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Affiliation of Dihydrolipoyl Dehydrogenase Allozymes in Mycorrhizae of European Forest Trees and Characterization of the Enzyme of the Matt Bolete (*Xerocomus pruinatus*) and the Bay Bolete (*X. badius*)

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Abstract

Mycorrhizal roots of the deciduous trees European beech (*Fagus sylvatica* (L.)) and Sessile oak (*Quercus petraea* (MattuschkaLiebl.)) and the conifers Norway spruce (*Picea abies* (L.) H. Karst.) and European larch (*Larix decidua* (Mill.)) associated with the ectomycorrhizal fungi matt bolete (*Xerocomus pruinatus* (Fries 1835)) or bay bolete (*X. badius* (Fries 1818)) were analysed with respect to the occurrence of dihydrolipoyl dehydrogenase (EC 1.8.1.4) allozymes. In root tissues of the two deciduous trees, two gene loci could be visualized after cellulose acetate electrophoresis while three loci were expressed in root tissues of the two coniferous species. The two fungal species and further ectomycorrhizal fungi expressed exclusively one dihydrolipoyl dehydrogenase gene. In *Xerocomus pruinatus* and *X. badius*, the dihydrolipoyl dehydrogenase gene consists of 1460 bp and 1370 bp, respectively, including five introns each consisting of 52 bp. Their DNA sequences correspond to 70 to 90% to other fungal dihydrolipoyl dehydrogenase genes. One monomer of the dimeric dihydrolipoyl dehydrogenase enzyme consists of 486 (*X. pruinatus*) or 454 (*X. badius*) amino acids which sum up to a molecular mass of 55 kDa (*X. pruinatus*), respectively 52 kDa (*X. badius*). The number of positively charged amino acid residues makes 79 (*X. pruinatus*) and 68 (*X. badius*) and the number of negatively charged amino acid residues was calculated to make 46 (*X. pruinatus*) and 48 (*X. badius*); isoelectric points make 9.99 (*X. pruinatus*) and 9.68 (*X. badius*). Calculated three dimensional structures reveal a short NADH binding site being part of a larger FAD-binding site and a binding/dimerization domain.

Keywords

Amino Acid Sequence, cDNA, Ectomycorrhizae, *Fagus sylvatica*, Gene Sequence, *Larix decidua*, mRNA, Dihydrolipoyl Dehydrogenase, *Picea abies*, *Quercus petraea*, *Xerocomus pruinatus*, *Xerocomus badius*

1. Introduction

Most European forest trees form at their root tips, a symbiosis with ectomycorrhizal fungi belonging to the ascomycota, basidiomycota or mitosporic fungi [1] [2]. The hyphae of ectomycorrhizal fungi associated with the root tips of their host trees inhibit the formation of long roots and cause instead the development of coralloid branched short roots [3] [4] [5]. The fungal hyphae partly grow between cells of the outer mantle of the root tips, forming a Hartig net, but also extend into the soil. This way they are able to mediate between the soil and their hosts supplying the trees with nutrients and water while obtaining organic nutrients from their hosts, especially carbohydrates. The difficulty is that in forest soils plant nutrients occur mostly in an organic form which cannot be taken up by ectomycorrhizal fungi [6]. That is why they excrete various enzymes to hydrolyze the corresponding components. Proteases serve to hydrolyze proteins [7], peroxidases split humus acids, and chitins are hydrolyzed by chitinases [8]. Phosphoric acid is set free from organic soil compounds either by excreted phosphomono- and diesterases hydrolyzing e.g. inositol phosphate, sugar phosphates and polyphosphates [8] [9] or by excreting organic acids such as oxalic acid or by excretion of protons [10]. Mycorrhizal roots are predominantly found within the upper soil horizon which indicates that they gain energy by use of oxygen processed in mitochondria to obtain energy in form of ATP. Mitochondria take up pyruvate, some amino acids and several fatty acids from the cytoplasm and transfer these metabolites to the citrate cycle where they are used to provide the basis for several molecular syntheses and to gain energy (GTP) and reduction equivalents ($NADH + H^+$, $FADH_2$). The latter are transmitted to the respiration chain where they are oxidized (NAD^+, FAD) and the arising electrons are transferred to oxygen while the protons set free are used for the generation of an electrochemical gradient providing the energy to synthesize ATP via the membrane bound enzyme ATP synthase [11]. The enzyme dihydrolipoyl dehydrogenase (EC 1.8.1.4) is part of two enzyme complexes of the citric acid cycle namely pyruvate dehydrogenase (EC 1.2.4.1) and α-ketoglutarate dehydrogenase (EC 1.2.4.2). After electrophoretic separations of native mycorrhizal extracts varying dihydrolipoyl dehydrogenase isozyme patterns result. In this study we investigated mycorrhizal roots of several European forest trees in order to allocate the various dihydrolipoyl dehydrogenase enzymes to the root tissues and the hyphae of the mycorrhizal fungi matte bolete (*Xerocomus pruinatus*) and bay bolete (*X. badius*). The fungal enzyme was studied in more detail de-

termining its DNA and cDNA sequence, amino acid sequence, molecular weight, isoelectric point and the putative secondary structure. The data and results presented here were collected during my doctoral thesis in the Department of Biology at the Johannes Gutenberg-University (Mainz, Germany).

