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An assessment of the value of nuclear and mitochondrial genes in elucidating the origin and evolution of *Isotoma klovstadi* Carpenter (Insecta, Collembola)

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Abstract: In order to infer the origin and the evolution of Antarctic Collembola, a correct phylogenetic analysis depicting relationships among Antarctic and non-Antarctic species is required. A preliminary assessment of the value of DNA sequences in reconstructing phylogenetic relationships among the Antarctic *Isotoma klovstadi* and other non-Antarctic species was carried out by sequencing one mitochondrial gene (Cytochrome *c* oxidase, subunit II) and two nuclear genes (a fragment of the 28S rDNA and the Elongation Factor-1α). Estimates of base composition heterogeneity revealed that in the two protein-coding genes (COII and EF-1α) 3rd codon position sites are compositionally very heterogeneous and the analysis of these two genes was therefore performed only on 1st and 2nd codon position sites. Phylogenetic analyses using Maximum Likelihood, Maximum Parsimony and Minimum Evolution revealed that the COII and the EF-1α genes are more suitable than the D3 fragment for the reconstruction of phylogenetic relationships within the Family Isotomidae to which *Isotoma* and several other genera of Antarctic Collembola belong.

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Introduction

Among the few groups of insects which are found in Antarctica, Collembola are the only free-living species inhabiting the continental area (excluding the Antarctic Peninsula). Together with mites, the springtails represent the major part of the Antarctic edaphic fauna. Recent accounts report the number of species in the Antarctic continent as approaching 25 (Greenslade 1995), but the faunistic catalogue is still incomplete. Although species diversity is poor, some collembolan communities are very abundant in terms of number of individuals, such as, for example, the isotomid species *Cryptopygus antarcticus* Willem, 1901 and *Gressittacantha terranova* Wise, 1967. From a faunistic point of view, a sharp difference has been observed between Western (Scotia Arc and Antarctic Peninsula) and Eastern Antarctica (Greenslade 1995), which seem to share only one species (*Friesea grisea* (Schäffer, 1891) of the family Neanuridae). Some species are endemic to the continent, but others belong to widely distributed genera. Such a distribution probably reflects a varied origin for Antarctic Collembola. The present collembolan fauna might be a combination of relic taxa present since the continent was located at a tropical latitude, and more recent immigrant species which may have been introduced into Antarctica from the southern continents (Wise 1967, Greenslade 1995).

The study of evolutionary relationships between Antarctic and subantarctic species, as well as species of the southern continents, would provide an insight into the history of the fauna of Antarctica, and provide indications on possible routes of colonization of the continent. Molecular markers are now well established for inferring such relationships, and, in particular, DNA sequencing has been found highly valuable (Simon *et al.* 1994, Swofford *et al.* 1996). Different genes have different rates of evolution: some of them, the most variable ones, are useful in studying relationships among closely related taxa, whereas others, the most conserved ones, are useful at a higher taxonomic level.

In this paper, we present the complete sequence of one mitochondrial gene and the partial sequence of two nuclear genes in the Antarctic species *Isotoma klovstadi* Carpenter, 1908 (Isotomidae). This species is endemic to North Victoria Land, where its southernmost record is at Cape Phillips (Frati *et al.* 1997a) (Fig. 1). The genus *Isotoma* is widely distributed all over the world, but *I. klovstadi* is the only species in eastern continental Antarctica. A congeneric species, *Isotoma (Folsomotoma) octooculata* Willem, 1901, is found in the Antarctic Peninsula. No formal designation of the subgenus assignment has been made yet for *Isotoma klovstadi*.

The sequences obtained from *I. klovstadi* for the target genes are compared with those from other closely and more distantly related collembolan species to assess whether these genes can be used in the reconstruction of a molecular phylogeny of Antarctic, subantarctic and circumantarctic species.

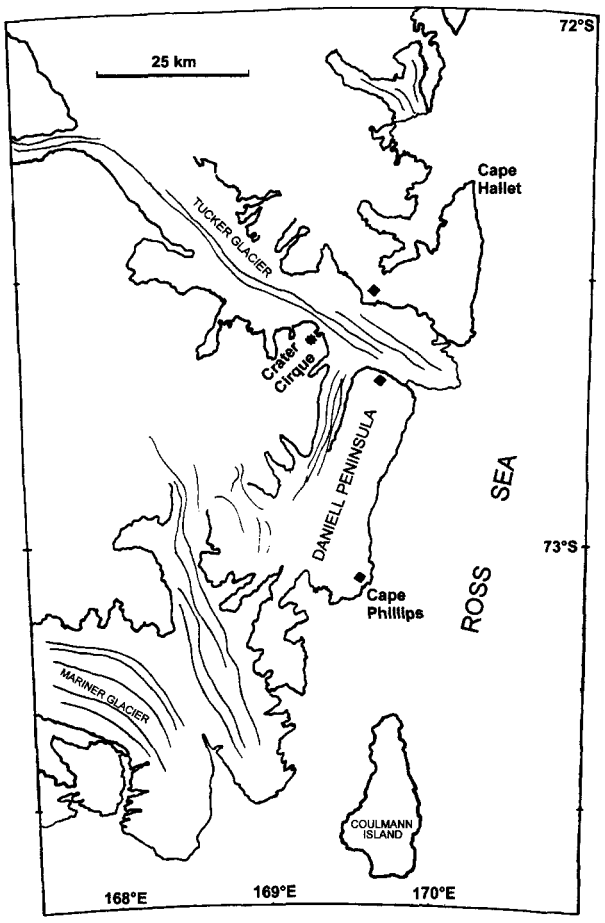

Fig. 1. Geographical position of the collecting locality (*). Additional sites where *Isotoma klovstadi* was collected are marked with ◆ (from Frati *et al.* 1997a).

Materials and methods

Several specimens of *I. klovstadi* were extracted using Tullgren funnels from mosses collected at Crater Cirque (72°37'S, 169°22'E: Fig. 1) during the period December 1995–January 1996, and brought to Italy frozen at -80°C. *Isotoma klovstadi* is abundant among mosses from the genera *Tortula* and *Bryum* on the shore of the small lake within the crater. They are usually found crawling on the ground, especially where humidity is higher due to snow melting (Frati *et al.* 1997a).

Total DNA was extracted from single individuals with the procedure described by Simon *et al.* (1991), with slight modifications due to the small size of the specimens. The whole specimen was ground in homogenizing buffer with the addition of SDS and proteinase K, extracting with phenol/chloroform and precipitating with ethanol. Extracted DNA was used as the template in the Polymerase Chain Reaction (PCR: Saiki *et al.* 1985), using the primers listed in Table I. Amplifications were carried out for 35 cycles with the following profile: denaturation at 94°C (1 min 10 sec), annealing at 45°C (1 min 10 sec) and extension at 72°C (1 min 30 sec). The amplified product was run on an agarose gel, the band excised from the gel and the DNA purified by phenol/chloroform extraction followed by ethanol precipitation. Double-stranded sequencing was performed following the protocol by Hsiao (1993) and using both the amplification primers, as well as other internal primers (Table I).

Three genes were amplified; the subunit II of the mitochondrial cytochrome *c* oxidase (COII) (for which the complete sequence was obtained), a fragment of the nuclear large ribosomal RNA subunit (D3) and a fragment of the nuclear gene encoding for the Elongation Factor-1α (EF-1α), which is involved in the translation of mRNAs into polypeptides. The sequences were aligned using the software CLUSTAL W (Thompson *et al.* 1994) with sequences obtained in other collembolan species of the genera *Isotomurus* and *Tetracanthella* (Isotomidae), and *Orchesella* (Entomobryidae). In the rDNA gene, the alignment was manually modified on the basis of conserved motifs in the secondary structure of the molecule. Sequence analysis was performed using a test version (4d64) of the program PAUP*, kindly provided by D. Swofford. Sequence analysis included a statistical assessment of compositional heterogeneity across taxa (divided by codon positions), nucleotide content, percentage and distribution of variable sites across nucleotide positions, estimate of among-site rate variation (ASRV), calculation of genetic distances under a variety of models of evolution and a preliminary phylogenetic analysis using the Minimum Evolution (ME), the Maximun Parsimony (MP) and the Maximum Likelihood (ML) methods. An additional estimate of base composition heterogeneity across taxa was obtained according to the method based on Z-scores, as described in Andrews *et al.* (1998).

The nucleotide sequences of *Isotoma klovstadi* reported in this paper have been deposited in the EMBL, GenBank and DDBJ Nucleotide Sequence Databases under accession numbers Y11703, Y11704 and AJ009853. New sequences of additional species used in this study were deposited under accession numbers AJ009821-2, AJ009850-2, AJ009854-7. Other sequences used in this study were retrieved from the above Databases (accession numbers: X80688, X80689, X95790, X95894, X84951, X84952, X84954, X84955).

Results and discussion

Mitochondrial COII gene

A total of 694 bp were sequenced in the COII gene of *Isotoma klovstadi*, beginning from its initiation codon. This sequence was aligned with those obtained in five species of the closely related genus *Isotomurus* (Isotomidae) and the more distantly related species *Orchesella villosa* (Geoffroy, 1762) (Entomobryidae). The latter species may be used as an outgroup reference in a phylogenetic analysis. The alignment is given in the Appendix I.

The initiation codon of *I. klovstadi* is ATA, which is used, among all other Collembola studied, only in the onychiurid

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Table I. List of primers used in this study for amplification and sequencing.

Primers[a]	Sequence	Location
COII (790bp)		
TL2-J-3037[b]	5'-AATATGGCAGATTAGTGCA-3'	Flanking: on the $tRNA^{Leu}$
C2-N-3389[b]	5'-TCAATATCATTGATGTCC-3'	Internal
C2-N-3661[b]	5'-GCTCCACAAATTTCTGAACA-3'	Internal
TK-N-3785[b]	5'-GTTTAAGAGACCAGTACTT-3'	Flanking: on the $tRNA^{Lys}$
D3 (350bp)		
D3a	5'-GACCCGTCTTGAAACACGGA-3'	Flanking
D3b	5'-TCGGAAGGAACCAGCTACTA-3'	Flanking
EF-1α (1230bp)		
M3	5'-CACAT(CT)AACAATGTCGT(GC)AT(CT)GG-3'	Flanking
rcM44.9	5'-CTTGATGAAATC(CT)CTGTGTCC-3'	Internal
M44-1	5'-GCTGAGCG(CT)GA(AG)CGTGGTATCAC-3'	Internal
rcM51-1	5'-CAT(AG)TTGTC(GT)CCGTGCCA(GT)CC-3'	Flanking

[a]Aproximate length of PCR-amplified fragment is given in parentheses.
[b]Modified from those reported in the appendix of Simon *et al.* (1994).

