is a vibrant and exciting research field where empiricists need to be aware of both pitfalls and potentials.

Despite the molecular support, it is important to not rely solely on a maternally inherited mitochondrial marker when testing species hypotheses, but to corroborate a hypothesis with multiple lines of evidence (de Queiroz 2007). Not the least because a number of potential pitfalls exist, including numts, (Moulton et al. 2010), Wolbachia infestation (Whitworth et al. 2007), introgressive hybridization (Sota et al. 2001), incomplete lineage sorting (Funk and Omland 2003), contamination in the lab, and more. In our case, except for specimen 9 of Piona variabilis, nuclear 28S is perfectly congruent with all of the COI-defined clusters. What specimen 9 of P. variabilis represents is uncertain, but variation in mitochondrial DNA not shown in nuclear or morphological data has been reported in other Acari groups (Leo et al. 2010). It highlights the need to corroborate hypotheses based on mitochondrial markers with nuclear loci and morphology.

The quantitative morphometric analysis supported all of the genetically defined species with a combination of morphological characters, except for the challenge represented by the new genetic clusters discovered in the P. pusilla and Unionicola crassipes-complexes. The variation in morphological characters and occurrence of intermediate specimens in these two groups are at the moment problematic for nonmolecular identification. The newly discovered and unnamed genetic clusters aside,

Figure 9. Sclerotization difference of genital acetabula of (A) Piona variabilis (specimen 1) (B) Piona variabilis specimen 9, and (C) P. dispersa (specimen 4). Notice the divided plates on each side of the genital opening in B.
the focus of the project was to test if form with or without already known taxonomical status were all supported as valid species by both COI and 28S. Even though there are morphological differences, numerous forms of water mites could not be recognized as species according to Lundblad (1920, 1924, 1962) due to the occurrence of intermediate specimen. Some of the characters’ variation between individuals (and occasionally within the same specimen) were congruently observed in this study, but not shown by the genetic data.

Is Unionicola minor a species or a species complex?

Many authors have expressed difficulty in classifying Unionicola crassipes-like specimens (Lundblad 1962; Conroy 1979, 1984; Crowell 1984). At present, U. minor is a valid species on Fauna Europaea (www.faunaeur.org) with the taxonomical comment that it is proposed as subspecies to U. crassipes (Gerecke 2011). Even though there are studies on life history, sexual biology, and morphological differences in all life stages providing evidence for two separate species (Hevers 1975, 1977, 1978, 1979a,b, 1980), there are alternative views (Conroy 1979, 1984). Conroy (1979) suggested first that U. minor should be discarded and synonymous with U. crassipes. However, after reexamination of North American U. laurentiana Crowell and Davids 1979 and U. nearctica Crowell and Davids 1979; Conroy (1984) concluded that the species complex can be represented by three subspecies; U. crassipes, U. minor, and U. laurentiana, despite the fact that the first two taxa occur sympatrically (Crowell 1979, 1984). The name implies, U. minor is smaller in size and were before 1972 treated only as a form. Our morphological analysis separated U. crassipes from U. minor, but no further divisions of U. minor was obvious. The GMYC analysis, however, gave multiple species delimitation alternatives (2–6 species), and large genetic variation indicated a complex of morphologically very similar species near U. minor. This exposes the sensitivity of the GMYC method to taxon sampling. The GMYC method has become quite popular and used in a number of studies (Monaghan et al. 2009; Fontaneto et al. 2011; Isambert et al. 2011), but a note of caution is warranted with regards to the effect of sampling on the outcome. This has only been explored so far with respect to sampling of populations within a species (Lohse 2009; Papadopoulou et al. 2009), but not with respect to the sampling of interspecific variation (see Fujisawa and Barraclough 2013). Analyzed separately, the GMYC confidence interval for Unionicola included solutions with between two and six separate units. When analyzed together with the Piona dataset, however, the confidence interval only included solutions with four to six units (not shown). However, the maximum likelihood solution in both cases was four units. Including the result of 28S, the genetic analyses indicated a complex of five species: U. crassipes, U. sp A nr minor, U. sp B nr minor, U. sp C nr minor, and U. sp D nr minor. This, together with an overlap in body size, creates uncertainty as to which of the U. minor clusters is the most suitable representative of the original description. Therefore, we treat all the clusters as “near U. minor”.

