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Convergence of molecular and morphological data reveals phylogenetic information on *Tetranychus* species and allows the restoration of the genus *Amphitetranychus* (Acari: Tetranychidae)

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Abstract

Nucleotide sequence variation and morphological characters were used to study the evolutionary relationships among nine tetranychid mites species. A phylogenetic study of this family based on mitochondrial cytochrome oxidase subunit I (COI) sequences had previously placed the species *Tetranychus viennensis* Zacher outside the other species analysed in the genus. Phylogenetic relations within the genus were re-examined with the addition of the species *Tetranychus quercivorus* Ehara & Gotoh, which is morphologically close to *T. viennensis*. Another region of the genome, the second internal transcribed spacer (ITS2) of ribosomal DNA, was also studied and proved to be of considerable interest at this taxonomic level. Both COI and ITS2 sequences indicated a close relationship between *T. viennensis* and *T. quercivorus*, which are grouped together and distinct from the other *Tetranychus* examined. The two species display morphological characteristics such as the absence of a medio-dorsal spur on all empodia of the legs of both sexes and the presence of anastomosing peritremes. This distinguishes them from the other members of the genus *Tetranychus*. The convergence of molecular and morphological data suggests that *T. viennensis* and *T. quercivorus* should not be classified in the genus *Tetranychus*. It is proposed that the genus *Amphitetranychus* Oudemans should be restored for classification of these species. Finally, a key to the Tetranychini tribe genera with one pair of para-anal setae is presented.

Introduction

Analysis of nucleotide sequences of a fragment of the mitochondrial cytochrome oxidase subunit I gene (COI) showed the phylogenetic relationship between 19 tetranychid species belonging to eight different genera of the family Tetranychidae (Acari) (Navajas *et al.*, 1996a). The resulting phylogeny based on COI sequences was compatible with classical systematics established from morphological characters. However, a number of potential taxonomic revisions were highlighted, including the possible polyphyly of the genus *Tetranychus*. The phylogenetic analysis showed that the species corresponding to the sample of *Tetranychus viennensis* Zacher should be separated from the other members of the genus *Tetranychus*. To examine the question in greater detail, the number of samples studied was increased and analysis of COI sequences was complemented by that of another region of the genome, the second internal transcribed spacer (ITS2) of ribosomal DNA. *Tetranychus quercivorus* Ehara & Gotoh was added to the range of species examined. This taxon displays morphological similarities to *T. viennensis*. In a general manner, analysis of the ITS2 would

seem appropriate for the study of closely related taxa (Hillis & Dixon, 1991) and preliminary data has confirmed their interest for defining phylogenetic relationships in mites of the genus *Tetranychus* (Navajas *et al.*, 1992). Several morphological characters used in the definition of the genus *Tetranychus* have been re-examined in parallel with these new comparisons of sequences. The two sets of data, both molecular and morphological, were then compared to obtain novel information on the systematics of these tetranychid mites.

Materials and methods

Biological material

Analyses were performed on the nine species of Tetranychidae listed in table 1. The origin, rearing conditions, and host plant were described in a previous publication (Navajas *et al.*, 1996a) for all the species except for *T. quercivorus*. The latter species is known only in Japan, where it was collected from deciduous oak, *Quercus mongolica*. Two different partially incompatible geographical strains (Gotoh *et al.*, 1995) were examined: Sapporo (Hokkaido) with sampling in 1991 and 1993 and Tsukuba-Ibaraki (Honshu) with sampling in 1994 and 1995. Four mites from each of the sampling dates were used for DNA studies.

Production and analysis of DNA sequences

The ITS2 of the ribosomal DNA and a 340 nucleotide long section of the gene coding for the COI mitochondrial were PCR amplifed and sequenced. In addition to the species presented in Navajas *et al.* (1996a), we included the sequence of *T. quercivorus* to the COI analysis. For the ITS2, we incorporated the species *T. quercivorus* to the sequences studied in Navajas *et al.* (1996b). DNA extraction, PCR primers, reactions and sequencing protocols were performed as previously described (Fournier *et al.*, 1994; Navajas *et al.*, 1996a), except for the *T. quercivorus* samples preserved in 100% ethanol and for which a preliminary step in the extraction procedures was added as follows: mites were hydrated by immersing each individual in 20 µl double distilled water three times for 10 sec. DNA was isolated from single adult females.