2. Materials and Methods

2.1. Stand Characteristics

Mycorrhizal samples and fruiting bodies were collected from European beech (*Fagus sylvatica* (L.)), Sessile oak (*Quercus petraea* (MattuschkaLiebl.)), Norway spruce (*Picea abies* (L.) H. Karst.) and European larch (*Larix decidua* (Mill.)) growing in pure stands at the south-side of the Taunus Mountains situated on the southern part of the state of Hesse, Germany (Table 1). Samples were taken in April and June and in September and October 50 cm to 1 m away from a trunk at a soil depth of 5 cm. Fruiting bodies were collected in autumn. The exact sampling periods are given in Table 1.

The collected mycorrhizae were put in marked plastic bags and transported at 4˚C in a cooled box to the lab where they were put in ice water and cleaned from adhering soil and humus particles under a microscope. Then, mycorrhizae of the same species were put in 1.5 ml Safelock Eppendorf tubes and stored at −20˚C.

2.2. Mycorrhizae with *Xerocomus* Species

The matt bolete (*Xerocomus pruinatus* (Fr. & Hoek)) and the bay bolete (*X. badius*) belonging to the Basidiomycetes are forming mycorrhizae with fine roots of conifers and deciduous trees [2] [12]. Their fruiting bodies are found all over Germany [13]. Mycorrhizae with *Xerocomus pruinatus* are silvery-white to light yellow. They are morphologically similar to *X. badius* (Fr.: Fr.) Kuhn.: Gilbert, *X. chrysenteron* (Bull.) Quil., *X. subtomentosus* (L.: Fr.) Quil. and *Boletus edulis* Bull.: Fr. [4] [5]. Therefore, *X. pruinatus* and *X. badius* were identified by ITS-RFLP-analyses using the endonuclease Hinf I [14] [15]. To approve the results of ITS analyses selected samples were used to sequence the ITS region.

2.3. Fruiting Bodies of the Two *Xerocomus* Species

The cap of the fruiting body of *X. pruinatus* can reach a diameter of 10 cm. Its

Table 1. Location of the investigated forest stands in the Taunus Mountains.

Forest district	Wiesbaden-Chausseehaus			Königstein
Near the city of	Taunusstein		Glashütten	Königstein
GPS	N50˚ 07.875' E08˚ 10.382'	N50˚ 07.875' E08˚ 10.705'	N50˚ 13.508' E08˚ 24.018'	N50˚ 12.623' E08˚ 25.992'
Tree species	Norway spruce (*Picea abies*)	European beech (*Fagus sylvatica*)	Sessile oak (*Quercus petraea*)	European larch (*Larix decidua*)
Sampling period	Apr-June/ Sept-Nov 2006-2010	Apr-June/ Sept-Nov 2006-2010	Sep-Oct 2009 June-July 2010	Sept. - Nov. 2005 June 2010

shape is at first hemispherical, later convex to flattened. Its colour varies from light brown to greyish or dark brown to sometimes olivaceous or reddish brown to almost black. The cap is dry, velvety or finely dusted. The stipe is cylindrical to almost club-shaped, sometimes hardly swollen in its lower part. The yellow stipe downwards gradually gets a reddish colour. Stipe and pale yellow flesh and tubes are bluing when bruised or injured. The diameter and the shape of the cap of the fruiting body of *X. badius* are similar to that of *X. pruinatus*. The colour of the cap of *X. badius* varies from dark reddish brown to chestnut brown to dark brick. The cap is smooth when dry, but distinctly viscid under wet weather. The stipe of the fruiting body is cylindrical, spindle-shaped or almost club-shaped and often tapered towards the base. Tubes and flesh of *X. badius* are whitish or yellowish and turn blue when injured (cf. http://boletales.com/genera/xerocomus/x-pruinatus/).

2.4. Protein Extraction

Native proteins were extracted from mycorrhizal roots associated with *X. pruinatus* or *X. badius*, non mycorrhizal fine roots of seedlings, mycorrhizal roots separated into root tissues and enclosing hyphae, and fruiting bodies. Mycorrhizae were separated into hyphae and central root-tissues under an enlargement of 25 × fixing a mycorrhizal root put in ice water with a fine tweezers and separating the outer hyphae with a needle or a preparation forceps [4]. A 1.5 ml Eppendorf tube was weighted, filled with a mycorrhizal sample and weighted again to determine the amount of fresh weight filled in. Then the sample was homogenized with a micropistill in fluid nitrogen. After homogenization the proteins were extracted on ice. To 100 mg of frozen and pulverized mycorrhiza 200 µl extraction medium and 7.5 mg PVPP (polyvinylpyrrolidone) were added. The extraction medium contained: 30 mg (2.5 mM) cysteine, 3.3 mg (0.2 mM) mercaptobenzothiazole, 95 mg (5 mM) Na-metabisulfite, 186 mg (5 mM) Na_2-EDTA, 102 mg (5 mM) $MgCl_2 \times 6H_2O$, 39.2 mg (0.5 M) NADP, 33.2 mg (0.5 M) NAD, 14 g (14% w/v) sucrose, 0.5 g (0.5% w/v) BSA and 0.5 g (0.5% w/v) TWEEN® 80 in 100 ml of 0.1 M sodium phosphate buffer of pH 7.0 (57.7 ml of 1M di-sodiumhydrogenphosphate and 42.3 ml of 1 M sodiumdihydrogen-phosphate [16]. Then, the mixture was centrifuged at 4˚C for 30 min at 5000 × g. The supernatant containing the native proteins was aliquoted, snap-frozen until further use, and then frozen at −20˚C, or directly used in cellulose acetate electrophoretic separations.