Tetrodontophora bielanensis (Waga, 1842) (Frati *et al.* 1997b). All other *Isotomurus* species have ATC as initiation codon. This variability of initiation codons among collembolan species (ATA, ATC and ATT), suggests that the translation machinery must have some mechanism to distinguish the correct position where translation should start.

The termination point of the gene is difficult to determine. While in *Orchesella* the gene is 690 bp long, Frati *et al.* (1997b) observed that no clear termination codon is found at the corresponding position in many collembolan species. *Isotoma klovstadi* has the codon ACA (Thr) in this position. In other cases, the presence of a T coupled with polyadenylation is believed to mark the termination of the gene (Anderson *et al.* 1981, Clayton 1984) and there are many examples of insect mitochondrial genes lacking a clear termination codon (Liu & Beckenbach 1992). There is no T in the 230th codon of *I. klovstadi*, but the two Ts located two bases downstream of the 230th codon might be used as a signal for termination. The 3'-end of the gene is also difficult to align due to its high variability and the possibility that it is overlapped with the sequence of the subsequent gene (the $tRNA^{Lys}$). The sequences were therefore truncated at position 672 for the present analysis.

As expected for insect mitochondrial genes, there is a remarkable bias towards Adenine (A) and Thymine (T) residues. In *I. klovstadi*, A+T content is 64.7% (Table II), comparable to what is observed in other collembolan species (Frati *et al.* 1997b). Also the percent of Guanine (G) in third codon positions (5.2% in *I. klovstadi*) reflects the observations made in other collembolan species. In all insects studied, an evolutionary trend towards the increasing of overall A+T content and the reduction of G in third codon positions in the more recently derived orders has been clearly demonstrated (Jermiin & Crozier 1994, Frati *et al.* 1997b).

The index of compositional bias (Irwin *et al.* 1991) measured on the whole data set is concordant with that observed in other Collembola (Table II) but, when the sequence is divided into the three codon positions, a slightly higher compositional bias at third codon positions is observed in *I. klovstadi* with respect to the collembolan average (Frati *et al.* 1997b).

Comparing the COII sequence of *I. klovstadi* with the sequence of other isotomid species, 40.3% of the sites are variable (Table III). Most of the variable sites between *I. klovstadi* and other *Isotomurus* species are, as expected, concentrated in 3rd codon positions, while only 6.6% of them occur in the very conserved 2nd codon positions. On the other hand, as many as 89.7% of the 3rd codon position sites are variable. Therefore, there is a high probability of occurrence of multiple hits in these variable sites even between closely related genera. The occurrence of multiple hits needs to be carefully evaluated when sequence data are used for phylogenetic reconstructions because they may cause a severe underestimation of genetic distances (Simon *et al.* 1994) and, by erasing the record of previous substitutions, they may prevent the correct establishment of synapomorphic changes.

When DNA sequences have to be used for reconstructing phylogenetic relationships among taxa, it is of crucial importance to collect all information from the data which allows us to understand the evolution of the sequences during time. One of the most important factors is to consider whether the sequences evolved under stationary conditions with respect to base composition and rates of variability across sites

Table II. Base composition in the COII gene and a fragment of the EF-1α gene of *Isotoma klovstadi*. Compositional bias is calculated according to Irwin *et al.* (1991).

	COII	EF-1α
A+T content (%)	64.7	56.3
% G in 3rd codon positions	5.2	18.4
Compositional bias		
overall	0.192	0.086
1st codon position	0.093	0.276
2nd codon position	0.168	0.183
3rd codon position	0.319	0.106

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Table III. Proportion of variable sites in the COII and EF-1α genes across codon positions in comparisons of *Isotoma klovstadi* with several *Isotomurus species*.

	A	B
COII: 40.3% variable sites		
COII 1st codon position	0.192	0.232
COII 2nd codon position	0.066	0.080
COII 3rd codon position	0.742	0.897
EF-1α: 13.2% variable sites		
EF-1α 1st codon position	0.048	0.019
EF-1α 2nd codon position	0.048	0.019
EF-1α 3rd codon position	0.904	0.357

A. Fraction of total variable sites that are found in each codon position.
B. Fraction of sites in each codon position that are variable.

Table IV. Test of base composition heterogeneity across taxa and estimates of the α-value of among-site rate variation (estimated *via* ML with the HKY model) for each subset of codon positions of the mtCOII gene.

	Chi-square test (*P*)		ASRV
codon positions	A	B	α value
All sites	0.8504	0.2375	0.2524
1st + 2nd	0.9999	0.9999	0.0809
1st	0.9998	0.9982	0.1507
2nd	1.0000	1.0000	0.0591
3rd	0.0769	0.0004	0.2283

A. *Isotoma klovstadi* + *Isotomurus* species
B. *Isotoma klovstadi* + *Isotomurus* species + *Orchesella villosa*

(Galtier & Gouy 1995). Insect mitochondrial genes are well known for their A+T bias, but special attention should be given to homogeneity of base composition across taxa. This can be done by a χ^2 test of independence (as implemented in PAUP*) which, in protein coding genes, can also be applied to each codon position separately. Table IV reports estimates of significance of base composition homogeneity across taxa including *Isotoma klovstadi* and the six species of *Isotomurus*. Although the complete data set (all sites) does not appear to suffer from a significant base composition heterogeneity across taxa (P = 0.8504), 3rd codon positions exhibit a relevant heterogeneity (P = 0.0769), showing that these sites evolved under non-stationary conditions. The same results were obtained when Z-scores (Andrews *et al.* 1998) were calculated for the three subsets of codon positions. Z-score distributions can be plotted in a graph (Fig. 2), allowing visual estimation of composition heterogeneity. While most of the comparisons based on 1st codon positions and all of those based on 2nd codon positions give negative Z-scores, the majority of comparisons based on 3rd codon positions give positive Z-scores, indicating the presence of substantial heterogeneity in base composition across taxa. Interestingly, the comparisons involving *Orchesella villosa* have the highest Z-scores. It is worthwhile noting that the method presented by Andrews *et al.* (1998) does not exclude invariate sites - it may therefore give a conservative picture of the compositional heterogeneity in this data.

Third codon positions are also the most variable ones as the level of divergence between the sequences at this position is relatively large (data not shown), and they may be more likely to have experienced multiple substitutions. Since these factors are known to confound phylogenetic reconstructions, this assessment suggests that it is advisable to remove 3rd codon positions from the analysis of the COII gene as was done in the study of a larger collembolan data set by Frati *et al.* (1997b).

Another very important aspect of sequence evolution, sometimes neglected in phylogenetic analyses, is the different rate of variability among sites (among-site rate variation: ASRV; Yang *et al.* 1994, Sullivan *et al.* 1995). This is directly correlated to different functional constraints on sites and to the occurrence of multiple hits in certain nucleotide positions. Such a feature can be estimated and incorporated in the phylogenetic analysis by assuming rates of variability among sites to follow a Γ-distribution. In particular, the shape parameter α provides a direct estimate of ASRV, with α being inversely related to the amount of ASRV (low α-values mean strong ASRV: Yang 1993). Estimates of the α-values were obtained by the Maximum-Likelihood method implemented in PAUP*, using the HKY model of evolution (Hasegawa *et al.* 1985: see below for the rationale underlying the selection of this model) and are shown in Table IV subdivided by codon positions. The α-value was estimated during the process of selecting the best tree for each subset of data using heuristic ML searches under the HKY model. There appears to be

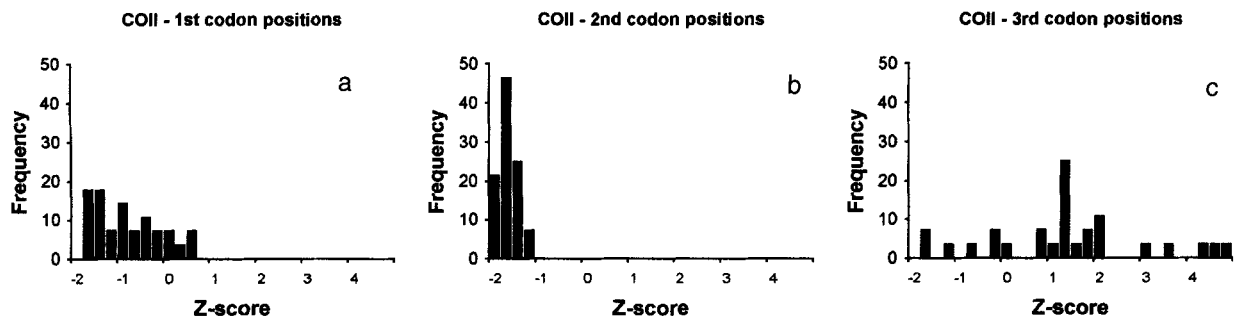

Fig. 2. Z-score distributions in comparisons (n = 28) based on the COII gene.

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strong among-site rate variation in all combinations of sites. The presence of strong among-site rate variation, especially in the 1st+2nd codon position data set, coupled with the occurrence of multiple hits, requires the data to be analysed incorporating correction for these factors.

Different substitution rates for sites and for different nucleotides affect phylogenetic methods based on genetic distances and parsimony. It is therefore particularly necessary to use a phylogenetic method based on a specific model of evolution, accounting for different substitution rates among different nucleotides, and incorporating a suitable correction for among-site rate variation. The Maximum Likelihood approach provides such a method and it allows the user to estimate directly from the data the most appropriate model of evolution for the data set under analysis (Swofford *et al.* 1996). We therefore chose four different models of evolution, and we separately incorporated, for each of them, four different ways of accounting for ASRV. The four different models, in increasing order of complexity, were:

a) the JC model (Jukes & Cantor 1969), where all types of substitutions have the same probability of occurrence and equal base composition within taxa is assumed,

b) the K2P model (Kimura 1980), assuming equal base composition but different probabilities for transitional vs transversional changes,

c) the HKY model (Hasegawa *et al.* 1985), assuming different probabilities for transitional vs transversional changes and also unequal base composition within taxa, and

d) the GTR model (Yang 1994), assuming unequal base composition and a different probability for each of the six possible nucleotide substitions.