The sequences obtained were aligned and phylogenetic analysis performed using several programs in J. Felsenstein's PHYLIP 3.5c package (Felsenstein, 1993). Two different procedures were utilized according to the distinct patterns of substitution of the two sequenced regions. COI sequence

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                               11233444455677788899900111223345556777888 00
                             429469256817628912471362517803284369214703614
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                              1123344445567778889990011122334555677788800
                             429469256817628912471362517803284369214703614

T. urticae                   GTTTTTTAATTGAATTATAAATTATAAGATTTTATCTTTCTTATA
T. kanzawai                  A..A......AA...........TC..........TA..T.....
T. mcdanieli                 AA..A.A....A..ACT......T....T..AA..TA.AT...A.
T. pacificus                 AA..A.A....A...CT......CC...TA..A..TA.AT.....
T. gloveri                   A..G..A...AA.T...CTT........T.A....A..GT...A.
T. neocaledonicus            AA.AGCA.G.GA.TA.......AT.G...AA....T..AT....G
T. viennensis                A..A......AA..A.....GA.T..T...A.AT.T...TAA.A.
T. quercivorus Tsukuba       ?A.AAC....AA..ACT..TG..T.....GA.....A..T.A...
T. quercivorus Sapporo       ?A.AAC....AA..ACT..TG..T.....GA.....A..T.A...
M. progresivus               A...A...TAAAG.......G.AT..TAT.....ATA....ATA.


                             222222222222222222222222222222223333333333333
                             001111122333344444455555566788890000011133333
                             780367925347902367901268948612810346925801246

T. urticae                   TATATTATTACTATTTTAATATTAAATACTAAATGATATATAATA
T. kanzawai                  ...T.A.CA.T..C.......CG......C.....G..C......
T. mcdanieli                 ...T....A.T....C......GT.......T.............
T. pacificus                 ....CA..A.T..C.......C.T...GA..G...........CT
T. gloveri                   .G.TAAT.AGTCGA..A............AGT.....TC......
T. neocaledonicus            ..AT.AT.A.T...CAA............A......ATCT...CT
T. viennensis                ...T.AT...T.....ATTAT.GTT.C....G.....T.......
T. quercivorus Tsukuba       C.A..AT...T.G...ATT.TA.TT....A..T..T.T.T.....
T. quercivorus Sapporo       C.A..AT...T.G...ATT.TA.TTG...A..T..T.T.T.....
M. progresivus               ...TCAT...T......GC............T.CAT...TCTC..
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Fig. 1. All variable nucleotide sites among COI sequences of nine tetranychid species. Dots indicate sequence matches to the first sequence. The position numbers refer to sequences published in Navajas *et al.* (1966a).

Table 1. Pairwise distances between tetranychid species based on differences in nucleotide sequences of the mitochondrial COI gene. Below the diagonal: number of nucleotide substitutions; above the diagonal: distances.

	T. urticae	*T. kanzawai*	*T. mcdanieli*	*T. pacificus*	*T. gloveri*	*T. neocaledonicus*	*T. viennensis*	*T. quercivorus* Tsukuba	*T. quercivorus* Sapporo	*M. progresivus*
Tetranychus urticae		0.0597	0.0729	0.0864	0.1000	0.1140	0.1000	0.1140	0.1175	0.1070
T. kanzawai	20		0.0763	0.0796	0.0966	0.1035	0.0966	0.1140	0.1175	0.1140
T. mcdanieli	24	24		0.0565	0.1105	0.1105	0.1000	0.1175	0.1210	0.1175
T. pacificus	28	25	18		0.1282	0.1175	0.1354	0.1282	0.1318	0.1390
T. gloveri	32	30	34	39		0.1105	0.1210	0.1354	0.1390	0.1463
T. neocaledonicus	36	32	34	36	34		0.1282	0.1140	0.1175	0.1574
T. viennensis	32	30	31	41	37	39		0.0898	0.0932	0.1210
T. quercivorus Tsukuba	35	35	36	39	41	35	28		0.0030	0.1390
T. quercivorus Sapporo	36	36	37	40	42	36	29	1		0.1427
Mononychellus progresivus	34	35	36	42	44	47	37	42	43	

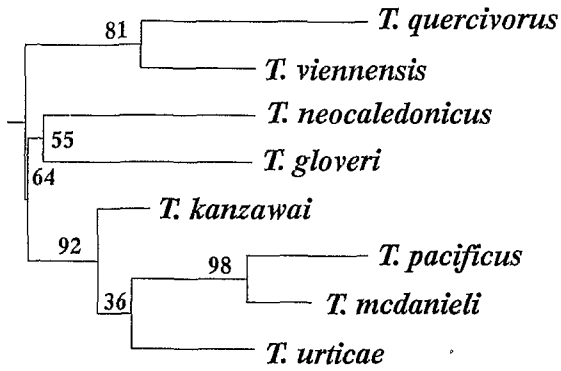

Fig. 2. Phylogenetic tree inferred from mitochondrial COI nucleotide sequences of various tetranychid species. The figures indicate the number of times (per 1000 replicates) that the group occurs together, calculated by bootstrap analysis.