2.5. Cellulose Acetate Electrophoresis

Cellulose acetate gels (Titan III, 7.6 cm × 7.6 cm, Helena Laboratories, Beaumont, Texas) were swollen under about 8˚C for 20 min in electrophoresis buffer which consisted of: 0.05 M Tris, 0.001 M Na_2-EDTA, 0.001 M $MgCl_2$ and 0.18 M maleic acid, pH 7.8 (modified according to [17]). Then the two chambers of the electrophoretic device were filled with buffer and two filter paper bridges (7 cm

long and 12 cm wide) were installed to connect the cellulose acetate gel with its gel side for 3 mm at each end. Then the gel was submitted for 5 min to a pre-electrophoresis at 200 V. After that the gel was taken off and 0.25 µl samples applied by use of a Super-Z-12 application kit (Helena Laboratories). Usually sample applications were repeated three times to gain enough enzyme activity. Then the gel side of the cellulose acetate gel was again put on the platform of the electrophoresis chamber, contacted to the buffer strips, and submitted to 200 V for 30 min [18].

2.6. Visualization of Dihydrolipoyl Dehydrogenase Allozymes

Immediately after electrophoresis dihydrolipoyl dehydrogenase activities were visualized covering gels with an agar overlay. The staining solution consisted of 1 ml 0.1 M Tris-HCl buffer, pH 8.5, 1.5 ml NADH solution (3 mg/ml), 5 drops DCIP (2,6-dichlorphenol-indophenol solution, 3 mg/ml) and 5 drops MTT (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazoliumbromid solution, 10 mg/ml) [19] [20]. To that mixture 2 ml of a boiling agar solution (40 mg agar agar in 2 ml distilled water) were added, mixed and poured on the dry-tipped gel. After the agar had set, the covered cellulose acetate gels were incubated in the dark at room temperature until enzyme bands were visible. Then the overlay was washed under running tape water and afterwards put into a shaking water bath up to several hours. If the staining solution is intensively coloured and enzyme activity is low, the staining solution must be washed from the gel for several hours until the weak enzyme bands are clearly visible. In the case of light staining solutions and high enzyme activity the colour bands can be seen after a few minutes.

The decolorized gels were photographed and put on a transmitted light plate to note the visible enzyme bands. Gels were then dried over night between several layers of dry tissue and then stored in welded polyethylene pockets.

2.7. DNA Extraction

Total DNA was extracted according to [14] with a modified CTAB (cetyltrimethyl ammonium bromide-protocol [21] as published recently [15].

2.8. PCR-RFLP Analysis

To identify mycorrhizal samples and fruiting bodies, the multicopy internal transcribed spacer (ITS) region of their ribosomal DNA (rDNA) was amplified and sequenced. The rDNA repeats, comprising the 18S rRNA gene, the ITS-1-spacer, the 5.8S rRNA, the ITS-2-spacer and the 28S rRNA gene, was amplified using the primer pair ITS1 [22] and ITS4b [23] (**Table 2**). Primer ITS1 binds to the 3'-end of the 18S rRNA gene and primer ITS4b binds to the 5'-end of the 28S rRNA gene. If no PCR product resulted, the primer pair ITS1F/ITS4 was used [23]. The detailed analysis followed the one published by Schirkonyer *et al.* [15].

Table 2. Primers used for PCR-RFLP.

ITS 1 (White *et al.* 1990):	5'-TCCGTAGGTGAACCTGCGG-3'
ITS1F (Gardes und Bruns 1993):	5'-CTTGGTCATTTAGAGGAAGTAA-3'
ITS4 (White *et al.* 1990):	5'-TCCTCCGCTTATTGATATGC-3'
ITS 4B (Gardes und Bruns 1993):	5'-CAGGAGACTTGTACACGGTCCAG-3'