Among-site rate variation was incorporated in four ways:

a) all sites evolving at the same rate (equal rates - E),

b) some sites assumed to be invariable and the remaining sites having equal rates (I),

c) variation across sites assumed to follow a Γ distribution represented by the α shape parameter (Γ), and

d) some sites assumed to be invariable and the remaining sites assumed to follow a Γ distribution ($I + \Gamma$).

The combination of the four substitution models and the four rate-distribution models produced 16 different models with which we measured the likelihood score of three competing trees: a Log-Det distance-based tree selected under the principle of Minimum Evolution and two equally parsimonious trees selected under the principle of Maximum Parsimony, with equal weights assigned to all sites.

The three trees had different topologies and one of the MP trees always showed the best likelihood scores under all 16 different models. Among those, the best scores were obtained with the GTR+I+Γ model (-lnL=3529.936), but the simpler HKY+I+Γ model also had a very good score (-lnL=3532.656). A likelihood-ratio test (χ^2-test with the degrees of freedom given by the difference between the free parameters in the models under comparison) demonstrated that the score obtained with the HKY+I+Γ model was not significantly different from the one obtained with the GTR+I+Γ model ($\chi^2 = 5.44$, $df = 4$, $P = 0.240$). Therefore, the HKY+I+Γ was selected because of the lower variance associated with the simpler model. The same approach to selecting the most appropriate model was used for the subset of data where 3rd codon positions were removed. In this case, the less parameter-rich HKY+Γ was preferred having a likelihood score non-significantly different from the HKY+I+Γ and the more complex GTR models. The HKY model was therefore used to compute the α-value discussed before (Table IV).

The HKY+Γ model was also used to compute genetic distances (Table V) based on 1st and 2nd positions only. These distances can be compared with uncorrected pairwise distances showing that these latter represent a strong underestimation of real genetic distances. HKY+Γ distances were also used to select a phylogenetic tree under the Minimum Evolution criterion.

A preliminary phylogenetic analysis using a heuristic ML search on 1st and 2nd codon positions provided the tree showed in Fig. 3. *Isotoma klovstadi* is correctly represented as the sister-taxon of all species of the genus *Isotomurus*, which is shown as a well-supported (82% bootstrap) monophyletic taxon. Within this genus, *Isotomurus fucicola* (Reuter 1891) is the basal species, with *I. maculatus* (Schäffer,

Table V. Genetic distance estimates based on 1st and 2nd codon positions of the COII gene. Uncorrected *P*-distances are below the diagonal while HKY+Γ-corrected distances are shown above the diagonal.

Taxon	1	2	3	4	5	6	7	8
1. *Isotoma klovstadi*	-	0.100	0.117	0.125	0.128	0.136	0.137	0.196
2. *Isotomurus fucicola*	0.083	-	0.084	0.061	0.064	0.064	0.067	0.225
3. *Isotomurus hadriaticus*	0.094	0.071	-	0.070	0.070	0.070	0.070	0.215
4. *Isotomurus maculatus*	0.098	0.054	0.060	-	0.026	0.036	0.037	0.241
5. *Isotomurus nebulosus*	0.100	0.056	0.060	0.025	-	0.029	0.029	0.247
6. *Isotomurus palustris*	0.105	0.056	0.060	0.034	0.027	-	0.040	0.229
7. *Isotomurus unifasciatus*	0.105	0.058	0.060	0.034	0.027	0.036	-	0.266
8. *Orchesella villosa*	0.136	0.150	0.145	0.156	0.159	0.152	0.165	-

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1896), *I. nebulosus* Lek & Carapelli, 1998, *I. palustris* (Müller, 1776) and *I. unifasciatus* (Börner 1901) being clustered in an unresolved group. The correct placement of *Isotoma klovstadi* as the sister-taxon to *Isotomurus* was also confirmed in the ME (with HKY+Γ distances) and the MP (with equal weights) analyses (on 1st and 2nd codon positions only) which differed from the ML tree for the presence, as the basal *Isotomurus* species, of *I. hadriaticus* sp.n. rather than *I. fucicola*. When 3rd codon positions are included into the analysis, the ML analysis using the HKY+I+Γ model select a tree where *Isotomurus fucicola* is replaced, as the basal *Isotomurus species*, by *I. hadriaticus*. In addition, bootstrap support values (100 replicates) in the all-site analysis are much lower, especially within *Isotomurus* species. The use of the highly variable 3rd codon positions seems to introduce a higher background noise in the analysis, making it difficult to recover phylogenetic relationships at the species level.

On the basis of this analysis, it is possible to conclude that the COII gene can be considered a reasonably good gene to study phylogenetic relationships at the genus level within and between Isotomidae genera, provided that potential sources of noise are removed and that the data are analysed according to the most likely model of evolution. The exclusion of 3rd codon positions, strongly affected by multiple substitutions and exhibiting substantial base composition heterogeneity across taxa, is advisable to remove potentially misleading effects. While it appears appropriate to use only 1st and 2nd codon positions for the phylogenetic analysis, this subset of sites has a strong among-site rate variation (Table IV), making it necessary to incorporate a more complex mode of analysis than is usually done (e.g. the JC model).

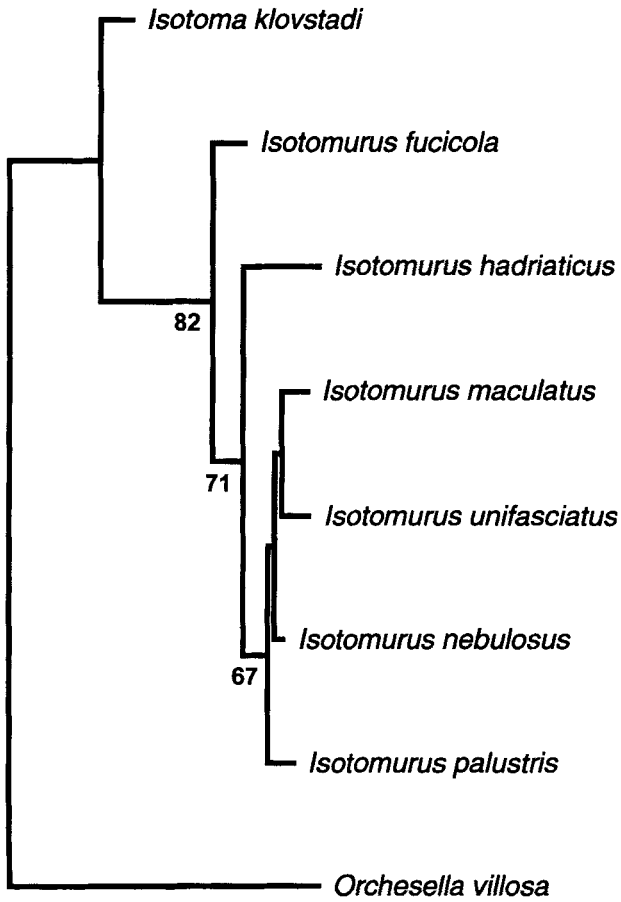

Fig. 3. Maximum likelihood tree depicting relationships among the species studied based on 1st and 2nd codon sites of the COII sequences (HKY+Γ model). Branch lengths were estimated *via* ML and bootstrap support values shown at the nodes and are based on 100 replications (with model parameters estimated in each replication).

Nuclear EF-1α gene

The nuclear EF-1α gene encodes a protein involved in the translation process. It is considered a slow evolving gene and it has been used at different phylogenetic levels in other taxa, such as tube worms (Kojima *et al.* 1993), moths (Cho *et al.* 1995), Lepidoptera (Mitchell *et al.* 1997), and to study relationships among arthropod classes (Regier & Shultz 1997).

A 504 bp fragment of the EF-1α gene, comprising its 5'-half, was amplified and sequenced in two specimens of *Isotoma klovstadi* and aligned (using CLUSTAL W) with three species of *Isotomurus* (Isotomidae) and the Entomobryidae *Orchesella cincta* (Linné, 1758) (see Appendix II). The two specimens of *I. klovstadi* differed by only five substitutions (one of which is non-synonymous) and only one sequence is included in the alignment and the analysis. The coding sequence of the EF-1 gene of all collembolan species is interrupted by an intron of variable length between codon positions 93 and 94. In *I. klovstadi* this intron is 97 bp long and has the same sequence in the two specimens studied.

Nuclear genes are not expected to have the same A+T bias as insect mitochondrial genes. Average A+T content in the five collembolan species studied here is only slightly higher (52.3%) than the unbiased state. However, *I. klovstadi* has a somewhat higher A+T content (56.3%) than the other species (Table II). The increase of A+T content in *I. klovstadi* with respect to *Isotomurus* species is almost completely due to a higher amount of A+T in 3rd codon positions, but the most heavily biased sites in all collembolan species are 2nd codon positions which always have A+T content higher than 60% (63.7% in *I. klovstadi*). Although overall compositional bias is fairly low (Table II), there is an interesting difference in the distribution of this bias when the EF-1α gene is compared to the mitochondrial COII gene. Compositional bias, in fact, is highest at 1st codon positions, which are biased towards As (33.8%) and Gs (36.9%), and lowest at 3rd codon positions.

A total of 62 variable sites (13.2%) were found among the Isotomidae species (Table III). The greatest variability was found on 3rd codon positions, with 1st and 2nd codon positions being extremely conserved. It appears clear that levels of variability in the EF-1α gene are remarkably lower than those observed in the faster-evolving mtCOII gene.