analysis was based on the maximum likelihood (ML) method as described in detail in Navajas *et al.* (1996a). The latter publication includes a description of the programs and the optimization strategy for the different parameters considered in the ML model (transition/transversion ratio, number of categories of sites and relative rate of substitution). A genetic distance matrix was generated using the Jukes & Cantor (1969) model for analysis of ITS2 sequences and the neighbour-joining method was used to construct the phylogenetic tree. In both cases, the phylogenetic tree was rooted at its midpoint and the confidence limits for nodes were estimated by using 1000 iterations in bootstrap analysis.

The sequences are deposited in the EMBL database under the following accesssion numbers: X77901; X80855-61; X99873-5 for the COI sequences and X99876-83 for the ITS2 sequences.

Results and discussion

Analysis of sequences

A region of 340 nucleotides in the central part of the mitochondrial COI gene was compared in eight *Tetranychus* species. Another tetranychid mite, *Mononychellus progresivus* Doreste, was also included for comparison. The alignment of sequences was straightforward and no insertions or deletions were found in this region. All the variable sites included in this portion of sequences are shown in fig. 1. The pair-wise genetic distances and number of substitutional differences among tetranychid species are presented in table 1. As expected, the largest genetic distances were found between *M. progresivus* and several of the other taxa examined. In addition, distances between species of the genus *Tetranychus* were in the same range, with the exception of the two clusters formed by the closely related species (1) *Tetranychus urticae* Koch, *T. kanzawai* Kishida and (2) *T. pacificus* McGregor and *T. mcdanieli* McGregor. The phylogenetic tree inferred from the COI nucleotide sequences is shown in fig. 2. The tree indicates that *T. viennensis* and *T. quercivorus* are clustered together and are outside the six other *Tetranychus* species. The confidence of this node (81%) reflects the evolutionary affiliation of these two species. An unique point mutation was detected between the two strains of *T. quercivorus*