2.9. RNA Extraction

Total RNA was extracted from mycorrhizal roots or fruiting bodies using the "NucleoSpin® RNA-Plant"-Kit, by Machery and Nagel (Düren, Germany). An amount of 50 to 100 mg of fresh material was homogenized in a 1.5 ml Eppendorf Safe tube with a micropistil under liquid nitrogen. The resulting homogenate was pipetted on ice into a microcentrifuge tube and 350 µl "RAP"-buffer (guanidine-HCl lysis buffer) and 3.5 µl 2-mercaptoethanol added, vortexing the mixture. The resulting lysate was pipetted on a "NucleoSpin®"-filter inserted into a collecting tube and then centrifuged for 1 min at 11,000 × g at room temperature. The filtrate was transferred into a microcentrifuge tube, 350 µl ethanol (70%) added and the mixture five times pipetted up and down. The resulting lysate was loaded on a "NucleoSpin®-RNA-Plant" column and the unit centrifuged for 1 min at 11,000 × g, to bind the total RNA (and DNA) to the silica membrane. Then the column was placed into a new collecting tube, 350 µl "Membrane Desalting" buffer added to the column and the unit centrifuged for 1 min at 11,000 × g, to desalt the membrane. Afterwards, 95 µl DNase reaction mixture were applied onto the silica membrane of the column and the unit incubated at room temperature for 15 min to digest the bound DNA. Then, the silica membrane was washed adding 200 µl "RA2" buffer to the column and centrifuging the unit for 1 min at 11,000 × g. Afterwards, the column was placed into a new collecting tube adding 600 µl "RA3" solution to the column and centrifuging the unit for 1 min at 11,000 × g. The flow-through was discarded and the column placed in the collecting tube again. Then, 250 µl "RA3" buffer was added and the unit centrifuged for 2 min at 11,000 × g. After that the column was put into a nuclease-free 1.5 ml microcentrifuge tube. Total RNA was eluted from the membrane by adding 60 µl RNase-free water followed by a 1 min lasting centrifugation at 11,000 × g.

2.10. cDNA Synthesis

A first strand cDNA was synthesized by use of "The First Strand cDNA Synthesis-Kit" of Fermentas (St. Leon-Rot, Germany), annealing an oligo(dT) primer to the poly(A) tail of mRNAs. Into a microcentrifuge tube 14 µl of purified total RNA and 1 µl of oligo(dT) primer (100 pmol) were added, the mixture briefly vortexed and centrifuged for 2 sec at 11,000 × g. The RNA-primer mix was denatured at 70˚C for 5 min and then placed on ice. Then, 5 µl M-"MulV-5x RTase-buffer (250 mM Tris-HCl (pH 8.3), 375 mM KCl, 15 mM $MgCl_2$), 2 µl

dNTP-mix (10 mM), 1 µl "Ribbolock-RNAse inhibitor" and 1 µl diethyl pyrocarbonate (DEPC)-water were added, and the mixture incubated for 60 min at 42˚C. A 10 minute incubation at 70˚C terminated the reaction.

2.11. Amplification of cDNA and DNA Dihydrolipoyl Dehydrogenase-Sequences

The DNA sequence of the enzyme NADH diaphorase of *Xerocomus badius* and *X. pruinatus* associated with European beech or Norway spruce was amplified by use of the primer pair P1 and P2 (Table 3) while the primers Dia1-fw and Primer-rev1 were applied to amplify the corresponding cDNA sequences.

Primers were purchased from Eurofins MWG Operon (Ebersberg, Germany). The used Primers were deduced from partial cDNA sequences published at the Genbank NCBI for the basidiomycetes *Ustilago maydis*, *Cryptococcus neoformans*, *Laccaria bicolor* and the diaphorase sequence of the two basidiomycetes *Xerocomus badius* and *Xerocomus pruinatus* gained via genome sequencing [24]. To avoid pcr-products of the diaphorase enzymes belonging to the host trees the sequence of the plant *Arabidopsis thaliana* was integrated into the primer construction. Primers were designed from the aligned sequences of the above named organisms using the software Primer Premier (PREMIER Biosoft International, Palo Alto, USA). The dihydrolipoyl dehydrogenase gene sequence was amplified using a 20 µl PCR mixture contained the following components: 2 µl of a 10 × PCR-buffer, 2 µl 2 mM dNTP mix, 1 µl 10 pM Primer-fw1 or Primer-fw2, 1 µl 10 pM Primer-rev1 or Primer-rev2, 3.8 µl HPLC-H_2O and 0.2 µl 5 U/µl polymerase. Amplifications were performed by applying the following temperature program: 1) denaturation (5 min 94˚C), 2) 35 cycles for amplification (30 sec at 94˚C, 1 min at 50˚C, 2 min at 72˚C), 3) final extension (30 sec at 94˚C, 1 min at 50˚C and 10 min at 72˚C) and 4) storage at 5˚C.

2.12. Sequencing of PCR Products and Genome Sequencing

Sequencing of PCR products and genome-sequencing of the fungi *Xerocomus badius* and *X. pruinatus* via Illumina HiSeq 2000 (Illumina 2006, San Diego, California, USA) was done by GENterprise-Genomics (Mainz, Germany). For fungal identification, BLAST searches were carried out against the public sequence databases NCBI (http://www.ncbi.nlm.nih.gov/) and UNITE (http://unite.ut.ee). Sequences were assigned to matching species names when the BLAST matches showed identities higher than 97% and scores higher than 900 bits.

Table 3. Sequences of the primer pairs P1 and P2 and the primer DIA1-fw.

