

Application of ubiquitin SSCP analysis in taxonomic studies within the subgenus *Orinocarabus* (Coleoptera: Carabidae: *Carabus*)

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Abstract. SSCP (single-strand conformation polymorphism) analyses of ubiquitin genes were used to investigate evolutionary relationships within the subgenus *Orinocarabus* of the genus *Carabus*. After SSCP electrophoresis of PCR-amplified ubiquitin copies, population-specific band patterns were obtained. Ubiquitin-SSCP-analyses of the six central European *Orinocarabus* species, including three subspecies and thirteen populations, resulted in a dendrogram that differed from that based on morphology. Phylogenetic analysis of mitochondrial DNA (mtDNA) did not support the SSCP dendrogram, but was in good accordance with the taxonomy based on morphological characters. The reason for the discrepancies seems to be evolutionary conservation of the ubiquitin genes. The time that elapsed since the evolution of the closely related *Orinocarabus* species is too short for concerted evolution of the ubiquitin genes.

INTRODUCTION

The subgenus *Orinocarabus* shows a disjunct distribution in the European Alps. The relatively high number of species, subspecies, microraces and hybrids within the genus *Carabus* has resulted in a diversity of views on the subgeneric and subspecific classification. The most recent revision of the genus (Deuve, 1991) is based on the system suggested by Ishikawa (1973, 1978, 1979), which uses endophallic structures as the main criteria for infrageneric classification. In recent years an analysis of DNA sequences was used for taxonomic studies (Prüser, 1996; Prüser & Mossakowski, 1998).

The classification at the species and subspecies level within the subgenus *Orinocarabus* is based on morphological data. The occurrence of many subspecies and races is controversial. In this study molecular techniques were used to obtain more information about the taxonomic relationships within this group.

Ubiquitin is a small 228 bp multifunction protein found in all eukaryotes. It is involved in the nonlysosomal proteolysis of proteins (Hershko & Ciechanover, 1986). Furthermore, it is important in other cellular processes, such as DNA repair (Jentsch et al., 1987), cell response to stress (Bond & Schlesinger, 1985), programmed cell death (Schwartz et al., 1990) and kinetochore function (Kopski & Huffaker, 1997). In the nucleus, ubiquitin is bound to histone 2A and 2B (Bonner et al., 1987), implicating its role in the regulation of gene expression.

Two different classes of ubiquitin genes are present in the eukaryotic genome. One class comprises two types of fusion genes, encoding a single ubiquitin joined to a ribosomal protein (Swindle et al., 1988; Cabrera et al., 1992; Barrio et al., 1994). The other class consists of polyubiquitin genes, which contain ubiquitin repeats in tandem (Wiborg et al., 1985). The number of repeats within such polyubiquitin genes varies from one in *Giardia lamblia*

(Krebber et al., 1994) up to 52 in *Trypanosoma cruzi* (Swindle et al., 1988). Examples of insect species with a relatively high number of repeats in polyubiquitin genes are *Manduca sexta*, with 15 repeats (Myer & Schwartz, 1996) and *Drosophila melanogaster*, with 18 repeats (Lee et al., 1988). Thus, SSCP analysis of ubiquitin genes yields information about numerous loci.

To investigate polymorphisms associated with ubiquitin genes we used SSCP (single strand conformation polymorphism) electrophoresis. This technique enables one to detect a polymorphism in a fragment of DNA due to as little as a single base substitution (Orita et al., 1989a, b). The differentiation of DNA fragments of the same length differing in their sequences is based on a sequence-dependent mobility shift of single-stranded DNA during electrophoresis. Sheffield et al. (1993) found that single base substitutions are detectable by SSCP analysis but that the sensitivity depends on the fragment length. Their data revealed a high sensitivity in a range between 95 and 212 bp (up to 76% of all single point mutations were detected among 29 different 212 bp fragments) and a strong decrease in mutation detection with increasing length above these values. We used a 210 bp PCR fragment of the whole 228 bp ubiquitin gene, which should enable a high resolution in SSCP analysis.

Although the amino acid sequence of the ubiquitin protein is highly conserved the degenerated genetic code enables variation on DNA sequence level. Therefore, the multicopy gene ubiquitin with its several repeats seems to have the potential for SSCP based analysis of population differentiation and in taxonomic studies.

Only a few examples of the use of SSCP analysis in taxonomic investigations have been reported. In most of these studies (Hiss et al., 1994; Tokue et al., 1995; Travis & Keim, 1995; Walsh et al., 1995; Stothard et al., 1998; Koekemoer et al., 1999) single gene loci were used,

which limited the information content of the SSCP patterns. Rarely have multiple loci been investigated (Ohsako et al., 1996, Nakamura et al., 1998).