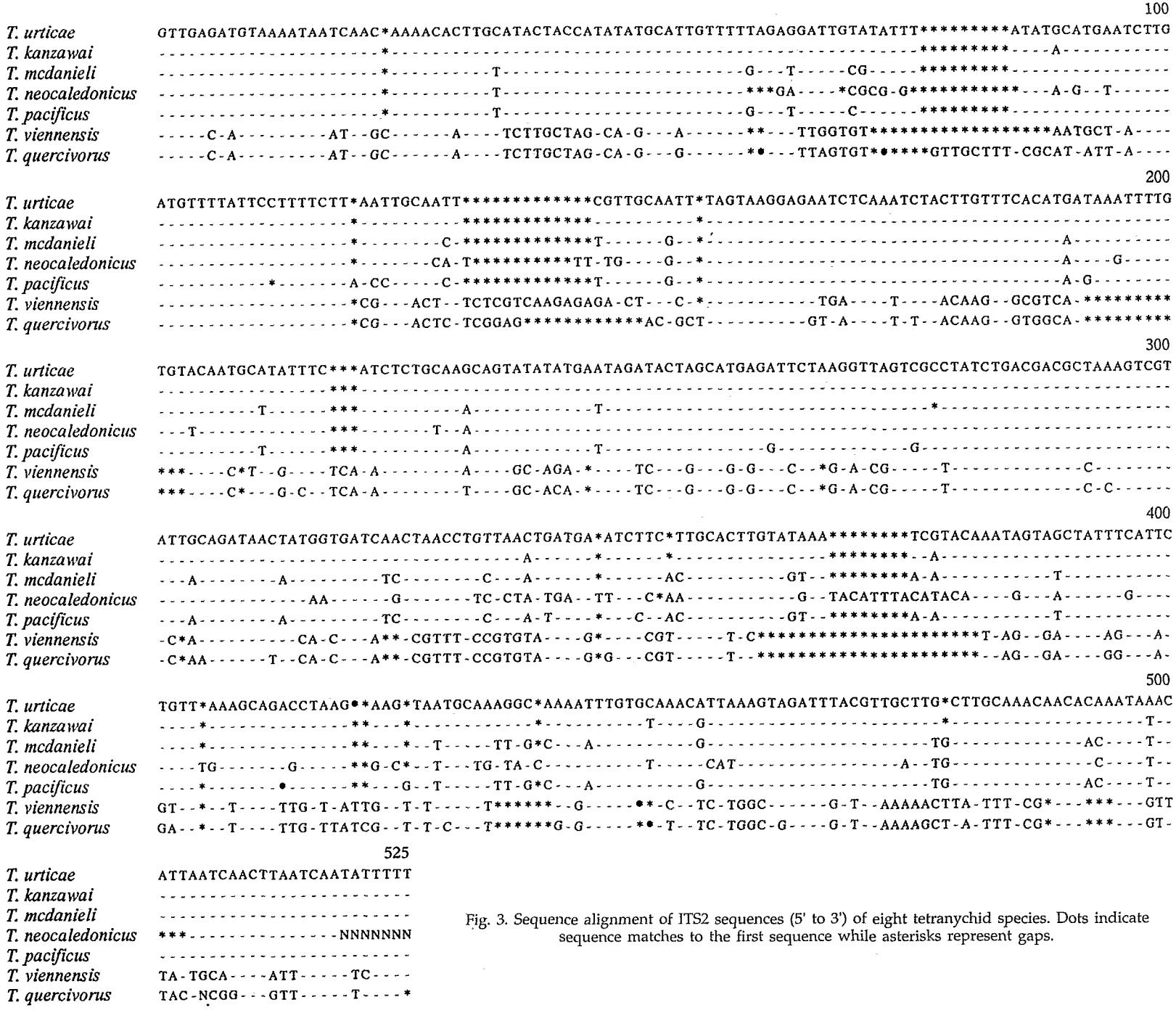

Fig. 3. Sequence alignment of ITS2 sequences (5' to 3') of eight tetranychid species. Dots indicate sequence matches to the first sequence while asterisks represent gaps.

Table 2. Pairwise distances between tetranychid species based on differences in nucleotide sequences of the ribosomal ITS2. Below the diagonal: number of nucleotide substitutions; above the diagonal: distances.

	T. urticae	*T. kanzawai*	*T. mcdanieli*	*T. neocaledonicus*	*T. pacificus*	*T. viennensis*	*T. quercivorus*
Tetranychus urticae		0.0251	0.0537	0.1174	0.0671	0.4962	0.5355
T. kanzawai	18		0.0564	0.1116	0.0725	0.4914	0.5254
T. mcdanieli	34	42		0.1031	0.0175	0.5254	0.5611
T. neocaledonicus	62	60	62		0.1174	0.5254	0.5663
T. pacificus	40	49	8	68		0.5507	0.5876
T. viennensis	159	158	167	164	172		0.1059
T. quercivorus	165	163	172	167	177	47	

examined. There were no differences between the four individuals represented by the four samples of *T. quercivorus* studied.

Analysis of ITS2 sequences gave more precise information about the phylogenetic relationship of *Tetranychus* mites. Extensive studies of the ITS2 sequences in tetranychid have been published elsewhere (Navajas *et al.*, 1996b) and only the characters of direct relevance to phylogenetic analysis are described here. Figure 3 shows the alignments of the ITS2 sequences of seven species of *Tetranychus*. The mites were also studied for COI, with the exception of the species *Tetranychus gloveri* Banks. The sequence of ITS2 of *M. progresivus* is not included in the alignment because of its dissimilarity with the sequences of *Tetranychus*. Table 2 shows the pair-wise genetic distances and number of base substitutions of these taxa in the ITS2 region. The relative distances were similar to those calculated from the COI sequences (table 1), with the noteworthy exception of the distances between both *T. viennensis* and *T. quercivorus* to all other *Tetranychus* that clearly establish the gap existing between the two clusters of species. The phylogenetic tree inferred from these alignments is shown in fig. 4. The topology of this tree is consistent with the one based on COI data (fig. 2). The tree separates the species into two sister groups, one of which unambiguously (100%) groups *T. viennensis* and *T. quercivorus*. The relatively longer branch to its common ancestor with the lineage of the other *Tetranychus* reflects an old divergence of these species. No interstrain or intrastrain polymorphism was detected in ITS2 sequences of samples of *T. quercivorus*.

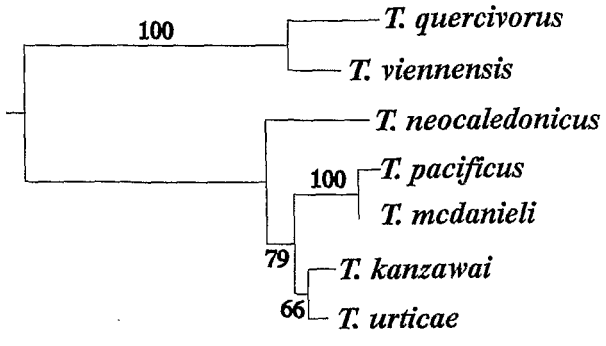

Fig. 4. Phylogenetic tree inferred from ITS2 nucleotide sequences (alignments in fig. 3) of seven tetranychid species. The figures indicate the number of times (per 1000 replicates) that the group occurs together, calculated by bootstrap analysis.