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The analyses of base composition heterogeneity using both PAUP* (Table VI) and the distribution of Z-scores (Fig. 4) show composition to be relatively homogeneous at 1st and 2nd codon positions and substantially more heterogeneous at 3rd codon positions. Although the complete data set (all sites) does not seem to suffer from base composition heterogeneity, the presence of heterogeneity in 3rd codon positions might suggest that it would be better to remove these sites from the analysis. However, the removal of 3rd codon positions would leave us with almost no variable sites (only six among Isotomidae, only nine including *Orchesella cincta*), dramatically reducing the amount of information and, consequently, the accuracy of the analysis.

The application of the same model-selection approach used for the COII gene, but based, in this case, on only one starting tree (both the ME and MP analyses, in fact, recovered the same topology), selected the GTR+Γ model as the most appropriate one to analyse this data set. The choice of a GTR model, where a specific probability is calculated for all the six possible types of substitutions, appears to be justified by a strong bias towards C<->T changes, which outnumber all other types of substitutions. Accommodation of among-site rate variation through the use of a Γ-distribution model was necessary because of the evidence that rate variability across sites is fairly high in this sequences ($\alpha = 0.215$). The GTR+Γ was therefore used to perform a Maximum Likelihood exhaustive search by estimating all model parameters during the search. The confidence of the nodes was estimated by 100 bootstrap replicates providing the tree in Fig. 5, where the genus *Isotomurus* constitutes a well-supported monophyletic taxon, with *I. maculatus* and *I. nebulosus* being sister-species. This tree has the same topology as the initial ME and MP trees, and it was selected also in a Minimum Evolution search based on GTR+Γ-corrected genetic distances (shown in Table VII).

Unfortunately, the EF-1α gene has been sequenced only in a small number of taxa to allow conclusive remarks. The use of 3rd codon positions (less variable than in the mtCOII gene) allows a more detailed analysis at the species level, as suggested by consistently high bootstrap values among *Isotomurus* species. When genetic distances become higher, such as between different genera, it will be necessary to assess the amount of variability affecting 1st and 2nd codon position as opposed to that affecting the more variable 3rd codon positions, which may result in the increase of the rate of multiple substitutions.

Table VI. Test of base composition heterogeneity across taxa and estimates for each subset of codon positions of the fragment of the EF-1α gene.

	Chi-square test (*P*)	
codon positions	A	B
All sites	0.8618	0.8653
1st + 2nd	0.9999	0.9999
1st	0.9999	1.0000
2nd	0.9999	1.0000
3rd	0.1652	0.0683

A. *Isotoma klovstadi* + *Isotomurus* species
B. *Isotoma klovstadi* + *Isotomurus* species + *Orchesella cincta*

Nuclear rRNA D3 fragment

The large nuclear rRNA molecule (28S) is composed of very conserved regions, presumably associated with important functional domains, which are separated by "expansion" fragments, where functional constraints are very relaxed and the degree of variability, both in sequence and in length, is much higher (Michot *et al.* 1984). One of these fragments is the D3 region, which has been sequenced in this work. Another feature of rRNA molecules is that they can be folded on themselves to form a secondary structure by the annealing of complementary regions. A typical rRNA secondary structure is therefore constituted by double-stranded portions (stems) intercalated with single-stranded connecting fragments (loops and bulges).

The primers used here gave an amplification product of about 350 bp, 303 of which were sequenced (see Appendix III). On the basis of the models drawn for other species (Michot *et al.* 1984, Hancock *et al.* 1988), we were able to reconstruct the presumed secondary structure of this fragment in *Isotoma klovstadi*, which is given in Fig. 6. Of the sequenced 303 bases, 276 (from position 20 to 295) were aligned with the sequences of five species of *Isotomurus* (Isotomidae), one undetermined species of the genus *Tetracanthella* (Isotomidae) and the Entomobryidae

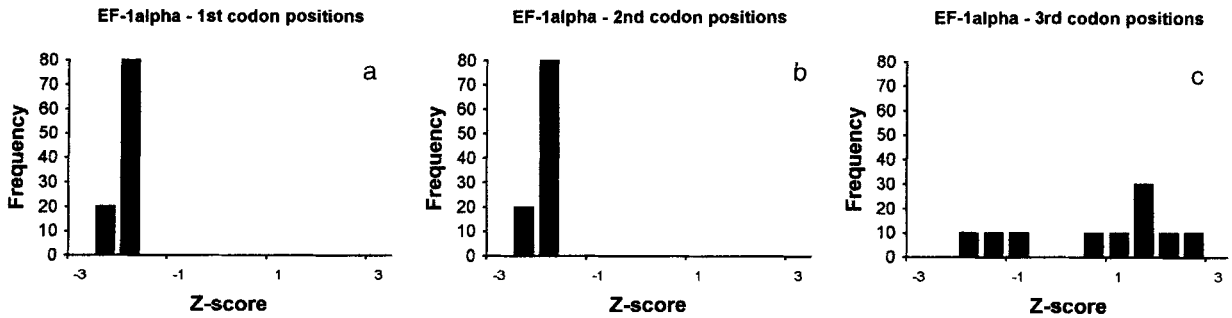

Fig. 4. Z-score distributions in comparisons ($n = 10$) based on the EF-1α gene.

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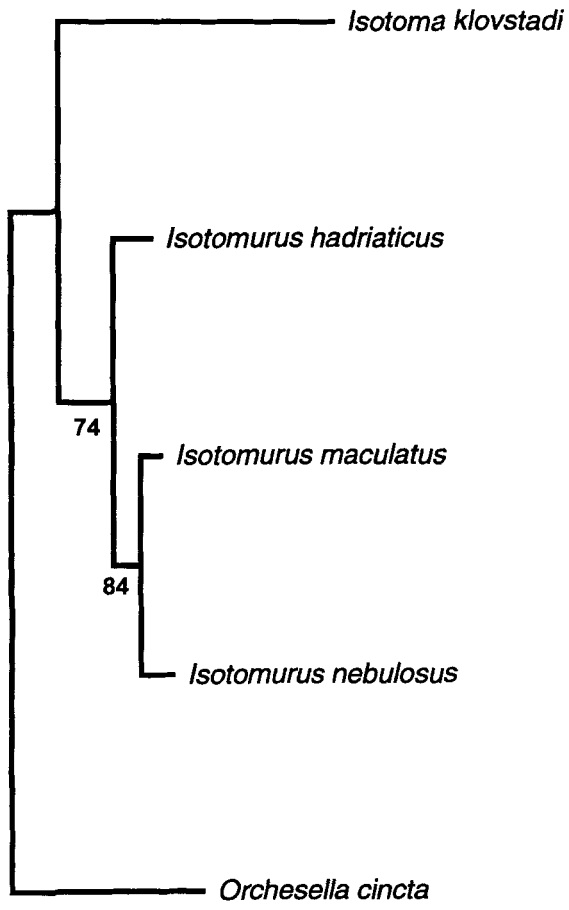

Fig. 5. Maximum likelihood tree depicting relationships among the species studied based on the fragment of the EF-1α gene. Branch lengths were estimates *via* ML and bootstrap support values shown at the nodes and are based on 100 replications (with model parameters estimated in each replication).

Orchesella ranzii Parisi, 1960. We used CLUSTAL W (Thompson *et al.* 1994) to align the sequences, but the alignment was then manually improved by taking into account the conservation of particular structural motifs (Kjer 1995).

In order to align all sequences, indels had to be added at 10 sites. Most of the indels, as well as most of the variable sites, were concentrated in the 5'-half part of the fragment; the 3'-half is the most conserved part of the molecule. The correct treatment of indels in phylogenetic studies is controversial and we therefore decided to remove them in the analysis. After removal of indels, a total of 51 variable sites (18.7%) were found among all collembolan species examined. The number of variable sites decreases to 41 (15%) if only the Isotomidae genera are considered, reflecting the closer relationships among these latter taxa. Most of the variable sites, together with 5 indels, were concentrated from positions 76 to 123 of the alignment, in a region containing the stem #D3.4. Interestingly, a closer look to the sequences suggests that the basic structure of this stem seems to be maintained

Table VII. Genetic distance estimates based on the fragment of EF-1α gene. Uncorrected *p*-distances are below the diagonal while GTR+Γ-corrected distances are shown above the diagonal.

Taxon	1	2	3	4	5
1. *Isotoma klovstadi*	-	0.160	0.155	0.176	0.219
2. *Isotomurus hadriaticus*	0.106	-	0.039	0.044	0.146
3. *Isotomurus maculatus*	0.104	0.034	-	0.025	0.140
4. *Isotomurus nebulosus*	0.114	0.036	0.023	-	0.147
5. *Orchesella cincta*	0.142	0.098	0.096	0.098	-

through compensatory substitutions in the two parts of it. In spite of the considerable amount of variability in the sequence of this stem among *I. klovstadi*, the five *Isotomurus* species and *Tetracanthella* sp., the structure of the stem could be easily drawn (Fig. 6). Therefore, according to the predictions, the general secondary structure of the molecule is conserved, even in those areas where variability is more intense.

Base composition in the D3 fragment is more uniform than in the mtCOII gene, but in *Isotoma klovstadi*, which has the highest A+T content with respect to all other species, A+T (56.8%) still outnumber G+C. The χ^2 test of independence of base composition implemented in PAUP* reveals that there is no heterogeneity across taxa ($P = 0.999$ both with and without *O. ranzii*).

In order to choose the most appropriate model of evolution for this data set, we used the same model-selection approach described above for the COII gene. Selection was performed on the basis of one ME (LogDet-corrected distances) and two MP (equal weights) trees; the second MP tree (showing *I. klovstadi* and *Tetracanthella* sp. as sister-species and *Isotomurus* as a paraphyletic taxon) always had the best scores with all the 16 combinations of substitution models and ASRV. In this case, even if the GTR+I+Γ model had the best likelihood score (-LnL = 766.76615), the score of the simpler HKY+I model (-LnL = 769.28619) was not significantly different, and it was therefore chosen to analyse these data. A more detailed analysis of ASRV showed that most of the sites (>76%) were invariable. When the HKY+I+Γ model is used, ASRV for the non-invariable sites is very low (α>3), suggesting these sites evolve at about the same rate. These sites are presumably represented by the fast evolving ones comprised between positions 76 to 123. On the other hand, when ASRV is accommodated without assuming invariable sites (HKY+Γ model), the α value is extremely low (< 0.015) reflecting the extreme difference in variability rates between the fast evolving and the invariable sites. This particular combination of very low and very fast evolving sites would explain why the analysis based on a model that assumes the presence of invariable sites has a better likelihood score than that based on a model that assumes a Γ-distribution of ASRV. This happens for both the HKY and the GTR models (data not shown).