It has been implied that size is not a suitable criterion to distinguish species because it might be influenced by environmental factors like nutrition during larval stages (Lundblad 1962; Conroy 1984). The size of the Unionicola minor specimens, we studied, does not exactly match the size delimitation of 945 by 734 μm postulated by Viets (1936) and overlaps with the smallest U. crassipes (Table 2). However, size differences may contribute to niche separation by affecting the selection of prey (Davids et al. 1981, 1985). Unionicola crassipes specimens are larger than U. minor, and are therefore able to select larger copepods as prey. Studies on the biology of U. crassipes (or crassipes-like species), indicate that nymphs and adults in both species prey on small crustaceans and are sponge-associated, while the larvae parasitize flying Chironomidae (Crowell and Davids 1979; Proctor and Pritchard 1989). Furthermore, previous studies have indicated that U. crassipes could be genetically isolated from U. minor due to the time between the appearance of the first-generation specimens in spring and that the males only deposit spermatophores in the presence of a conspecific female (Hevers 1978; Davids et al. 1985). Except body size, it is important to note the characteristic palpal femur (Lundblad 1962). In the literature, it is stated that U. crassipes has a straight palpal femur, while the femur is convex in U. minor (Lundblad 1962). However, the palpal femur of the genetically defined species examined here shows a more or less continuous gradient from a straight to a convex profile (Fig. 6). Based primarily on the genetic data, which very clearly separate U. crassipes and U. minor, we confirm U. minor as valid species. However, it is likely that U. minor in fact is composed of a minimum of four species, as judged from our restricted sample of specimens. Further studies on the U. minor-complex, with material from a wider geographic area, are needed to fully elucidate the delimitation and diagnostics of the species in this complex.

Confirming species statuses in Piona

Numerous species in the large family Pionidae, as well as in other water mite families, are rich in variation (Viets 1936; Lundblad 1962; European Water Mite Research
2009; van Haaren and Tempelman 2009). The investigation of Davids and Kouwets’s (1987) is the reason why several former varieties are seen as separate species in recent work (Gerecke 2011). They (1987) added morphological characters including larval morphology as an important factor, and these conclusions have also been corroborated by others (Biesiadka 1977; see Viets 1987). Earlier they were each treated as a form or as in Piona stjordalensis divided up into multiple taxa (Koenike 1920; Viets 1987). Piona stjordalensis was first described as a separate species (Thor 1897). Specialists have later seen it as a form of P. coccinea (Sokolow 1940; Láska 1954; Lundblad 1962). The additional forms P. coccinea f. confertipora and P. coccinea f. hankensis were treated as synonyms to P. stjordalensis (Lundblad 1962). Davids and Kouwets (1987) suggested raising P. stjordalensis to species level. Furthermore, they contested the opinion by Lundblad (1962) regarding P. coccinea f. confertipora. In fact, they synonymized the form, together with Piona coccinea f. recurva and P. coccinea f. gracilipalpis with P. imminuta, due to similar shape of palpal femur. The P. imminuta specimens in this study were therefore classified as “P. imminuta s. lat.” using the wide concept of imminuta sensu Davids and Kouwets (1987) (i.e., including confertipora, recurva and gracilipalpis). Piona rotundoides was treated as a form by Lundblad in 1956 and Thor (1897) commented already in the original description that the new species might be a variant or subspecies of P. pusilla. However, it is very clear that Davids and Kouwets (1987) were right in their conclusion that Piona rotundoides is a valid species, as confirmed by our study.

Regarding identification, we want to point out that the characteristic diagnostic feature of two small papillae on the palpal tibia (P-IV) on Piona imminuta s. lat. stated by Piersig (1897) were only present in two females and one of which clearly belonged to another species (P. stjordalensis specimen 2). Other authors have noticed that the presence or absence of these papillae vary (Lundblad 1962; Davids and Kouwets 1987). However, a better character seems to be the straight ventral side of the palpal femur (Fig. 8). Davids and Kouwets (1987) mention a smaller palp size in comparison with P. coccinea and P. stjordalensis, but we found no distinct difference in this study. The distinction of P. coccinea, P. stjordalensis, and P. imminuta s. lat. were very clear due to shape difference of the tarsus and claws of the males’ third leg and palpal femur in both males and females. While P. coccinea have a red colour, P. stjordalensis and P. imminuta s. lat. are much paler.