The advantage of SSCP analysis of the multicopy gene ubiquitin is that the simultaneous PCR amplification of several loci gives SSCP banding patterns with numerous bands. Thus, the information content of these patterns is greater than that obtained from single-copy genes. A first attempt to analyse the multicopy gene ubiquitin by SSCP was made by Boge et al. (1994). SSCP analysis of ubiquitin genes of four *Carabus* species showed numerous bands for each specimen, which differed between the species. But no further analysis of the ubiquitin SSCP banding pattern was made.

In this study we examined the suitability of SSCP analysis of the ubiquitin genes, using new beetle-specific primers, for taxonomic research. Our aim was to improve the identification and differentiation of *Orinocarabus* subspecies and populations (races) using SSCP electrophoresis, and to obtain further relevant data for taxonomic studies.

MATERIAL AND METHODS

Samples

Six species of ground beetles of the subgenus *Orinocarabus*, including three conspecific subspecies were collected at twelve locations in the central European Alps (Fig. 1): *Carabus (Orinocarabus) alpestris dolomitanus* Mandl, 1956, *C. (O.) alpestris hoppei* Germar, 1824, *C. (O.) alpestris rottenmannicus* Sokolar, 1907, *C. (O.) bertolini* Kraatz, 1878, *C. (O.) carinthiacus* Sturm, 1815, *C. (O.) concolor concolor* F., 1792, *C. (O.) linnei* Panzer, 1813 and *C. (O.) sylvestris nivosus* Heer, 1837.

DNA extraction

Total DNA was extracted from the thorax and femora of the beetles as described by Boge et al. (1994).

Sequencing of an ubiquitin dimer

To obtain sequence information from beetle specific ubiquitin genes, an ubiquitin dimer of *C. alpestris dolomitanus* was

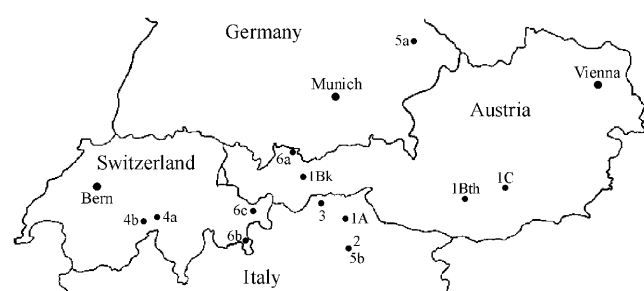


Fig. 1. Map of the locations from which the beetles were collected. Names of the locations and the number of specimens analysed are given in parentheses. 1A – *C. alpestris dolomitanus* (Mte Plose, 3); 1Bk – *C. alpestris hoppei* (Kühtai, 5); 1Bth – *C. alpestris hoppei* (Turracher Höhe, 1); 1C – *C. alpestris rottenmannicus* (Zirbitzkogel, 4); 2 – *C. bertolini* (Rollepaß, 4); 3 – *C. carinthiacus* (Penser Joch, 6); 4a – *C. concolor concolor* (Furkapaß, 7); 4b – *C. concolor concolor* (Grimselfaß, 3); 5a – *C. linnei* (Kohlachtal, 6); 5b – *C. linnei* (Rollepaß, 3); 6a – *C. sylvestris nivosus* (Ammerwald, 2); 6b – *C. sylvestris nivosus* (Berninapaß, 6); 6c: *C. sylvestris nivosus* (Flüelapaß, 4).

sequenced. For this purpose ubiquitin genes were amplified by PCR as described by Boge et al. (1994), using degenerate ubiquitin primers because no corresponding sequence data were available for Coleoptera. The PCR products were separated using a 1.5% agarose gel. Due to the tandem organisation of the polyubiquitin repeats, which are not separated by introns, ubiquitin PCR results in monomeric as well as multimeric ubiquitin fragments. The dimeric ubiquitin copies were extracted from the gel using the USBioclean MP-Kit (USB). These extracted dimeric PCR products were cloned using the pCR Script SK(+) Cloning Kit (STRATAGENE) according to the supplier's protocols. Plasmids containing the ubiquitin insert were isolated using a QIAprep Spin Plasmid Miniprep-Kit (QUIAGEN) and a dimeric ubiquitin insert was sequenced commercially in both directions (Sequserve, Germany). The sequence (Fig. 2) has been deposited at the EMBL data library under the accession number X94621.