P1	Primer-fw1:	5'-CTT CGG TCA CAC GTA TCC T-3'
	Primer-rev1:	5'-CTC GCT GAG TGT GGG CTA-3'
P2	Primer-fw2:	5'-CCA GTG ACA CCA CTT ACA-3'
	Primer-rev2:	5'-TGA GTG TGG GCT AGA ATA GA-3'
	Dia1-fw:	5'-G(AG)T TGA GGC (AC)AA GAA C(AG)T-3'

The name suggested by UNITE, a curated database for ectomycorrhizal fungi [25], was used preferentially and that of NCBI only if there was no entry in UNITE.

3. Results and Discussion

3.1. Allozymes and Affiliation of Dihydrolipoyl Dehydrogenases

Separation of native proteins extracted from mycorrhizae of European beech by Cellulose Acetate electrophoresis resulted in up to seven isozymes of the enzyme dihydrolipoyl dehydrogenase. The various isozymes were adjoined to four gene loci A, B', B and C (**Figure 1**). At the loci A, B' and C a single isozyme was observed that migrated, depending on the sample from which it was taken, somewhat faster or slower. Consequently, two alleles were adjoined to each of the three loci. The dihydrolipoyl dehydrogenase enzymes expressed at these loci are assumed to be homodimers. At locus B either one faster or slower migrating allozyme or three isozymes could be visualized after electrophoresis. The homozygotic states of the corresponding gene locus are expressing two homodimeric forms of the dihydrolipoyl dehydrogenase enzyme (B1 B1 and B2 B2) while the heterozygotic form leads to two homodimeric allozymes and one heterodimeric allozyme (B1 B1, B1B2 and B2 B2) (**Figure 1**).

In order to find out the affiliation of dihydrolipoyl dehydrogenase allozymes to tree roots and fungal hyphae respectively, ectomycorrhizae from European beech, Sessile oak, Norway spruce and European larch associated with the fungus *Xerocomus pruinatus* were investigated as well as rhizomorphae and fruiting bodies of that fungus. Additionally, non mycorrhizal root tips of European beech were analysed. It results that allozymes at locus C exclusively stem from the fungus *X. pruinatus* (**Figure 2**). The fungus specific affiliation of locus C was

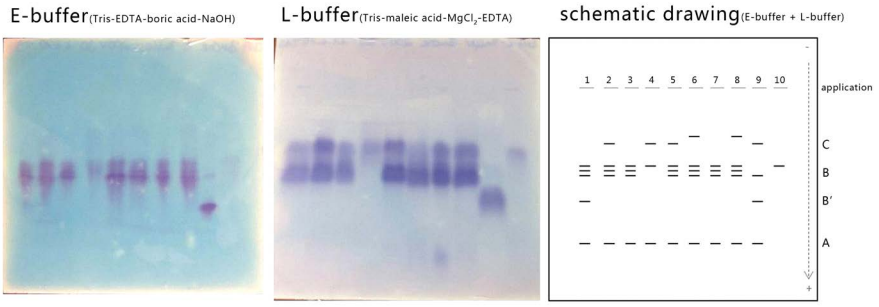


Figure 1. Allozyme patterns of dihydrolipoyl dehydrogenase enzymes visualized after Cellulose Acetate electrophoresis. Enzymes were extracted from ectomycorrhizae of European beech (*Fagus sylvatica*) associated with hyphae of the following fungi: *Lactarius hepaticus*, 2: *Xerocomus pruinatus*, 3: *Paxillus* spp., 4: *Russula ochroleuca*, 5: *Fagirhiza* spp. and 6, 7, 8, 10: *Xerocomus pruinatus*. Sample 9 represents an extract of root tips of Norway spruce (*Picea abies*) in symbiosis with *Xerocomus badius*. Single enzyme bands separated better upon electrophoresis in a Tris-EDTA-borate buffer of pH 8.9, while the enzymes forming a complex of three enzymes were better resolved in a Tris-maleate-$MgCl_2$-EDTA buffer of pH 7.8.