Another taxonomical problem is the character of the amount of genital acetabula. Several species are distinguished by their count, but the intraspecific variation can be substantial (Viets 1936; Lundblad 1962, 1968). Despite this, the number of genital acetabula of Piona rotundoides in this study corresponds well with the original description of 30–40 per genital plate (Thor 1897). Moreover, P. pusilla is regarded to have 15–22 genital acetabula (Davids and Kouwets 1987), but the females of P. sp. A-B nr pusilla in this study were slightly over the marginal of 22 acetabula per plate (Table 2). Piona sp A-B nr pusilla probably also represent two distinct species, although the morphological differences recognized to date are subtle and the sampling quite small. This, together with the results from U. minor, indicates that there are more species or species complexes present than previously thought.

**Piona variabilis and Piona dispersa**

Until this study, Piona dispersa was treated as a synonym of the nominate species P. variabilis (European Water Mite Research 2009; Gerecke 2011). However, there are no detailed taxonomical studies and P. dispersa has been irregularly treated as a synonym, as a form or as a species (Lundblad 1962; Böttger and Ulrich 1974; etc. see Viets 1987). Böttger and Ulrich (1974) commented after collecting in Germany that they believed these two taxa are conspecific, and it was originally described as a variety of P. variabilis due to unsclerotized genital acetabula and no distinguishing features between males (Sokolow 1926). However, the genetic and morphometric data in this study are unequivocal, showing large distances between P. variabilis and P. dispersa. The molecular analyses point out that the variation in sclerotization around the genital acetabula is intraspecific, yet not evidence of conspecificity with P. variabilis (Lundblad 1962). In contrast to the occurrence of incomplete sclerotization connecting some genital acetabula in P. dispersa, the opposite pattern is displayed in P. variabilis with single acetabula arranged freely without sclerotization. The divided genital plates shown in specimen 9 of P. variabilis (Fig. 9) coincide with the divergent COI sequence (Figs 2, 4). However, we refrain from assigning COI sequence to this character because of (1) the lack of genetic differentiation in 28S, (2) the coherence of the P. variabilis cluster in the morphometric PCA analysis, and (3) the same feature detected on two other specimens, but on only one side of the genital opening (a total of three plates). Despite the intraspecific and overlapping variation, this character alone can well be used to identify P. dispersa. We imagine the two species can coexist, perhaps due to small differences in prey selection, behaviour or habitat preference which often explains species coexistence (Davids et al. 1981). Some 30–40 species of over 5000 parasitengonine mites are recorded to have a loss of larval parasitism, including the P. coccinea and the P. pusilla group (Smith...
1998). This could also explain how two closely related species can occur sympatrically, one with typical parasitic larvae and the other with nonfeeding larvae (Smith 1998).

**Taxonomic changes**

Following our results with both molecular and morphological data, *Piona dispera* is a valid species, separated from the nominate species *P. variabilis*. We propose that *P. dispera* is raised to species level. Our results also show that *Unionicola minor* and *P. pusilla* consist of at least three cryptic species each, which we refrain from formally naming here in the anticipation of future morphological studies uncovering reliable diagnostic characters separating these species.

**Conclusions**

Species statuses as suggested by Davids and Kouwets (1987) based on morphological comparison of *Piona imminuta s. lat.*, *P. stjordalensis*, and *P. rotundoides*, are now strongly supported with both molecular and morphometric analyses with this study. Likewise, the new species level status of *P. dispera* is established with the same criteria. We make the assumption that more diversity is present in water mites than thought in the past, not only uncovered when described “forms” are shown to be valid species but also through molecular data revealing cryptic species complexes. Even if our study clarifies only a fragment of this really problematic topic, it has demonstrated the utility of explicit species delimitation methods to test taxonomic questions at the species-to-population level. The sympatric occurrence, a convergence toward a unified species concept (de Queiroz 2007) and implementation of the coalescent process model as a null hypothesis are key elements to species delimitation for the future.

**Acknowledgments**

We are grateful for the support of this study from the Swedish Taxonomy Initiative (The Swedish Species Information Centre). The staff of the Molecular Systematics Laboratory (MSL, Swedish Museum of Natural History) is acknowledged for their valuable advice on sequencing, especially the help from Bodil Cronholm, Martin Irestedt, and Keyvan Mirbakhsh. We also want to express our gratitude to Marianne Espeland (Museum of Comparative Zoology and Department of Organismic and Evolutionary Biology, Harvard University) and Tobias Malm (University of Eastern Finland, Joensuu, Finland) who were at the time PhD students at the Entomology Department, Swedish Museum of Natural History, Stockholm, for their advice on molecular and morphological analysis. Last, but not least, we are thankful for the taxonomic discussion with Ulf Lettevall (Växjö, Sweden).