Ubiquitin SSCP analysis

PCR of ubiquitin was performed with primers designed according to the sequenced ubiquitin dimer of *C. alpestris dolomitanus* (Fig. 2). Sequences of the commercially (Pharmacia) synthesised primers, which enclose a 210 bp section of the whole 228 bp ubiquitin gene, were: 5'-TCT TCG TCA AGA CCC TCA CT-3' and: 5'-GAC GGA GGA CCA ACT GAA GA-3'.

PCR reactions were set up in 25 µl reaction volumes containing 0.5 µg genomic DNA, 5 nmol of each dNTP, 25 pmol of each primer, 2.5 µl 10× reaction buffer and 0.8 units DNA polymerase (DynaZyme, Biometra). The thermocycling profile consisted of an initial step at 94°C for 2 min, 9 cycles of 30 sec at 94°C and 45 sec at 50°C, followed by 27 cycles of 30 sec at 94°C and 45 sec at 55°C and was completed by a final step at 72°C for 5 min.

A

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1 atgcagatct ttgtgaagac tctgactggc aagaccatca ctttgaagt
51 cgagccgtct gacaccatcg aaaacgtcaa ggctaagato caagataagg
101 aaggtattcc tccagatcag caacgcttga tcttcgccgg taagcagctt
151 gaagatggcc gtactctgag tgattataac attcagaaag aatctactct
201 tcacttggtc ctcgctctgc gtggtggtAT gcaaatcttc gtcaagaccc
251 tcactggcaa aactattact ctggagggtg agccttcoga tactattgag
301 aacgttaag caaaaattca ggacaaagaa ggcaccccto cagatcagca
351 gcgtotgato ttcgctggca aacaactgga agatggctgc actotttcgg
401 actacaacat ccagaag

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B

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1 atgcagatct ttgtgaagac tctgactggc aagaccatca ctttgaagt
229 atgcaaatct tctgcaagac c-c-c----- -a-a-t-t- -c-c-g-
51 cgagccgtct gacaccatcg aaaacgtcaa ggctaagato caagataagg
279 g-----t-t- -t-t-t-t- -g-----t- a-a-a-a-t- -g-g-c-a-
101 aaggtattcc tccagatcag caacgcttga tcttcgccgg taagcagctt
329 -----c-c- ----- -g-tc- -----t- c-a-a-a-g-
151 gaagatggcc gtactctgag tgattataac attcagaaag aatctactct
379 -----t- -c-----ttc ggactacaac atccagaag
201 tcacttggtc ctcgctctgc gtggtggt 228

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Fig. 2. Sequence of a cloned ubiquitin dimer of *C. alpestris dolomitanus* (417 bp). A – the entire sequence consists of a whole ubiquitin unit (1–228) and the first 189 bp of the following repeat (229–417). PCR-primers are indicated by bold letters. B – base substitutions between ubiquitin repeats of the cloned dimer. Comparable sequences differ by 25.2%, but there was no amino acid substitution between the two repeats (data not shown).

After separation of resulting PCR products on a 1.5% agarose gel the band containing the monomeric ubiquitin fragments (210 bp) was cut out of each lane. The gel slices were homogenised in 20 µl 1× PCR buffer and centrifuged for 10 min at 12,000 rpm. To reamplify the monomeric ubiquitin fragments, 3 µl of the liquid phase was introduced into a second PCR for 2 min at 94°C and 24 cycles of 30 sec at 94°C and 45 sec at 55°C followed by 5 min at 72°C. The isolation of the monomeric ubiquitin fragments from other PCR products after the first PCR minimises the occurrence of non specific products during SSCP electrophoresis, which could have been amplified during the first PCR from genomic DNA.

SSCP electrophoresis of the reamplified monomeric ubiquitin fragments was carried out using the Multiphor II system (Pharmacia). 4.5 µl PCR product was mixed with an equal volume of formamide and denatured at 95°C for 5 min. After denaturation, samples were loaded immediately onto a 15% polyacrylamide gel (MiniCleanGel 15% DNA-HP; ETC, Germany). Running conditions were 20 min, 120 V, 23 mA, 5 W and 60 min, 600 V, 30 mA, 18 W using the DNA Disc Buffer Kit (ETC). After electrophoresis, gels were silver-stained according to the supplier's protocol.