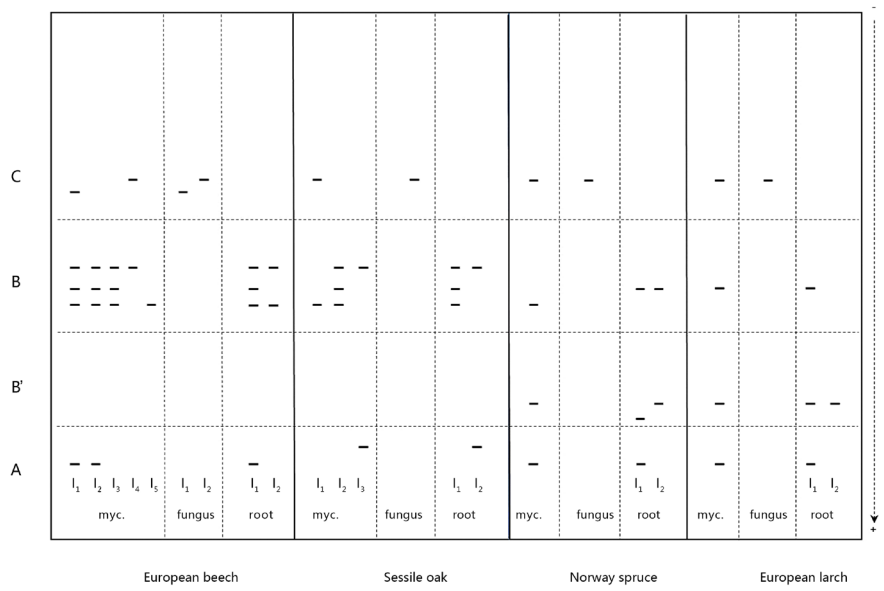


Figure 2. Scheme of dihydrolipoyl dehydrogenase patterns visualized on the separation medium Cellulose Acetate after electrophoresis. Myc: mycorrhizal root tips associated with *Xerocomus pruinatus*. Fungus: fruiting bodies, peeled off hyphae or rhizomorphae. Root: root tips of European beech seedlings grown in hydroculture or peeled fine roots without fungal hyphae.

also observed for the ectomycorrhizal fungi *Lactarius* spp., *Paxillus involutus*, *Russula ochroleuca* and *Xerocomus badius* in association with the same four host trees (**Figure 3**).

The loci A, B' and B are belonging to each of the four host trees. The deciduous trees European beech and Sessile oak are expressing loci A and B whereas the conifers Norway spruce and European larch possess in addition the active gene locus B' (**Figure 2** and **Figure 3**).

In extracts of the ectomycorrhizal fungi *Boletus edulis*, *Laccaria amethystina*, *Russula ochroleuca*, *Tylopilus felleus*, *Xerocomus badius* and *Xerocomus pruinatus* only one active dihydrolipoyl dehydrogenase enzyme and one corresponding enzyme gene was observed. We assume that the enzyme is part of the two mitochondrial enzyme complexes pyruvate dehydrogenase (EC 1.2.4.1) and alpha-ketoglutarate dehydrogenase (EC 1.2.4.2). In contrast to these results we conclude the presence of two active dihydrolipoyl dehydrogenase genes within the deciduous tree species European beach and Sessile oak. Here, each of the two mitochondrial enzyme complexes pyruvate dehydrogenase and alpha-ketoglutarate dehydrogenase may contain a slightly differing form of the enzyme dihydrolipoyl dehydrogenase. The two conifers Norway spruce and European larch which function as hosts for ectomycorrhizal fungi are expressing three dihydrolipoyl dehydrogenase gene loci. Provided the corresponding enzyme forms result from two, respectively three different enzyme loci, upon the evolution of the deciduous trees from conifers one of the corresponding enzyme genes may have been silenced. Histochemical stainings that served to visualize dihydrolipoyl

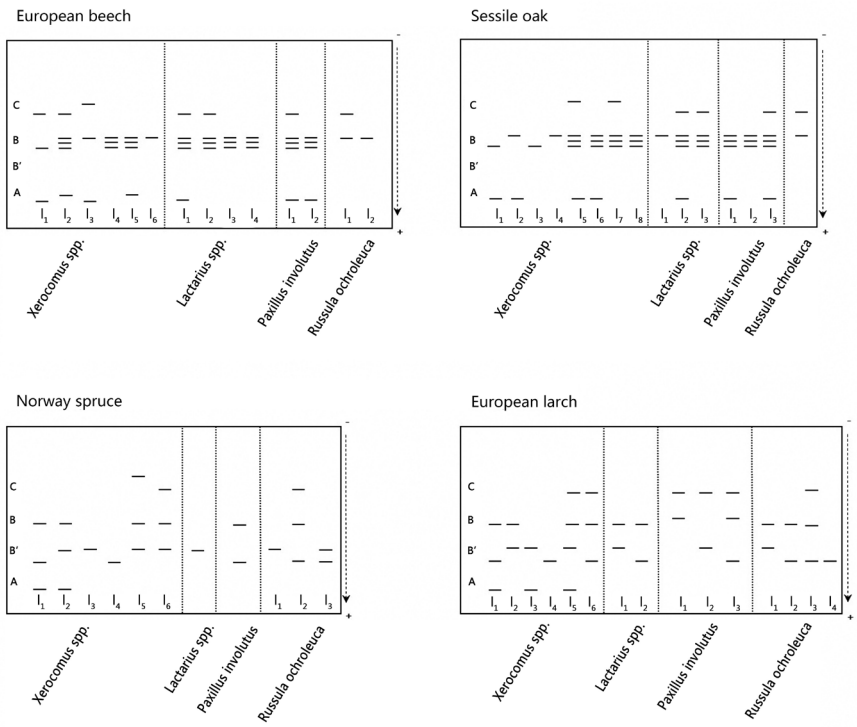


Figure 3. Allozyme pattern of NADH-diaphorase in different ectomycorrhizal fungi in symbiosis with beech, oak, spruce and larch. (I1, I2... = individuals).

dehydrogenase activities showed that the enzyme was more active in hyphae of *Xerocomus badius* than in those of *X. pruinatus*. Kinetic analyses lead to corresponding results. Differing activities were also observed between the ectomycorrhizal species *Cenococcum geophilum*, *Scleroderma citrium*, *Paxillus involutus* and *Pisolitus tinctorius* [26].