**Conflict of Interest**

None declared.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Characters (length = l, width = w) used in the principal component analyses of Unioniola and Piona and loadings of each character on principal components 1 and 2.
Appendix 1 Details of the 94 specimens included in the study. Vouchers have the collection numbers NHRS-ACAR 000000001-94, and are deposited at the Swedish Museum of Natural History (NHRS). In the table ‘F’ stands for female and ‘M’ for male specimen. Localities (site 1–6) are described in Figure 1 and Table 1. Each specimen has a Morphbank accession number and each sequence a Genbank accession number.

| Specimen | Sex | Voucher       | Site Sampled | Genbank  | COI   | 28S   | Morphbank |
|----------|-----|---------------|--------------|----------|-------|-------|-----------|
| Unionicola crassipe-complex | | | | | | |
| Unionicola crassipes | | | | | | |
| 1 | F | NHRS-ACAR 000000001 | 2008-06-17 2 | JN034803 JN034883 | 647460 |
| 2 | F | NHRS-ACAR 000000002 | 2008-06-17 2 | JN034802 JN034882 | 647461 |
| 3 | F | NHRS-ACAR 000000003 | 2008-07-22 5 | JN034801 JN034881 | 647462 |
| 4 | F | NHRS-ACAR 000000004 | 2008-06-17 2 | JN034800 JN034880 | 647463 |
| 5 | F | NHRS-ACAR 000000005 | 2008-07-22 5 | JN034799 JN034879 | 647464 |
| 6 | F | NHRS-ACAR 000000006 | 2008-07-22 5 | JN034798 JN034878 | 647465 |
| 7 | F | NHRS-ACAR 000000007 | 2008-06-17 2 | JN034797 JN034877 | 647466 |
| 8 | F | NHRS-ACAR 000000008 | 2008-06-17 2 | JN034796 JN034876 | 647467 |
| 9 | F | NHRS-ACAR 000000009 | 2008-06-17 2 | JN034795 JN034875 | 647468 |
| 10 | F | NHRS-ACAR 000000010 | 2008-04-05 4 | JN034794 JN034874 | 647470 |
| 11 | F | NHRS-ACAR 000000011 | 2008-06-17 2 | JN034793 JN034873 | 647471 |
| 12 | F | NHRS-ACAR 000000012 | 2008-06-17 2 | JN034792 JN034872 | 647472 |
| 13 | F | NHRS-ACAR 000000013 | 2008-06-17 2 | JN034791 JN034871 | 647473 |
| 14 | F | NHRS-ACAR 000000014 | 2008-06-17 2 | JN034790 JN034870 | 647474 |
| Unionicola sp. A nr minor | | | | | | |
| 1 | F | NHRS-ACAR 000000015 | 2008-04-05 4 | JN034814 JN034895 | 647475 |
| 2 | F | NHRS-ACAR 000000016 | 2008-04-05 4 | JN034813 JN034894 | 647476 |
| 3 | F | NHRS-ACAR 000000017 | 2008-04-05 4 | JN034812 JN034893 | 647477 |
| 4 | F | NHRS-ACAR 000000018 | 2008-04-05 4 | JN034811 JN034892 | 647478 |
| 5 | F | NHRS-ACAR 000000019 | 2008-04-05 4 | JN034810 JN034891 | 647479 |
| Unionicola sp. B nr minor | | | | | | |
| 1 | F | NHRS-ACAR 000000020 | 2008-06-17 2 | JN034809 JN034890 | 647480 |
| 2 | F | NHRS-ACAR 000000021 | 2008-06-17 2 | JN034808 JN034889 | 647481 |
| 3 | F | NHRS-ACAR 000000022 | 2008-07-22 5 | JN034807 JN034888 | 647482 |
| Unionicola sp. C nr minor | | | | | | |
| 1 | F | NHRS-ACAR 000000023 | 2008-07-22 5 | JN034806 JN034887 | 647483 |
| 2 | F | NHRS-ACAR 000000024 | 2008-07-22 5 | JN034805 JN034886 | 647484 |
| 3 | F | NHRS-ACAR 000000025 | 2008-07-22 5 | JN034804 JN034885 | 647485 |
| Unionicola sp. D nr minor | | | | | | |
| 1 | F | NHRS-ACAR 000000026 | 2008-07-22 5 | JN034884 | 647486 |
| Piona coccinea-complex | | | | | | |
| Piona coccinea | | | | | | |
| 1 | M | NHRS-ACAR 000000027 | 2008-06-17 1 | JN034738 JN034825 | 647306 |
| 2 | M | NHRS-ACAR 000000028 | 2007-06-01 1 | JN034737 JN034824 | 647307 |
| 3 | M | NHRS-ACAR 000000029 | 2007-06-01 1 | JN034736 JN034823 | 647308 |
| 4 | M | NHRS-ACAR 000000030 | 2008-06-17 2 | JN034735 JN034822 | 647303 |
| 5 | M | NHRS-ACAR 000000031 | 2007-06-01 2 | JN034734 JN034821 | 647304 |
| 6 | M | NHRS-ACAR 000000032 | 2008-06-17 2 | JN034733 JN034820 | 647305 |
| 7 | M | NHRS-ACAR 000000033 | 2008-06-17 1 | JN034732 JN034819 | 647309 |
| 8 | M | NHRS-ACAR 000000034 | 2008-06-17 1 | JN034818 | 647310 |
| 9 | M | NHRS-ACAR 000000035 | 2008-06-17 1 | JN034817 | 647311 |
| 10 | M | NHRS-ACAR 000000036 | 2008-07-22 5 | JN034816 | 647312 |
| Piona stjordalensis | | | | | | |
| 1 | M | NHRS-ACAR 000000037 | 2008-06-17 1 | JN034780 JN034863 | 647313 |
| 2 | F | NHRS-ACAR 000000038 | 2008-06-17 1 | JN034779 JN034862 | 647314 |
| 3 | M | NHRS-ACAR 000000039 | 2008-06-17 1 | JN034778 JN034861 | 647315 |
| 4 | F | NHRS-ACAR 000000040 | 2008-06-17 1 | JN034777 JN034860 | 647316 |
| 5 | F | NHRS-ACAR 000000041 | 2008-06-17 1 | JN034776 JN034859 | 647317 |
| 6 | M | NHRS-ACAR 000000042 | 2008-06-17 1 | JN034775 JN034858 | 647318 |
### Appendix 1. Continued.