SSCP banding patterns were digitised using the Image Master video system (Pharmacia) and analysed using ONE-Dscan (Scanalytics) to obtain the corresponding R_f-values. The bands were matched on a basis of maximum divergence of 1% between two compared bands. Band sharing rates of Nei & Li (1979) between the SSCP patterns and the corresponding distances were calculated. The resulting distance matrix was analysed with the RESTSITE package (Miller, 1990) using UPGMA (Sneath & Sokal, 1973).

Sequencing of mtDNA

A fragment of 559 bp of mtDNA (Fig. 5), consisting of the 3' end of NADH-dehydrogenase subunit 1 (ND1), a tRNA for Leucine and the 5' end of the 16 S rRNA was amplified from at least one specimen from each location representing all detected ubiquitin banding patterns. The following primers were used: 5'-TAG AAT TAG AAG ATC AAC CAG C-3' (Weller & Pashley, 1995) and 5'-ACA TGA TCT GAG TTC AAA CCG G-3' (Vogler & DeSalle, 1993). PCR products were sequenced commercially (TOPLAB) by direct sequencing in both directions.

The 3' end of the ND1 gene, which is the most variable part of this sequence, consisting of 350 bp, was analysed using PAUP 3.1.1 (Swofford, 1993). A maximum parsimony cladogram was generated after 1000 bootstrap replications using the branch and bound search option. *C. carinthiacus* was used as the outgroup to root the tree, because this taxon has clearly more base substitutions than the other taxa (data not shown).

Furthermore, the same data set was analysed using the PHYLIP package 3.573 (Felsenstein, 1993) and the maximum likelihood method. The ratio of transitions to transversions (T/V) was set to 2.57 as this is the calculated average T/V ratio of the sequenced ND1 fragments of the relevant taxa (data not shown). A phylogenetic tree was generated after 1,000 bootstrap replications.

RESULTS

Ubiquitin

Sequencing of a cloned ubiquitin dimer of *Orino-carabus alpestris dolomitanus* resulted in a sequence of 417 bp (Fig. 2), consisting of two joined ubiquitin repeats. The end of the first repeat and the beginning of

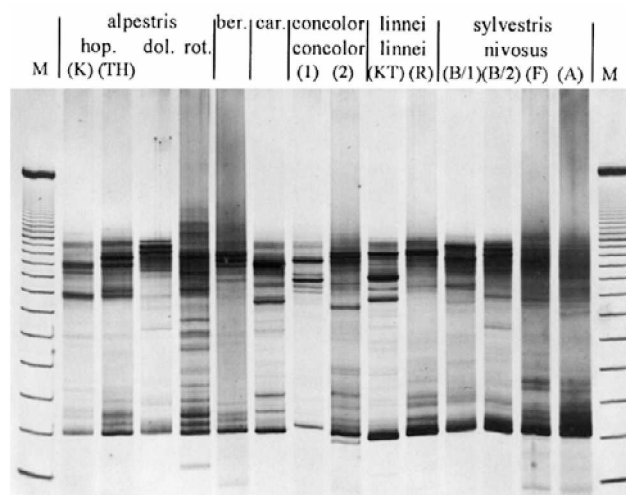


Fig. 3. SSCP patterns of all different ubiquitin types. The letters in parentheses refer to different locations. K – Kühtai; TH – Turracher Höhe; KT – Kohlbachtal; R – Rollepaß; B – Berninapaß; F – Flüelapaß; A – Ammerwald. The numbers indicate the SSCP patterns within *O. concolor concolor* and within the Berninapaß population of *O. sylvestris nivosus*. The first and the last lane (M) show a 100 bp ladder (Pharmacia)

the second were utilised for primer design to optimise PCR of the ubiquitin fragments of the beetles under investigation. The new primers enclosed 210 bp of a 228 bp-ubiquitin unit, rather than the 189 bp of the primers used initially (Boge et al., 1994).