3.2. Molecular Genetic Analyses

The DNA of *Xerocomus pruinatus* and *X. badius* was analyzed by use of "The Next-Generation-Illumina sequencing-method" (Solexa/Illumina, Berlin, (performed by GENterprise-Genomics, Mainz University). After the genome had been sequenced, localized primers were deduced as described in chapter 2.11 in order to amplify the gene sequence of the dihydrolipoyl dehydrogenase gene.

The full length of the dihydrolipoyl dehydrogenase gene has a length of 1631bp (cDNA: 1370 bp + (5 Introns = 261 bp) in *Xerocomus badius* and 1721 (cDNA: 1460 bp + 261 bp) in *X. pruinatus* (cf. sequences listed at the **Appendix**). The DNA sequences of the dihydrolipoyl dehydrogenase gene isolated from *Xerocomus pruinatus* and *X. badius* resemble those of other fungi deposited at the NCBI-gene bank to 70% to 78% (**Table 4**).

Five introns, each having a length of 52 bp, could be localized comparing the full gene length with that of the cDNA length (**Table 5**).

The gene sequences of the dihydrolipoyl dehydrogenase enzymes existing within the ectomycorrhizal fungi *Boletus edulis*, *Laccaria amethystina*, *Paxillus*

Table 4. Similarity of the genomic sequence of the dihydrolipoyl dehydrogenase of *Xerocomus pruinatus* and *X. badius* in comparison to other fungi.

Xerocomus pruinatus	% similarity compared to		*Xerocomus badius*	% similarity compared to	
	X. pruinatus	99		*X. pruinatus*	90
	X. badius	90		*X. badius*	98
	Cryptococcus neoformans	78		*Cryptococcus neoformans*	78
	Coprinus cinerea	75		*Ustilago maydis*	76
	Laccaria bicolor	75		*Coprinus cinerea*	74
	Cryptococcus gatti	71		*Laccaria bicolor*	74
	Ustilago maydis	70		*Cryptococcus gatti*	71

Table 5. Position and length of the five introns of the dihydrolipoyl dehydrogenase gene in *Xerocomus badius* and *X. pruinatus.*

Intron number	Sequence range	Size (bp)
1	295 - 347	52
2	485 - 538	53
3	985 - 1037	52
4	1172 - 1224	52
5	1691 - 1743	52

involutus and *Russula ochroleuca* also include five 52 bp long introns located at the regions of the *Xerocomus* gene. These observations are in accordance with reports concerning the gene structure of the basidiomycetes *Cryptococcus gatti*, *Cryptococcus neoformans*, *Coprinus cinerea*, *Ustilago maydis* and *Laccaria bicolor* and the ascomycetes *Candida albicans*, *C. orthopsilosis*, *Mycosphaerella graminicola* and *Trichophytum rubrum* deposited at the Gene Bank (NCBI). The coding sequence of the gene of *X. pruinatus* deviates at 144 positions from that of *X. badius*. Besides the single nucleotide polymorphisms, the *X. pruinatus* gene contains a 48 bp long sequence at the positions 200 to 248 that could not be proved for the DNA and cDNA sequences of the gene from *X. badius*. Altogether, the two gene sequences deviate at 192 positions, which makes 11%. The number of single nucleotide polymorphisms of the five introns of *X. badius* and *X. pruinatus* sum up to 74 bp, corresponding to a deviation of 28.5%. Consequently, the nucleotide deviations in the five intron areas are about three times higher than those within the coding regions. The host trees European beech and Norway spruce did not influence the dihydrolipoyl dehydrogenase gene sequences in the two *Xerocomus* species.

3.3. Protein Structures

The cDNA sequences of the dihydrolipoyl dehydrogenases from the two *Xero-*

comus species served to determine their amino acid sequences (Figure 4 and Figure 5).

The number of positively charged amino acid residues (Arg and Lys) within the enzyme of *X. pruinatus* makes 79 while it makes 68 in *X. badius*. The number of negatively charged amino acids (Asp and Glu) makes 46 in *X. pruinatus* and 48 in *X. badius*. Molecular weights and isoelectric points were determined by use of the software ExPASy-"Protparam" (https://web.expasy.org/protparam/) (Table 6).