Biological and morphological data

The definition of the genus *Tetranychus* Dufour has been modified several times since its creation in 1832. The currently accepted definition of Pritchard & Baker (1955) is based on a number of morphological and biological characters, enabling the grouping of 136 species. In the genus *Tetranychus* mites live generally on the upperside of leaves and the species are characterized morphologically by the following features: one pair of para-anal setae (setae h2 being absent), empodium with clawlike dorsal member much shorter than proximoventral hairs or else rudimentary, aedeagus bent sharply dorsad, peritreme curved distally or rarely anastomosing and duplex setae widely spaced on tarsus I. This definition covered three species originating from Eastern Asia, with similar aedeagi (distal knob modified as a small anterior angulation near the base of the bent portion, with the caudal angulation very attenuated and tapering) and with an anastomosed peritreme: *Tetranychus viennensis*, *T. quercivorus* and *T. savenkoae* Rekk. This ancestral peritreme configuration is more widespread in the subfamily Bryobiinae, which is considered to be less evolved than that of the Tetranychinae, to which the genus *Tetranychus* belongs (Gutierrez & Helle, 1985). Additionally, a careful re-examination of morphological characters shows the absence of a medio-dorsal spur in *T. viennensis* and *T. quercivorus* in all empodia of specimens of both sexes. This spur is not included in the description of *T. savenkoae* either. Gutierrez & Helle (1985) proposed for the Tetranychini different lineages based on the evolution of the shape of the empodium. This character of phylogenetical importance is linked to the mite's life type and the nature of the surface on which it moves or marks an adaptation to locomotion along silken strands or on a web that is of varying density. The absence of a mediodorsal spur in the three species with anastomosed peritreme suggests that their empodium is formed by a different process to that of the other *Tetranychus* and enhances the idea of membership of another lineage.

The data provided by molecular analysis thus confirm and reinforce morphological information. A larger number of specimens sequenced might help to better assess intraspecific variation of species. Although powerful, molecular techniques are expensive and time consuming. In our study, morphological characters were checked on numerous specimens, whereas sequencing data was confined to a few individuals but revealed strong homogeneity.

The separation of *T. viennensis* and *T. quercivorus* from other species of *Tetranychus* appears justified. It is proposed that the genus *Amphitetranychus* created by Oudemans in 1931 should be restored. First defined for the species *T.*

viennensis alone and used by Geijskes (1939) and Ehara (1956), *Amphitetranychus* was subsequently considered a sub-genus by Wainstein (1960) and then a simple group by Pritchard & Baker (1955). This genus would include the three morphologically homogeneous species *Amphitetranychus viennensis* (Zacher), *A. savenkoae* (Rekk) and *A. quercivorus* (Ehara & Gotoh).

The *Amphitetranychus* and *Tetranychus* that are all found on the undersides of leaves display several common morphological characters, and especially a single pair of para-anal setae and duplex setae widely spaced on tarsus I.

They can be separated by the complete absence of an empodial spur on all the legs in both sexes, similar aedeagi and the formation of an anastomosed peritreme in *Amphitetranychus*, whereas the empodial spur is more or less visible but always present on the legs of one of the sexes and the peritreme ends in a distal curve in *Tetranychus*.

Key to the Tetranychini genera with one pair of para-anal setae

1. Empodium clawlike with proximoventral hairs; duplex setae of tarsus I distal and adjacent 2
– Empodium split distally, usually into 3 pairs of hairs; duplex setae of tarsus I well separated 5
2. 2 pairs of anal setae 3
– 1 pair of anal setae ..... *Atrichoproctus* Flechtmann
3. Opisthosoma with 9 dorsal setae (c2 absent) ..... *Xinella* Ma and Wang
– Opisthosoma with 10 dorsal setae 4
4. All or most legs bearing empodial claws as long as or longer than the proximoventral hairs ..... *Oligonychus* Berlese
– All or most legs with empodial claws about half as long as the proximoventral hairs ..... *Hellenychus* Gutierrez
5. Empodial spur generally visible, peritreme curved distally ..... *Tetranychus* Dufour
– Empodial spur absent, peritreme anastomosed distally ..... *Amphitetranychus* Oudemans

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