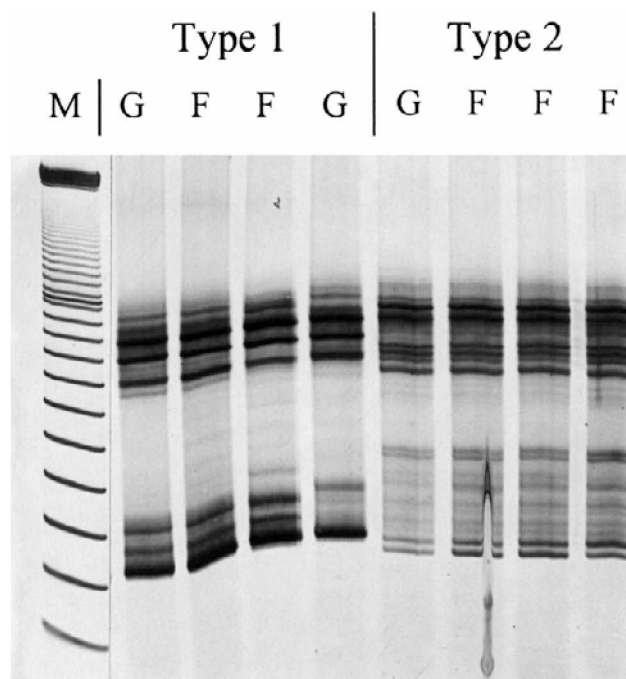


Fig. 4. The two different ubiquitin SSCP types of *O. concolor concolor* (four of the six patterns of type 2 are shown). The ubiquitin SSCP pattern of individuals from the two locations Furkapaß (F) and Grimselpaß (G), of two distinct types, with little variation within type 1 and no detectable variation within type 2. Both types occur at both locations. M – 100 bp ladder (Pharmacia).

SSCP-analysis of ubiquitin genes of six central European *Orinocarabus* species, including three conspecific subspecies and 13 populations, resulted in 14 different banding patterns (Fig. 3). The number of bands varied from 28 to 37, with an average of 31 bands. Within populations there was little divergence between banding patterns. Only *C. concolor concolor* specimens from both locations (Fig. 4) and those of *C. sylvestris nivosus* from one (Berninapaß) of three locations showed two distinct banding patterns. In the case of *C. concolor concolor* two fingerprints (type 1 and type 2) were found in habitats that were close to one another. Of the seven Furkapaß specimens, two showed type 1 and five type 2 fingerprints. Of the three Grimselpaß specimens two showed type 1 and one type 2 fingerprints.

Mitochondrial DNA

C. concolor concolor at both locations showed two distinct different SSCP patterns (Fig. 4). An explanation for this could be that two species coexist at each location, which are indistinguishable morphologically. To elucidate this, 559 bp of mtDNA of one individual of each SSCP type of *C. concolor concolor* from both locations were sequenced. This revealed identical sequence data for the two SSCP types within each habitat. Comparison of specimens from the two habitats shows sequence differences of 2.0% (Fig. 5). Hence, the coexistence of two species at Furkapaß and Grimselpaß was refuted. Similarly, the two SSCP types from the Berninapaß population of *C. sylvestris nivosus* showed identical mtDNA sequences (data not shown).

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1 TTACAGTATA AACCCCTAAA CTAGTACAAG CTAAAAAAA TAACATTCTCT
1 -----G-----

51 AAATTAAAT TAAATAAAC TAAAAAATAA GGAATAATTA CTCATAATA
51 -----C-----

101 TAATGATAAA AATAAACTTA TAATTGGAGA ATAATAATAT ATAATATAAT
101 -----C-----

151 TAGATAATAA GGGATAAGTC TGTTCITTAG AGAATAATTT AATGGCATCA
151 -----A-----

201 CAAAAGGTT GGGGAATACC TATAAATCCT ACTTTATTTG GACGTTTACG
201 -----A-----

251 AATTTGAATA TAACCTAATA CCTTCGTTT CAATAAAGTC AAAAAGGCAA
251 -----T-----

301 CTCCCACTAA AACACAAATA ATTAACAATA ATATACAAAT TAATGAAAT
301 -----T-----

351 ATTACATCTA GATAAAACAA GTATTACTTG TAAATAATTT ACATAAATGA
351 -----T-----

401 ATCTAAATTT CATTGCACTA ATCTGCCAAA ATAATAAACT TTAATAACAT
401 -----C-----

451 TCAATAAATT AAATATAAAT ATATCAAATA TTTGGTCCTT TCGTACTAAA
451 -----C-----

501 ATATTTTATT TTATTAAGA TAGAAACGGA CCTGGCTTAC ACCGTTTGA
501 -----C-----

551 ACTCAGATC
551 -----

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Fig. 5. Comparison of 559 bp of mtDNA from the two populations of *C. concolor*. 1–370 – ND1; 380–435 – tRNA^{Leu}; 436–559 – 16S rRNA. The first sequence was obtained from an individual from Grimselpaß, the second from a Furkapaß specimen.