| Specimen | Sex | Voucher       | Site Sampled | Site Collected | Genbank COI   | Genbank 28S | Morphbank |
|----------|-----|---------------|--------------|----------------|--------------|-------------|-----------|
| 7 M      |     | NHRS-ACAR 000000043 | 2008-06-17 1 | 1 JN034774     | JN034857 647319 |
| 8 M      |     | NHRS-ACAR 000000044 | 2008-06-17 1 | 1 JN034773     | JN034856 647320 |
| 9 M      |     | NHRS-ACAR 000000045 | 2008-06-17 1 | 1 JN034772     | JN034855 647321 |
| 10 F     |     | NHRS-ACAR 000000046 | 2008-06-17 1 | 1 JN034854     | 647322      |

**Piona imminuta s. lat.**

1 M NHRS-ACAR 000000047 | 2008-06-17 1 | JN034750 | JN034834 | 647326 |
2 F NHRS-ACAR 000000048 | 2008-07-22 5 | JN034749 | JN034833 | 647327 |
3 M NHRS-ACAR 000000049 | 2008-06-17 1 | JN034748 | JN034832 | 647328 |
4 M NHRS-ACAR 000000050 | 2008-07-22 5 | JN034747 | JN034831 | 647329 |

**Piona longipalpis**

1 F NHRS-ACAR 000000051 | 2008-06-17 1 | JN034760 | JN034839 | 476669 |
2 F NHRS-ACAR 000000052 | 2008-06-17 1 | JN034759 | JN034838 | 464445 |
3 F NHRS-ACAR 000000053 | 2008-06-17 2 | JN034758 | JN034837 | 467690 |
4 F NHRS-ACAR 000000054 | 2008-06-17 2 | JN034757 | JN034836 | 476692 |
5 F NHRS-ACAR 000000055 | 2008-06-17 1 | JN034756 | JN034835 | 476673 |
6 F NHRS-ACAR 000000056 | 2008-06-17 1 | JN034755 |             | 476670 |
7 F NHRS-ACAR 000000057 | 2008-06-17 1 | JN034754 |             | 476675 |
8 F NHRS-ACAR 000000058 | 2008-06-17 2 | JN034753 |             | 476678 |
9 F NHRS-ACAR 000000059 | 2008-06-17 2 | JN034752 |             | 476694 |
10 F NHRS-ACAR 000000060 | 2008-06-17 2 | JN034751 |             | 476696 |