The HKY+I model was used to conduct a heuristic search yielding a tree (Fig. 7) having the score of -LnL = 769.28619 and the same topology of the best MP tree. This tree shows

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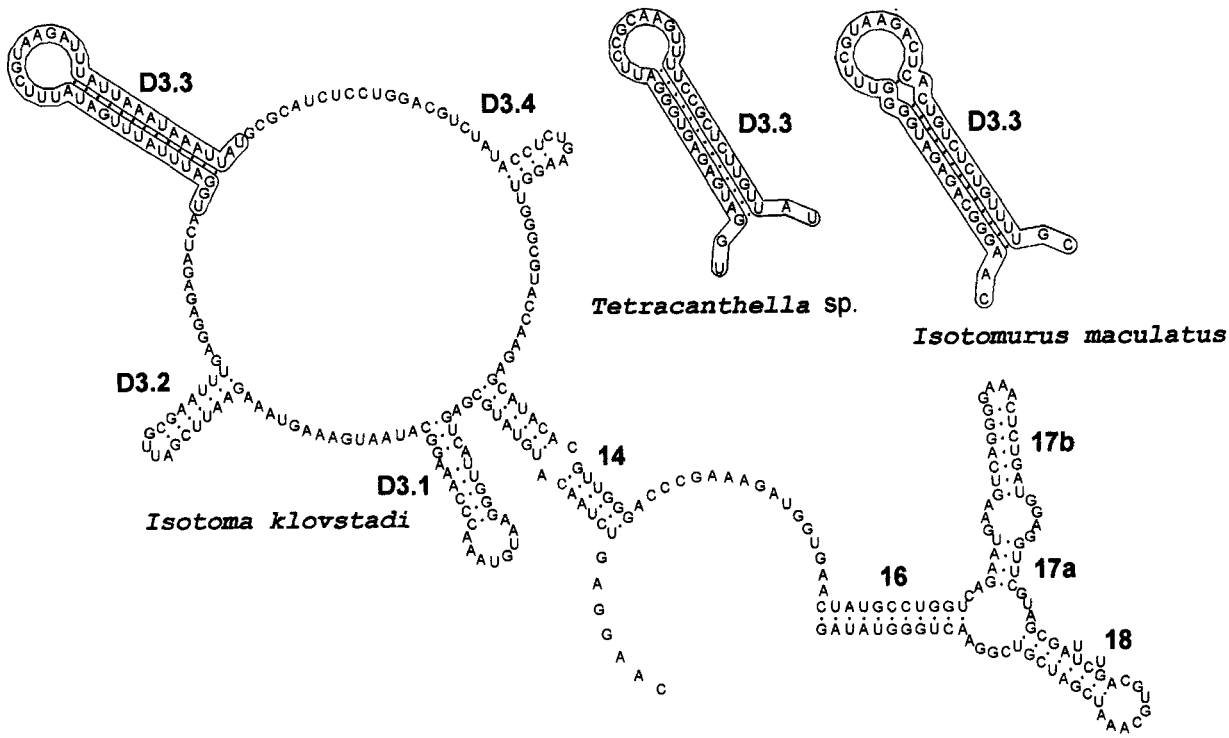

Fig. 6. Proposed secondary structure of the rRNA D3 fragment in *Isotoma klovstadi* and comparison of the D3.3 stem between *Isotoma klovstadi*, *Isotomurus maculatus* and *Tetracanthella* sp.

Isotoma klovstadi and *Tetracanthella* sp. being sister-species and they appear to come out from within the *Isotomurus* group, with *Isotomurus fucicola* being their closest species. The genus *Isotomurus* would therefore be paraphyletic with respect to *Isotoma klovstadi*. The Kishino-Hasegawa test (Kishino & Hasegawa 1989) as implemented in PAUP* was used to test whether the tree depicting a monophyletic genus *Isotomurus* can be rejected or not with these DNA sequences. It turned out that, under the HKY+I model, a monophyletic taxon *Isotomurus* can be rejected, with respect to the best ML tree, with a probability $P < 0.05$. Bootstrap support was very high for the placement of *Isotomurus fucicola* together with *Isotoma klovstadi* and *Tetracanthella* sp. (Fig. 7), without resolving their reciprocal relationships.

The same HKY+I model, with parameters estimated *via* Maximum Likelihood, was used to calculate genetic distances (Table VIII) and to select a phylogenetic tree under the principle of Minimum Evolution. This tree had the same topology as the ML and MP trees described above, with the exception of the relative position of *Isotomurus maculatus* and *I. nebulosus*.

It is concluded that the D3 fragment is characterized by a combination of extremely conserved positions and really rapidly evolving sites. These latter positions are likely to have experienced multiple substitutions, introducing the possibility of misleading information. On the other hand, if highly variable sites are removed, we are left with highly conserved positions, containing little or no information at the genus level. The use of an appropriate model of evolution and of a likelihood approach does not allow us to construct

Table VIII. Genetic distance estimates based on the D3 fragment of the rRNA gene. Uncorrected *p*-distances are below the diagonal while HKY+I-corrected distances are shown above the diagonal.

Taxon	1	2	3	4	5	6	7	8
1. *Isotoma klovstadi*	-	0.126	0.150	0.129	0.132	0.095	0.112	0.312
2. *Isotomurus nebulosus*	0.084	-	0.024	0.057	0.011	0.052	0.108	0.177
3. *Isotomurus unifasciatus*	0.088	0.022	-	0.083	0.028	0.078	0.130	0.183
4. *Isotomurus palustris*	0.088	0.048	0.062	-	0.062	0.076	0.120	0.242
5. *Isotomurus maculatus*	0.088	0.011	0.026	0.051	-	0.057	0.113	0.185
6. *Isotomurus fucicola*	0.073	0.044	0.059	0.062	0.048	-	0.072	0.232
7. *Tetracanthella* sp.	0.077	0.084	0.095	0.088	0.088	0.059	-	0.246
8. *Orchesella ranzii*	0.125	0.106	0.103	0.125	0.110	0.110	0.121	-

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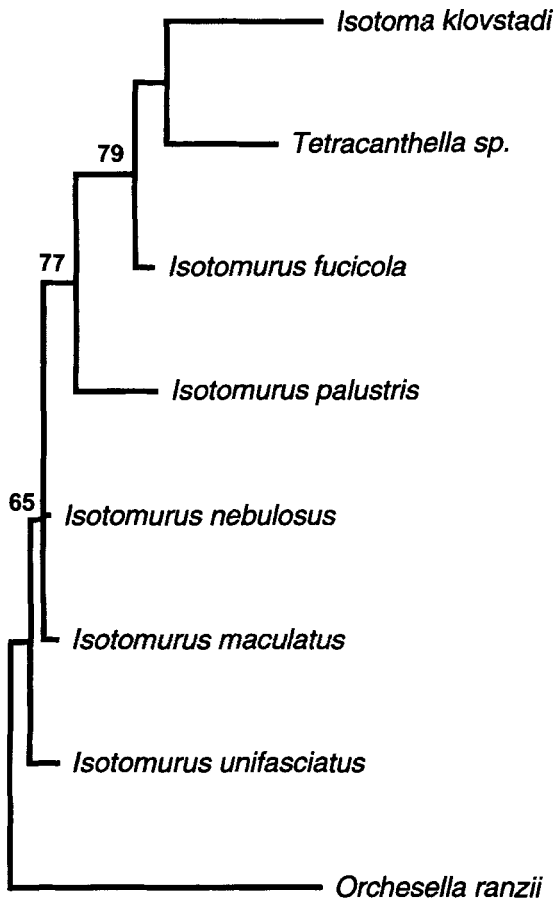

Fig. 7. Maximum likelihood tree depicting relationships among the species studied based on the rRNA D3 fragment. Branch lengths were estimates *via* ML and bootstrap support values shown at the nodes and are based on 100 replications (with model parameters estimated in each replication).

a solid hypothesis and the position of *Isotoma klovstadi* with respect to the other Isotomidae genera remains ambiguous. It appears that when variable sites are so saturated, even the appropriate model of evolution could fail to recover the correct phylogeny. It is possible to conclude that this gene might be more useful at a higher taxonomic level, when hypervariable sites can be removed but more conserved positions might still contain some clear phylogenetic signal (see Friedrich & Tautz (1995, 1997a, 1997b) for the use of 28S rRNA among Diptera, Insecta and Arthropoda).

Conclusions and perspectives

The results here indicate that both the mitochondrial COII and the nuclear EF-1α genes appear to be useful at this taxonomic level. They provide concordant phylogenetic reconstructions, which are also in agreement with the accepted taxonomy. In addition, they show appropriate levels of variability to infer evolutionary relationships at the species and genus level. From these data, the ribosomal D3 fragment seems to be less useful than the other two genes to give information on phylogenetic relationships of Antarctic Collembola, especially within genera. This is probably due to the presence of very conserved regions interspersed with portions exhibiting an extremely high rate of variation, both in length and sequence.

One important aspect is the need for a careful examination of the pattern of evolution of the genes under study in order to assess whether they contain potentially misleading factors, such as evolution under non-stationary conditions. Once the sequences have been screened for these factors, it is of major importance that the analysis is conducted under the most appropriate approach. More powerful computers available today allow the application of the computationally-intensive Maximum Likelihood approach which can perform the analysis under the most appropriate model of evolution for the data under study.

All genes studied here showed remarkable among-site rate variation. This underlines once more the importance of incorporating a suitable correction, as is possible by the use of a Γ distribution and/or incorporating an estimate of invariable sites. Failure to do so would result in biased and possibly erroneous phylogenetic analysis.