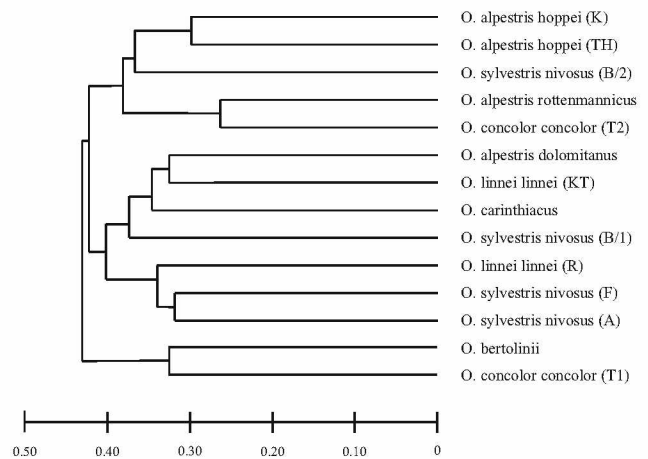


Fig. 6. Cluster analysis of all the ubiquitin SSCP types based on similarity indices of Nei & Li (1979). The tree was constructed by the UPGMA method (Sneath & Sokal, 1973). Numbers refer to similarity indices. The letters and numbers in parentheses refer to different locations and SSCP types, respectively. The abbreviations are explained in Fig. 3.

Taxonomic classification

Ubiquitin-SSCP-analysis

Analyses of all 14 different SSCP patterns resulted in a dendrogram (Fig. 6) that differs from the classification based on morphological data. This is illustrated, for example, by the distant branching of the two SSCP types of *C. sylvestris nivosus* from Berninapaß (B1, B2) and of the two types of *C. concolor concolor* (T1, T2), respectively. Furthermore, the two populations of *C. linnei linnei* and all three subspecies of *C. alpestris* are clearly separated.

On the other hand, the combining position of both populations of *C. alpestris hoppei* and of two populations of *C. sylvestris nivosus* (A, F), respectively, accords with the morphological classification. In general, however, the tree reveals obvious inconsistencies with the conventional taxonomic classification.

DNA sequencing

Maximum parsimony analysis of 350 bp of the mitochondrial ND1 gene was performed using PAUP 3.1.1 (Swofford, 1993). The resulting tree (Fig. 7a) is clearly different from the dendrogram based on ubiquitin-SSCP-analysis, but is in good agreement with the taxonomy based on morphological characters. All populations of *C. alpestris* are grouped together as well as both the populations of *C. linnaei linnaei* and two of the three populations of *C. sylvestris nivosus*. The third one is at the previous node. Only the two populations of *C. concolor concolor* are clearly separated.

The maximum likelihood tree (Fig. 7b), calculated with PHYLIP 3.573 (Felsenstein, 1993) is similar to the maximum parsimony tree. Its topology is even more similar to the taxonomy based on morphology, because the two populations of *C. concolor concolor* are not separated but grouped together in the same subtree with the *C. sylvestris nivosus* populations.