**Piona pusilla-complex**

**Piona pusilla**

1 F NHRS-ACAR 000000061 | 2008-06-17 1 | JN034767 | JN034846 | 647435 |
2 F NHRS-ACAR 000000062 | 2008-06-17 1 | JN034766 | JN034845 | 647436 |
3 F NHRS-ACAR 000000063 | 2008-06-17 1 | JN034765 | JN034844 | 647437 |
4 F NHRS-ACAR 000000064 | 2008-06-17 1 | JN034764 | JN034843 | 647438 |
5 F NHRS-ACAR 000000065 | 2008-06-17 1 | JN034763 | JN034842 | 647439 |
6 F NHRS-ACAR 000000066 | 2008-06-17 1 | JN034762 | JN034841 | 647440 |
7 F NHRS-ACAR 000000067 | 2008-06-17 1 | JN034761 | JN034840 | 647441 |

**Piona C. nr pusilla**

1 F NHRS-ACAR 000000068 | 2008-07-22 6 | JN034771 | JN034853 | 647444 |
2 F NHRS-ACAR 000000069 | 2008-07-22 5 | JN034770 | JN034852 | 647445 |

**Piona rotundoides**

1 F NHRS-ACAR 000000071 | 2008-06-17 1 | JN034789 | JN034869 | 647331 |
2 F NHRS-ACAR 000000072 | 2008-07-22 5 | JN034788 | JN034868 | 647332 |
3 F NHRS-ACAR 000000073 | 2008-07-22 5 | JN034787 | JN034867 | 647333 |
4 M NHRS-ACAR 000000074 | 2008-06-17 3 | JN034786 | JN034866 | 647334 |

**Piona variabilis-complex**

**Piona variabilis**

1 F NHRS-ACAR 000000075 | 2008-06-17 1 | JN034789 | JN034869 | 647331 |
2 F NHRS-ACAR 000000076 | 2008-06-17 1 | JN034788 | JN034868 | 647332 |
3 F NHRS-ACAR 000000077 | 2007-06-01 1 | JN034787 | JN034867 | 647333 |
4 F NHRS-ACAR 000000078 | 2007-06-01 1 | JN034786 | JN034866 | 647334 |
5 F NHRS-ACAR 000000079 | 2007-06-01 1 | JN034785 | JN034865 | 647335 |
6 F NHRS-ACAR 000000080 | 2008-06-17 1 | JN034784 | JN034864 | 647336 |
7 F NHRS-ACAR 000000081 | 2008-06-17 1 | JN034783 |             | 647337 |
8 F NHRS-ACAR 000000082 | 2008-06-17 1 | JN034782 |             | 647338 |
9 F NHRS-ACAR 000000083 | 2008-06-17 1 | JN034781 | JN034864 | 647339 |

**Piona dispersa**

1 F NHRS-ACAR 000000084 | 2007-06-01 1 | JN034746 |             | 647340 |
2 F NHRS-ACAR 000000085 | 2007-06-01 1 | JN034745 |             | 647341 |
### Appendix 1. Continued.

| Specimen | Sex | Voucher         | Site Sampled | Site | Genbank     |
|----------|-----|-----------------|--------------|------|-------------|
|          |     |                 |              |      | COI          |
|          |     |                 |              |      | 28S          |
|          |     |                 |              |      | Morphbank   |
| 3        | F   | NHRS-ACAR 0000000086 | 2007-06-01 | 1    | JN034744    | 647342 |
| 4        | F   | NHRS-ACAR 0000000087 | 2007-06-01 | 1    | JN034743    | 647343 |
| 5        | F   | NHRS-ACAR 0000000088 | 2007-06-01 | 1    | JN034742    | 647344 |
| 6        | F   | NHRS-ACAR 0000000089 | 2007-06-01 | 1    | JN034741    | JN034830 | 647345 |
| 7        | F   | NHRS-ACAR 0000000090 | 2007-06-01 | 1    | JN034740    | JN034829 | 647346 |
| 8        | F   | NHRS-ACAR 0000000091 | 2008-06-17 | 1    | JN034828    | 647347 |
| 9        | F   | NHRS-ACAR 0000000092 | 2007-06-01 | 1    | JN034739    | JN034827 | 647348 |
| 10       | F   | NHRS-ACAR 0000000093 | 2008-06-17 | 1    | JN034826    | 647349 |
| Arrenurus suecicus | |                 |              |      |             |
| 1        | M   | NHRS-ACAR 0000000094 | 2008-06-17 | 2    | JN034731    | JN034815 | 659537 |

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