Most Antarctic Collembola belong to the Family Isotomidae, including the genera *Isotoma*, *Cryptopygus*, *Gressittacantha*, *Neocryptopygus*, *Antarctophorus*, *Antarcticinella*, *Folsomia* and *Archisotoma* (Greenslade 1995), many of which are endemic. Future investigations will be dedicated to comparing *Isotoma klovstadi* with representatives of these genera in order to establish the evolutionary pathway of isotomid species in Antarctica. The study of other species from the Southern continents will help to understand the origin of these Antarctic species and, in combination with palaeogeographic data, will provide estimates on times of divergence between different taxa.

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Appendix I. Alignment of the mitochondrial COII gene.

Isotoma klovstadi	ATA	GCA	ACA	TGA	TCA	GCA	ATC	GGT	TTT	CAA	AAC	GCA	GCT	TCC	CCG	45
Isotomurus fucicola	. . C	. . G	. . .	. . .	AT .	. . C	C . A	. . A	. . C	. . .	. . .	. . C	. . C	. . .	. . C	
Isotomurus hadriaticus	. . C	. . .	. . .	. . .	AG .	. . T	. . T	. . .	. . .	. . .	. . .	. . T	. . C	. . T	. . A	
Isotomurus maculatus	. . C	. . .	. . .	. . .	AT .	. . .	. . .	. . G	. . C	. . .	. . T	. . .	. . .	. . .	. . A	
Isotomurus nebulosus	. . C	. . .	. . .	. . .	AT .	. . .	. . T	. . G	. . C	. . G	. . .	. . .	. . .	. . T	. . A	
Isotomurus palustris	. . C	. . .	. . .	. . .	AT .	. . .	. . T	. . G	. . C	. . .	. . .	. . C	. . C	. . T	. . T	
Isotomurus unifasciatus	. . C	. . .	. . .	. . .	AT .	. . .	. . .	. . G	. . C	. . .	. . .	. . C	. . A	. . .	. . C	
Orchesella villosa	. . C	T.T	. . .	.T.	G .C	. . G	C . A	AA .	. . .	. . .	. . .	. G .	. . A	. . A	. . T	
Isotoma klovstadi	GTT	ATA	GAA	CAA	TTA	ATC	TTT	TTT	CAT	GAT	CAC	TCA	ATA	ACA	ATT	90
Isotomurus fucicola	C . .	. . G	. . G	. . .	C . .	. . T	. . .	. . .	. . C	. . C	. . .	G . .	. . .	. . .	. . .	
Isotomurus hadriaticus	T . G	. . .	. . .	. . .	. . .	. . T	. . .	. . .	. . C	. . C	. . T	G . T	. . .	. . T	. . .	
Isotomurus maculatus	C . A	. . .	. . .	. . .	. . G	. . .	. . .	. . C	. . .	. . C	. . .	G . C	. . .	. . .	. . .	
Isotomurus nebulosus	C . .	. . .	. . G	. . .	. . .	. . T	. . .	. . C	. . .	. . C	. . .	G . T	. . .	. . .	. . .	
Isotomurus palustris	C . .	. . G	. . G	. . .	C . .	. . T	. . .	. . .	. . .	. . C	. . .	G . C	. . .	. . .	. . .	
Isotomurus unifasciatus	C . G	. . .	. . .	. . G	. . .	. . T	. . .	. . C	. . .	. . .	. . .	G . C	. . .	. . T	. . .	
Orchesella villosa	T . A	. . .	. . .	. . G	C . .	. . T	. . .	. . .	. . .	. . C	. . T	. . T	. . .	. . C	. . C	
Isotoma klovstadi	TTA	ATT	CTA	ATT	ATC	ACT	ATC	GTT	GGG	TAT	AAT	CTT	TTT	TCC	ACT	135
Isotomurus fucicola	. . G	. . .	. . T	. . .	. . T	. . C	. . T	. . A	. . C	. . C	. . .	T.A	G.C	. . T	. . C	
Isotomurus hadriaticus	. . .	. . .	T . .	. . .	. . T	. . .	. . T	. . .	.CT	. . C	. . .	. . .	. . .	. . A	. . C	
Isotomurus maculatus	. . G	. . .	. . T	. . .	. CT	. . .	. . T	. . G	TCT	. . C	. . .	T.A	. . C	. . A	. . .	
Isotomurus nebulosus	. . G	. . C	. . .	. . C	. CT	. . A	. . .	. . C	TCA	. . C	. . .	. . .	. . C	. . A	. . .	
Isotomurus palustris	. . G	. . .	. . T	. . .	. CT	. . C	. . T	. . G	TCT	. . .	. . C	T.A	. . .	G.A	. . .	
Isotomurus unifasciatus	. . .	. . .	. . T	. . .	. CA	. . A	. . T	. . G	TCA	. . .	. . C	. . A	. . .	. . A	. . C	
Orchesella villosa	. . .	. . .	T . .	. . .	. . T	. . G	. . T	. . A	A.C	.T.	. . .	T.A	. . .	. . A	. . .	
Isotoma klovstadi	TGT	TTT	AAC	ATT	AAC	ATT	GAT	CAA	CAC	ATA	CTT	GAG	TCT	CAA	AGC	180
Isotomurus fucicola	. . C	. . .	. GA	. C .	. . .	. . .	. . C	. . .	. . T	. . .	. . .	. . .	. . C	. . .	GAA	
Isotomurus hadriaticus	. . C	. . .	. G .	TC .	. . T	. . C	. . .	. . .	. . T	. . .	T . A	. . A	. . A	. . .	G . A	
Isotomurus maculatus	. . C	. . .	GGA	. C .	. . .	. . C	. . C	. . G	. . .	. . G	T . A	. . .	. . C	. . .	G . G	
Isotomurus nebulosus	. . .	. . .	GGG	GCA	. . .	. . .	. . .	. . .	. . .	. . G	. . .	. . .	. . C	. . G	G . A	
Isotomurus palustris	. . C	. . .	GGA	GC .	. . .	. . .	. . .	. . .	. . T	. . .	. . .	. . A	. . .	. . G	G . .	
Isotomurus unifasciatus	. . .	. . .	GGA	GC .	. . .	. . C	. . C	. . .	. . .	. . G	. . A	. . A	. . C	. . .	G . A	
Orchesella villosa	. . .	ACA	. . T	. G .	. . .	. . .	. . .	. . .	. . T	. . .	. . A	. . A	. . C	. . G	CCT	
Isotoma klovstadi	CTA	GAG	TTA	TTC	TGA	ACA	ATT	GTC	CCA	GCG	TTT	ATC	TTA	TTA	TTT	225
Isotomurus fucicola	. . .	. . A	. . .	. . T	. . G	. . .	. . C	. . T	. . T	A . T	. . .	. . T	. . .	. . .	. . C	
Isotomurus hadriaticus	T . .	. . A	. . .	. . .	. . .	. . C	. . C	. . A	. . T	A . T	. . .	. . T	. . G	. . .	. . .	
Isotomurus maculatus	. . .	. . .	. . .	. . T	. . .	. . .	. . .	. . .	. . .	A . T	. . .	. . T	C . .	C . .	. . .	
Isotomurus nebulosus	. . T	. . A	. . .	. . T	. . G	. . .	. . .	. . .	. . C	A . A	. . .	. . T	C . .	C . .	. . .	
Isotomurus palustris	T . .	. . .	. . .	. . .	. . G	. . .	. . C	. . G	. . T	A . T	. . .	. . .	. . .	C . .	. . C	
Isotomurus unifasciatus	. . T	. . A	C . .	. . T	. . .	. . C	. . .	. . A	. . .	A . A	. . .	. . T	C .T	. . .	. . C	
Orchesella villosa	T . .	. . A	. . .	. . T	. . .	. . T	. . .	A . T	. . T	AGC	. . .	. . .	. . .	A.T	. . .	
Isotoma klovstadi	ATC	GGT	CTA	CCC	TCA	ATT	CGT	CTC	CTA	TAC	CTC	TTA	GAT	GAA	GTT	270
Isotomurus fucicola	. . .	. . G	. . .	. . A	. . .	. . .	. . .	. . G	T . .	. . .	. . A	. . .	. . C	. . .	. . C	
Isotomurus hadriaticus	. . T	. . G	T . .	. . T	. . .	. . .	. . A	. . A	. . T	. . .	. . T	. . .	. . C	. . .	. . C	
Isotomurus maculatus	. . T	. . G	. . .	. . .	. . .	. . .	. . C	. . A	. . T	. . .	. . .	. . .	. . .	. . .	. . .	
Isotomurus nebulosus	. . .	. . A	. . .	. . .	. . C	. . .	. . A	. . T	. . T	. . .	. . T	. . .	. . C	. . .	. . A	
Isotomurus palustris	. . T	. . G	. . .	. . .	. . T	. . .	. . .	. . A	. . C	. . .	. . T	. . .	. . .	. . .	. . A	
Isotomurus unifasciatus	. . T	. . C	T . .	. . T	. . .	. . .	. . .	. . A	. . T	. . T	T . A	. . .	. . .	. . .	. . C	
Orchesella villosa	. . T	. . .	. . C	. . A	. . .	. . .	. . .	. . G	T . .	. . T	. . A	. . .	. . .	. . .	. . .	
Isotoma klovstadi	TAT	AAC	CCG	GCT	ATT	ACA	CTA	AAA	ACC	GTA	GGC	CAC	CAA	TGG	TAT	315
Isotomurus fucicola	. . C	. . .	. . C	. . C	. . C	. . .	. . .	. . .	. . A	. . .	. . .	. . .	. . .	. . A	. . C	
Isotomurus hadriaticus	. . .	. . .	. . .	T . A	. . C	. . T	. . .	. . .	. . A	. . G	. . G	. . .	. . .	. . A	. . C	
Isotomurus maculatus	. . .	. . .	. . T	. . A	. . .	. . C	. . .	. . G	. . A	. . .	. . A	. . .	. . G	. . A	. . C	
Isotomurus nebulosus	. . .	. . .	. . T	. . A	. . C	. . .	. . .	. . .	. . .	. . C	. . A	. . .	. . G	. . A	. . C	
Isotomurus palustris	. . C	. . .	. . C	. . C	. . C	. . T	T . .	. . .	. . A	. . G	. . T	. . .	. . .	. . .	. . C	
Isotomurus unifasciatus	. . C	. . .	. . T	. . .	. . .	. . G	. . .	. . .	. . A	. . G	. . A	. . .	. . G	. . A	. . C	
Orchesella villosa	. . C	C . A	. . T	T . .	. . .	. . T	A . T	. . .	. . A	A . .	. . A	. . T	. . .	. . .	. . .	
Isotoma klovstadi	TGG	TCA	TAC	GAA	TAC	TCA	GAT	TTT	TTA	AAC	GCA	GAA	TTT	GAT	TCT	360
Isotomurus fucicola	. . .	. . T	. . T	. . G	. . .	. . T	. . C	. . .	. . .	. . .	. T .	. . .	. . .	. . C	G . .	
Isotomurus hadriaticus	. . A	. . T	. . .	. . .	. . .	. . .	. . .	. . .	A . .	. . T	. TT	. . .	. . C	. . .	G . G	
Isotomurus maculatus	. . .	. . C	. . .	. . .	. . .	. . T	. . C	. . .	. . .	. . T	. TC	. . G	. . .	. . C	G . A	
Isotomurus nebulosus	. . A	. . C	. . .	. . .	. . .	. . T	. . C	. . C	. . G	. . .	CT.	. . G	. . .	. . .	G.A	
Isotomurus palustris	. . A	. . T	. . .	. . G	. . .	. . C	. . .	. . C	C . .	. . T	. T .	. . .	. . C	. . C	G . .	
Isotomurus unifasciatus	. . A	. . T	. . .	. . .	. . .	. . .	. . C	. . .	C.T	. . T	. T .	. . .	. . C	. . C	G.A	
Orchesella villosa	. . A	. . T	. . T	. . .	. . T	. . .	. . .	. . .	A . .	. . T	CT.	. . .	. . C	. . .	. . .	