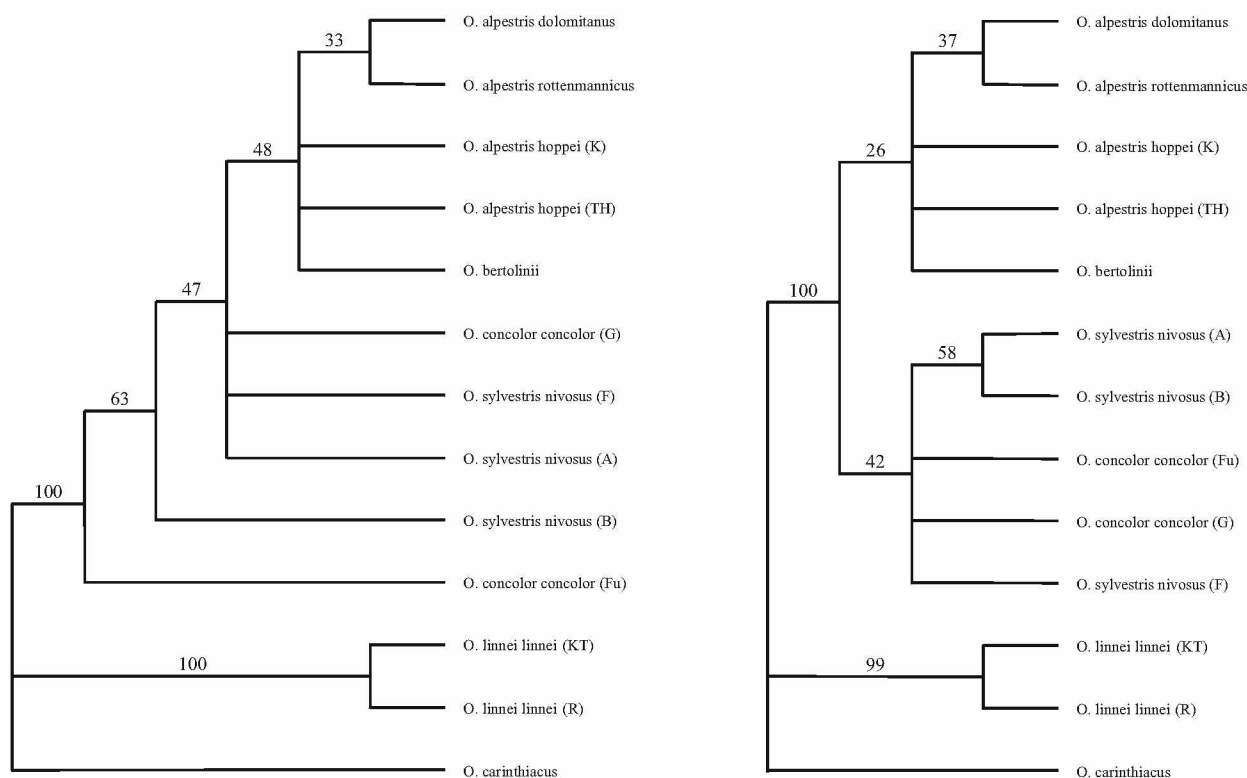


Fig. 7. Phylogenetic trees based on 350 bp of ND1. Abbreviations for the sampling locations given in parentheses are explained in Fig. 3, with two exceptions: Fu – Furkapaß; G – Grimselpaß. 7a (left) – maximum parsimony tree using PAUP 3.1.1 (Swofford, 1993). Bootstrap values were obtained from 1,000 replicates using the branch and bound search option and are indicated on the branches. All nodes with bootstrap values above 25% are shown. 7b (right) – maximum likelihood tree using PHYLIP 3.573 (Felsenstein, 1993). T/V ratio was set to 2.57. Bootstrap values of 1,000 replications are indicated on the branches. All nodes with bootstrap values above 25% are shown.

DISCUSSION

SSCP analysis of ubiquitin genes of six species from thirteen populations resulted in 14 different banding patterns. The number of bands in each pattern varied between 28 to 37. One reason for this variation could be a different number of ubiquitin repeats in these beetles. The minimum and maximum number of bands are represented by the two SSCP types of *C. concolor concolor*. Since these extremes are found in specimens from the same population, specific variation in the number of ubiquitin copies could be excluded. However, the variation could be due to allelic polymorphism. Such a polymorphism was found in *Trypanosoma brucei* (Wong et al., 1992), where two polyubiquitin alleles of the same locus consist of 13 and 30 repeats, respectively. Alternatively it could be due to sequence differences between polyubiquitin repeats. The sequences of the two repeats of the sequenced ubiquitin dimer (Fig. 2) differ by 25.2%. It can not be excluded that primer annealing during PCR is influenced by sequence variability within the different ubiquitin repeats, resulting in different numbers of SSCP bands.

The observed difference of 25.2% is very high compared with that between conspecific ubiquitin repeats in other species. Sequence data from 20 polyubiquitin genes from 14 eukaryotic species (Tan et al., 1993) revealed a maximum difference of 25.0% between polyubiquitin

repeats within a species. In this context, the two insect species, *Drosophila melanogaster* and *Manduca sexta*, exhibited a maximum difference of 8.3% and 17.5%, respectively (Tan et al., 1993).

In this study the ubiquitin SSCP patterns are almost identical, with two exceptions. The Berninapaß population of *C. sylvestris nivosus* and the two populations of *C. concolor concolor* (Furkapaß and Grimselpaß) show two distinct different SSCP patterns. In *C. concolor concolor* the two patterns exist in each population (Fig. 4). Sequences of 559 bp of mtDNA reveal no differences between specimens from the same *C. concolor concolor* population but with different ubiquitin-SSCP types. However, the sequences for individuals from the two populations of *C. concolor concolor* differ by 2.0%. This is a high value for variation in mtDNA between populations compared with other insect species. Sequence divergence between the *C. concolor* populations is in the range of values reported for populations of *Drosophila silvestris* (DeSalle & Templeton, 1992), but greater than that reported for several other insect populations (DeSalle et al., 1987; Satta & Takahata, 1990; Düring & Mosakowski, 1995) and between insect species (Vogler et al., 1993, 1998; Sperling & Hickey, 1994). The studies cited above used a combination of sequences from mitochondrial protein and RNA genes (rRNA and tRNA). Our data suggest that the gene pools of the two populations of

C. concolor concolor have been separated for a relatively long time. The occurrence of the same two ubiquitin-SSCP patterns in both populations reveals that these two ubiquitin types of *C. concolor concolor* existed before the populations were separated. Hence, the relevant ubiquitin genotypes have been conserved, at least during the period of population separation.

Comparison of different SSCP patterns was based on band sharing rates of Nei & Li (1979). Due to the separation by sequence differences during SSCP electrophoresis, fragments from different specimens at the same position in the gel should share the same sequence. Therefore, the matched band positions, which are used for calculating these similarity indices, should belong to homologous gene loci. Analysis of all the ubiquitin-SSCP patterns resulted in a dendrogram that differed greatly from that resulting from morphological systematics. The discrepancies are most probably caused by the conservation of the ubiquitin genes. Therefore the reliability of a dendrogram based on ubiquitin-SSCP types depends on the evolutionary age of the genotypes. In the UPGMA dendrogram (Fig. 6) SSCP types from conspecific populations (*C. linnaei linnaei*) are well separated as are the SSCP types from the same population (B1, B2; T1, T2). From the close relationship of these types to SSCP types of other species it is concluded, that these genotypes were already present before the recent split of these species from their ancestors. In contrast, the genotypes of the SSCP types of both populations of *C. alpestris hoppei* and of two populations of *C. sylvestris nivosus* (A, F), which are united in the dendrogram in each case, may have been generated in recent times after these species separated. As the ubiquitin genes are conserved the SSCP electrophoresis of these genes did not reflect the speciation process within the subgenus *Orinocarabus*.

Although no conclusions can be drawn from analyses of the ubiquitin SSCP data about the phylogeny of *Orinocarabus*, something can be concluded about the evolution of the ubiquitin genes. Sharp & Li (1987) postulated concerted evolution within polyubiquitin loci to explain the higher level of homogenisation between repeats within a polyubiquitin gene than between repeats compared across species. They assumed that concerted evolution between ubiquitin loci is much less effective than within polyubiquitin genes. Tan et al. (1993) analysed additional polyubiquitin genes of other species and included ubiquitin fusion protein loci in their study. They concluded that concerted evolution is an effective force for homogenising ubiquitin repeats not only within but also between loci. As a result of concerted evolution, polyubiquitin genes should evolve in accordance with the organisms in which they exist. Hence polyubiquitin genes of organisms belonging to the same taxon should differ less in their DNA sequences than the genes of different taxa. This effect of concerted evolution of ubiquitin genes was recognised in two other studies, dealing with higher vertebrates (Vrana & Wheeler, 1996; Nenoï et al., 1998).

This study shows that within *Orinocarabus* it is likely that concerted evolution has not occurred in general. Only

the similarity of the ubiquitin SSCP patterns of individuals from both populations of *C. alpestris hoppei* and from two populations of *C. sylvestris nivosus* (A, F) implies concerted evolution of the corresponding ubiquitin genes. In all other cases, where conspecific populations and subspecies were investigated, no evidence for concerted evolution of the ubiquitin genes was found.

The absence of concerted evolution between the ubiquitin genes in the taxa studied is supported by the phylogenetic analysis of mtDNA (Fig. 7a, b). In contrast to those obtained from the ubiquitin data, these cladograms group most of the conspecific populations together and are thus in great accordance with the taxonomy based on morphology. Some of the nodes in these trees are characterised by weak bootstrap values below 50%. However, as the main focus of this work was on the SSCP analysis of ubiquitin, these nodes are shown to illustrate the close relations of the conspecific populations in these analyses of mtDNA.

The main reason for the absence of concerted evolution across the ubiquitin loci is probably the close relationship of the species studied compared to previous investigations where the species belonged to different higher taxonomic levels, like different orders or phyla. Due to the highly conserved character of the ubiquitin genes, and the recent splitting of these species from one another, there has been too little time for concerted evolution to affect the homogenisation of the ubiquitin repeats within these species. To obtain more detailed knowledge about concerted evolution of ubiquitin genes in insects, it is necessary to study more species at different taxonomic levels, like genera, families or orders.

Ubiquitin SSCP analysis was not suitable for taxonomic studies of *Orinocarabus* species. Nevertheless, it could be useful for investigations of phylogenetic relations of higher taxa within a wide range of different species as ubiquitin is present in all eukaryotes.

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