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Appendix I. Alignment of the mitochondrial COII gene.

Isotoma klovstadi	TAC	ATA	ATC	CCT	ACT	AAT	GAA	CTA	AAC	ACA	GAC	ATG	TTT	CGT	CTT	405
Isotomurus fucicola	...	...	...	..G	..A	...	..G	...	TCT	.AC	TCT	..A	...	...	...	
Isotomurus hadriaticus	...	...	...	..G	T.A	..C	..G	A.G	TCA	.AT	..A	..A	...	...	T.A	
Isotomurus maculatus	..T	...	...	..C	T..	...	...	A..	.CA	.AT	TCT	..A	..C	...	..C	
Isotomurus nebulosus	...	..G	..T	..C	T..	..C	...	A.G	TCT	.AC	TC.	...	..C	...	...	
Isotomurus palustris	..T	...	...	..A	T.A	...	...	A..	TC.	.AC	TCT	...	..C	...	..G	
Isotomurus unifasciatus	..T	...	...	..A	T..	..C	...	A..	.CG	.AT	TCA	..A	...	..C	T.A	
Orchesella villosa	..T	...	...	...	.GC	.CA	..C	T..	.CT	.AT	..A	TCC	...	...	T.A	
Isotoma klovstadi	TTA	GAT	GTA	GAT	AAC	CGA	ACA	GCT	ATC	CCC	ATA	AAC	TCC	CAA	GTG	450
Isotomurus fucicola	C..	...	...	...	...	..C	...	.TA	G.A	..T	...	...	...	...	..T	
Isotomurus hadriaticus	C..	..C	...	..C	..T	..C	..T	.TA	G.T	..A	...	...	..T	G..	..A	
Isotomurus maculatus	C..	..C	..C	..C	..T	..T	...	.TC	G.G	...	...	...	..T	..G	..T	
Isotomurus nebulosus	C.T	..C	...	..C	...	..C	..T	.TA	G.A	..T	G..	..T	..G	...	..A	
Isotomurus palustris	C..	...	...	..C	..T	...	..G	.T.	G.G	..A	G.T	..T	..G	...	A.T	
Isotomurus unifasciatus	C.T	..C	...	..C	..T	...	..C	.TA	G.A	..T	G..	...	..A	...	..C	
Orchesella villosa	...	..C	...	...	..T	..T	...	.TA	..T	...	TAC	C..	A.T	...	A.T	
Isotoma klovstadi	CGT	ACT	TTA	ATC	TCT	GCC	GCA	GAT	GTT	CTT	CAT	TCA	TGA	ACT	GTG	495
Isotomurus fucicola	..A	...	...	...	A.A	..A	..T	..C	..A	..A	..C	...	...	..A	...	
Isotomurus hadriaticus	..A	...	...	...	A.C	..T	...	...	..A	...	..C	...	..G	..A	..C	
Isotomurus maculatus	..A	...	C.T	...	A.A	..T	...	...	...	T.A	..C	..T	...	..G	..A	
Isotomurus nebulosus	..A	...	C.T	..T	A.A	..A	..T	..C	..A	...	..C	..G	...	..A	..C	
Isotomurus palustris	..C	..A	C.T	...	A.A	..A	...	..C	...	...	...	..T	...	..G	...	
Isotomurus unifasciatus	..G	..A	C.T	...	A.A	..A	...	..C	..C	..A	...	..T	...	..A	...	
Orchesella villosa	..A	..A	C..	...	..A	..T	...	..C	..A	T.A	...	..T	...	..G	..T	
Isotoma klovstadi	CCA	TCC	CTA	GGA	GTT	AAA	GCA	GAC	GCT	GTA	CCT	GGG	CGA	TTA	AAT	540
Isotomurus fucicola	..T	...	A..	...	..A	..G	...	...	..A	...	..A	..A	..G	...	...	
Isotomurus hadriaticus	..T	..T	A..	...	..A	..G	..T	...	..A	..T	..A	..A	...	C..	...	
Isotomurus maculatus	..C	..A	A..	...	..G	..G	..T	...	..A	..C	...	..A	..C	C.C	..C	
Isotomurus nebulosus	..C	..T	A.G	...	...	..G	.TT	...	..C	...	..G	...	...	C..	...	
Isotomurus palustris	...	...	A..	..T	..C	...	..T	...	..A	...	...	...	...	C.C	..C	
Isotomurus unifasciatus	..C	..A	A..	..G	..A	...	...	..T	..A	..G	..C	..A	...	C.C	..C	
Orchesella villosa	...	G.T	T.G	..T	..A	...	..C	..T	..A	...	..A	..T	...	C..	...	
Isotoma klovstadi	CAA	GTA	AGA	ATT	TAC	TGC	AAT	CGC	CCA	GGC	TTA	TTC	TTT	GGG	CAA	585
Isotomurus fucicola	...	...	..T	T..	...	GCG	.C.	...	...	...	G..	.A.	..C	...	...	
Isotomurus hadriaticus	...	...	...	T.C	...	.CT	.CC	...	T..	...	...	CA.	...	..T	..G	
Isotomurus maculatus	...	..T	..T	T..	...	GC.	.C.	..A	..G	...	...	.A.	...	..C	..G	
Isotomurus nebulosus	...	..T	..T	T.C	...	GCT	.C.	..T	...	..A	..G	.A.	...	...	...	
Isotomurus palustris	...	...	...	T..	...	GC.	.C.	..G	...	..A	...	.AT	..C	...	..G	
Isotomurus unifasciatus	...	..T	..C	T..	...	GCA	.C.	...	..T	..T	C..	.AT	...	..T	...	
Orchesella villosa	...	..T	.AT	T..	...	.CA	...	...	..G	..T	...	..T	...	..T	...	
Isotoma klovstadi	TGT	TCA	GAA	ATT	TGC	GGA	GCA	AAT	CAT	AGA	TTT	ATA	CCT	ATT	GTT	630
Isotomurus fucicola	..C	...	...	..C	..T	..T	...	...	..C	...	...	..G	..C	..C	..A	
Isotomurus hadriaticus	...	...	...	..C	..T	...	..C	..C	..C	..C	...	...	..C	...	...	
Isotomurus maculatus	..C	..T	...	..C	...	..G	...	..C	...	..T	...	..G	..G	...	...	
Isotomurus nebulosus	..C	...	...	...	..T	..G	..T	..C	..C	..T	...	...	...	..C	..A	
Isotomurus palustris	..C	...	...	...	..T	..T	..T	..C	..C	..T	...	...	..A	..C	...	
Isotomurus unifasciatus	..C	...	...	...	...	...	...	..C	..C	..C	..C	...	..A	...	...	
Orchesella villosa	...	..T	...	...	...	..G	...	...	...	..C	..C	...	..G	..C	TC.	
Isotoma klovstadi	ATC	GAA	AGA	GTA	CCT	ACT	AAA	ACA	TTT	ATT	TCA	TGA	ATT	AAA		672
Isotomurus fucicola	G.A	...	GCT	..C	..A	T..	...	..T	..C	..C	...	...	G..	...		
Isotomurus hadriaticus	..T	...	TC.	..G	...	T..	...	...	...	...	AA.	...	...	...		
Isotomurus maculatus	G.A	..G	TCC	..C	...	T.A	...	.AT	...	..C	AGC	...	...	...		
Isotomurus nebulosus	..T	..G	TCT	..C	..A	T.A	...	..T	..C	...	AGC	..G	G.A	...		
Isotomurus palustris	..T	..G	TC.	...	...	T.A	...	..T	...	..C	A.C	...	...	...		
Isotomurus unifasciatus	G.A	..G	TCT	..T	..G	T..	...	...	...	...	A.T	...	G..	...		
Orchesella villosa	..T	...	...	..C	...	C..	T.T	.TT	..C	...	AAT	...	G..	...		

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Appendix II. Alignment of the fragment of the nuclear EF-1a gene.

Isotoma klovstadi	TTG	ATC	TAC	AAG	TGT	GGA	GGT	ATT	GAC	AAA	CGT	ACC	ATT	GAA	AAG	45
Isotomurus hadriaticus	...	...	...	...	..C	...	...	..C	...	..G	..A	...	..C	..G	...	
Isotomurus maculatus	...	...	...	...	..C	...	...	..C	...	..G	...	...	..C	..G	...	
Isotomurus nebulosus	...	...	...	...	..C	...	...	..C	...	..G	...	...	..C	..G	...	
Orchesella cincta	...	...	...	...	..C	...	...	..C	...	..G	...	...	..C	..G	...	
Isotoma klovstadi	TTC	GAG	AAG	GAA	GCT	CAA	GAA	ATG	GGC	AAA	GGA	TCC	TTC	AAA	TAT	90
Isotomurus hadriaticus	...	...	...	...	..C	..G	...	...	..A	...	...	...	...	..G	..C	
Isotomurus maculatus	...	...	...	...	..C	..G	...	...	..A	...	...	...	...	..G	..C	
Isotomurus nebulosus	...	...	...	...	..C	..G	...	...	..A	...	...	...	...	..G	..C	
Orchesella cincta	...	...	...	...	..C	..G	...	...	..T	...	...	...	...	..G	..C	
Isotoma klovstadi	GCC	TGG	GTT	TTG	GAC	AAA	TTG	AAG	GCT	GAG	CGA	GAA	CGT	GGT	ATC	135
Isotomurus hadriaticus	..T	...	..C	...	...	...	C..	...	...	...	...	...	...	...	...	
Isotomurus maculatus	..T	...	..C	...	...	...	C..	...	...	...	...	...	...	...	...	
Isotomurus nebulosus	..T	...	..C	...	...	...	C..	...	..A	...	...	...	...	...	...	
Orchesella cincta	..T	...	..C	...	...	...	C..	...	...	...	...	...	...	...	...	
Isotoma klovstadi	ACC	ATC	GAC	ATT	GCC	CTC	TGG	AAA	TTC	GAA	ACT	GCA	AAG	TAC	TAT	180
Isotomurus hadriaticus	...	...	...	...	...	...	...	...	...	...	..C	.GC	...	...	..C	
Isotomurus maculatus	...	...	...	...	..T	...	...	..G	...	...	..C	..C	...	.T.	..C	
Isotomurus nebulosus	...	...	...	...	...	...	...	..G	...	...	..C	..C	...	.T.	..C	
Orchesella cincta	...	..T	..T	...	..T	...	...	..G	...	...	...	..T	.GA	...	...	
Isotoma klovstadi	GTC	ACC	ATC	ATT	GAC	GCT	CCT	GGA	CAC	AGA	GAT	TTC	ATC	AAG	AAC	225
Isotomurus hadriaticus	...	...	...	...	..T	..C	..A	...	...	...	...	...	...	...	...	
Isotomurus maculatus	...	...	...	...	...	...	..A	...	...	...	...	...	...	...	...	
Isotomurus nebulosus	...	...	...	..C	..T	..C	..A	...	...	...	...	...	...	...	...	
Orchesella cincta	...	...	...	...	..T	..C	..C	...	...	...	...	..T	...	...	...	
Isotoma klovstadi	ATG	ATT	ACT	GGA	ACC	TCT	CAG	GCC	GAT	TGT	GCC	GTA	TTG	ATC	GTT	270
Isotomurus hadriaticus	...	...	...	...	...	...	...	..T	...	...	...	..C	...	..T	..C	
Isotomurus maculatus	...	...	...	...	...	...	...	..T	...	...	..T	..C	...	..T	..C	
Isotomurus nebulosus	...	..C	...	...	..A	...	...	..T	...	...	...	..C	...	..T	..C	
Orchesella cincta	...	..C	...	..T	..T	...	...	..T	...	...	..T	..G	...	..T	...	
Isotoma klovstadi	GCT	GCT	GGT	ACC	GGA	GAG	TTT	GAA	GCT	GGT	ATT	TCC	AAG	AAT	GGT	315
Isotomurus hadriaticus	...	...	...	...	...	...	..C	...	...	...	...	...	...	..C	...	
Isotomurus maculatus	...	...	...	...	...	...	..C	...	...	...	...	...	...	..C	..A	
Isotomurus nebulosus	...	...	...	...	...	...	..C	...	...	...	...	...	...	..C	..A	
Orchesella cincta	...	..C	...	..T	..T	...	..C	...	...	...	..C	...	...	..C	...	
Isotoma klovstadi	CAA	ACT	CGA	GAA	CAC	GCT	CTT	TTG	GCT	TAC	ACC	TTG	GGA	GTT	AAG	360
Isotomurus hadriaticus	...	..C	...	...	...	...	...	...	..C	...	..T	..A	...	..G	...	
Isotomurus maculatus	...	..C	...	...	...	...	...	...	..C	...	...	..A	...	..G	...	
Isotomurus nebulosus	...	..C	...	...	...	...	...	...	..C	...	..T	...	...	..G	...	
Orchesella cincta	...	...	..T	..G	...	..A	..C	...	...	...	...	...	..T	..G	...	
Isotoma klovstadi	CAA	CTG	ATT	GTT	GGA	GTT	AAT	AAA	ATG	GAC	TCT	ACC	GAA	CCA	CCA	405
Isotomurus hadriaticus	..G	..C	..C	...	...	...	..C	..G	...	...	..C	..T	..G	...	..T	
Isotomurus maculatus	..G	..C	..C	..C	...	..G	..C	..G	...	...	..C	..T	...	...	...	
Isotomurus nebulosus	..G	..C	..C	..C	...	..G	..C	..G	...	...	..C	..T	...	...	...	
Orchesella cincta	..G	..C	..C	...	..T	..C	..C	..G	...	...	..C	..T	..G	...	..G	
Isotoma klovstadi	TAC	TCT	GAA	ACC	CGA	TTC	GAG	GAA	ATC	AAG	AAG	GAA	GTC	GGA	AAT	450
Isotomurus hadriaticus	...	...	..G	G.T	...	...	...	...	...	...	...	...	..G	TCC	..C	
Isotomurus maculatus	...	...	..G	T.T	...	...	...	...	...	...	...	...	...	TCC	..C	
Isotomurus nebulosus	...	...	..G	T.T	...	...	...	...	...	...	...	...	...	TCC	..C	
Orchesella cincta	...	..C	..G	T.T	..T	...	...	...	...	...	...	...	..A	A.C	GCA	
Isotoma klovstadi	TAT	ATC	AAG	AAG	ATC	GGA	TAC									471
Isotomurus hadriaticus	..C	...	...	...	...	...	...									
Isotomurus maculatus	..C	...	...	...	...	...	...									
Isotomurus nebulosus	..C	...	...	...	...	..G	...									
Orchesella cincta	..C	...	...	...	...	..T	...									

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Appendix III. Alignment of the D3 fragment of the nuclear 28S rRNA.

```
Isotoma klovstadi          GCGA---GTCATTGGGAATGTAAACCCAAAGGCATAAT 35
Isotomurus fucicola        ..A----.....................GG... 
Isotomurus maculatus       ..A-----.....................
Isotomurus nebulosus       ..A----.....................
Isotomurus palustris       ..A----.....................GG...
Isotomurus unifasciatus    ..A----.....................
Tetracanthella sp          ....GTT.....A.....................
Orchesella ranzii          .......----.....A.....G....

Isotoma klovstadi          GAAAGTAAAGAATTCGATTGCGAATTTGAGGAGAGAT- 72
Isotomurus fucicola        ...................................-
Isotomurus maculatus       ...................-...............-
Isotomurus nebulosus       ...................................-
Isotomurus palustris       ..................G................-
Isotomurus unifasciatus    ...................................-
Tetracanthella sp.         ...................................-
Orchesella ranzii          .......G....-.....................A

Isotoma klovstadi          CATGGATTTATTTGATA-TTTCGTAAGATTTATTAAAT 109
Isotomurus fucicola        .....GCC.GG..GGG-.....C....C.C.C.GCC.
Isotomurus maculatus       ..A..GCAG.GA..GGG-..........C.C.C.GTC.
Isotomurus nebulosus       ..A..GCAG.GA..GGG-..........C.C.C.GTC.
Isotomurus palustris       ..AT...A..GA..GGG-..CT....AGA.C.C.GTC.
Isotomurus unifasciatus    ..A..GCAG.GAG.GGG-..........C.CC..CTC.
Tetracanthella sp.         .......GAGAG..GG.-..C..C....-...TCCGCTC
Orchesella ranzii          .CGTAGCGGTG.G.TGGC..C......G-..G..GCCA

Isotoma klovstadi          AAATTATG-CGCATCTCCTGGACGTCTA-TACCTCTGA 145
Isotomurus fucicola        GTGC....-.....................-.....
Isotomurus maculatus       CTG..T...-.....................-.....
Isotomurus nebulosus       CTG..T...-.....................-.G...
Isotomurus palustris       .TG.....-.....................-.....
Isotomurus unifasciatus    CTG..T...-.....................-.....
Tetracanthella sp.         TTG.....-.....................A.....
Orchesella ranzii          C.GC.TG.T.........G.....-.....

Isotoma klovstadi          AGGTTGGGCGTACCAAGAGCATACACGTTGGGACCCGA 183
Isotomurus fucicola        .....................................
Isotomurus maculatus       ..................T..................
Isotomurus nebulosus       ..................T..................
Isotomurus palustris       ..................T..................
Isotomurus unifasciatus    ..................T..................
Tetracanthella sp.         .....................................
Orchesella ranzii          ....A.....T.....G....................

Isotoma klovstadi          AAGATGGTGAACTATGCCTGGTCAGAATGAAGTCAGGG 221
Isotomurus fucicola        ..................C..................
Isotomurus maculatus       ...G..............C..................
Isotomurus nebulosus       ..................C..................
Isotomurus palustris       ..................C..................
Isotomurus unifasciatus    ..................C..................
Tetracanthella sp          ..................C..................
Orchesella ranzii          ..................C..................

Isotoma klovstadi          GAAACTCTGATGGAGGTTCGTAGCGATTCTGACGTGCA 259
Isotomurus fucicola        .....................................
Isotomurus maculatus       .....................................
Isotomurus nebulosus       .....................................
Isotomurus palustris       .....................................
Isotomurus unifasciatus    .....................................
Tetracanthella sp.         .....................................
Orchesella ranzii          .....C...............................

Isotoma klovstadi          AATCGATCGTCGGAACT 276
Isotomurus fucicola        .................
Isotomurus maculatus.      .................
Isotomurus nebulosus.      .................
Isotomurus palustris.      .................
Isotomurus unifasciatus.   ...C.............
Tetracanthella sp.         .................
Orchesella ranzii.         .................
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