The length of cDNA of the dihydrolipoyl dehydrogenase gene of the basidiomycete *Coprinopsis cinerea* makes 1527 bp, corresponding to 494 amino acids [27]. The cDNA length of the gene of the fungus *Laccaria bicolor* makes 1593 bp, which equals 514 amino acids [28]. The dihydrolipoyl dehydrogenase enzyme of the yeast *Saccharomyces cerevisiae* comprises 487 amino acids and its molecular

```
                10          20          30          40          50          60          70
           ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
X. pruinatus QRTTQRVRLM KANDFSFLHV ARRAACQRTR SMASSGLDTF RVVRCHYWWR WWLCRSHQGR ATRFEDCLHR

                80          90          100         110         120         130         140
           ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
X. pruinatus EAWFAWWNVF ERRMHTVKGH AQQLTYLPPN EAPKTWYLRF TQPASDVEGQ GSIGRRAYKG YGDLQAKQGR

                150         160         170         180         190         200         210
           ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
X. pruinatus LHKGYRFFRF PNAYRCTTSR GWRDGNRKER HHCYRLRLRH SQGAPFKDEK QIVSSTGALE LQEVPDKMVV

                220         230         240         250         260         270         280
           ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
X. pruinatus IGGGIIGLEM GSVWSRLGAE VTVVEFWWHR RCWHRGGRKQ FQKILAKQGI KFKLGTKVLS ADKADGKVLV

                290         300         310         320         330         340         350
           ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
X. pruinatus KRSAKGDKEE TSKPTSFWFR SVVVHTEGLN LEALGVEKDN KGRIIIDDQF STSVKNIKCI GDVTFGPMLA

                360         370         380         390         400         410         420
           ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
X. pruinatus HKAEEEGIAA VEYLKSGHGH VNYNGIPSVV YTHPEVAWVG QTEQDLKATG VQYNIGKFPF AANSRAKTNL

                430         440         450         460         470         480
           ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|.
X. pruinatus DSEGFVKFLS EKETDRILGV HIIGLTLVKS RKPSLLWSTV QARKISLVQL TLPTLSEAFK EAAMSA
```

Figure 4. Amino acid sequences of the dihydrolipoyl dehydrogenase gene of *Xerocomus pruinatus*.

```
                10          20          30          40          50          60          70
           ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
X. badius  QDCYRSESMA RRGLDIFWVV RRRYRWRWWL CGSHQGRATR FEDCLHREAW FAWWDVFERR LHPVKGDAQQ

                80          90          100         110         120         130         140
           ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
X. badius  LTPLPPNDPP REAWHRLRFA QLASNVEGQG PIGRWADGYD LQAKQGRLHQ GERFFCFSNT HRCTTSRGWR

                150         160         170         180         190         200         210
           ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
X. badius  DGNKERHHCY RLRRLHRSQE APFKLDEKQI VSSTGALELQ EVPNKMVVIG GGIIGLEMGS VWSRLGAEVT

                220         230         240         250         260         270         280
           ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
X. badius  VVEFWWHRRC WHRGGCKQFQ KILAKQGLKF KLNTKVMSAD NVDGKVLVKQ SPKGDKEETS RQTLFWSRSV

                290         300         310         320         330         340         350
           ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
X. badius  VVHTEGLNLA YVVENKGRII IDDQLNTSVK NIKCIGDVTF GPMLAHKAEE EGIAAVEYIK SGHGHVNYNG

                360         370         380         390         400         410         420
           ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
X. badius  IPSVVYTHPE VAWVGQTEQD LKAAGVQYNV GKFPFAANSR AKTNLDSEGF VKFLSEKETD RILGVHIIGL

                430         440         450
           ....|....| ....|....| ....|....| ....
X. badius  TPVRSRRRFL LRLAQARWIS LAQHTLSSIT DMVA
```

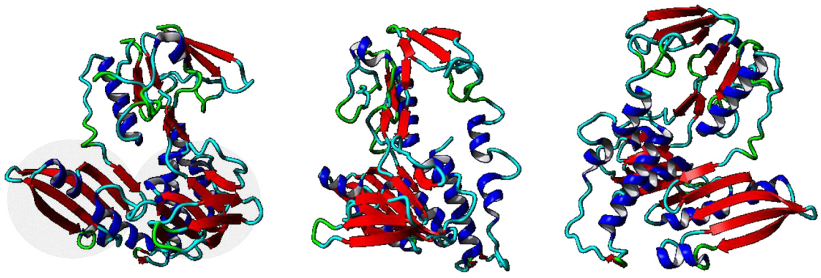
Figure 5. Amino acid sequences of the dihydrolipoyl dehydrogenase gene of the fungus *Xerocomus badius*.

mass makes 51558 Da [29]. In most organisms, the enzyme represents a homodimer with a monomeric molecular weight of 50 to 55 kDa (Data Bank BRENDA, https://www.brenda-enzymes.org/index.php). Crystallographic studies of the human enzyme dihydrolipoyl dehydrogenase lead to the conclusion that its amino acid sequence contains four functional domains: an NADH domain within a larger FAD domain, a central domain and a dimerization domain at its C-terminal end [30]. This result was confirmed for the bacterial, fungal and plant enzyme [31]. Comparing the amino acid sequences of the dihydrolipoyl dehydrogenase enzymes of several fungi with those of the two *Xerocomus* species, we conclude that within the latter the range from amino acid 207 to 281 represents a short NADH binding site being part of a larger FAD-binding domain. The second highly conserved region is located at the C-terminal end and ranges from amino acid 375 to 485 making the binding/dimerization domain. These domains are characteristic for pyridine nucleotide-disulfide oxidoreductases (InterPro Protein sequence analysis & classification; http://www.ebi.ac.uk/interpro/entry/IPR012999). By use of the SWISS-MODEL a fully automated protein structure homology-modelling server, accessible via ExPASy web server, the 3D-structure of the *Xerocomus badius* enzyme (454 amino acids) and *X. pruinatus* (486 amino acids) could be evaluated (**Figure 6**). The amino acid chains deviate by 32 amino acids but the 3D structures are congruent.

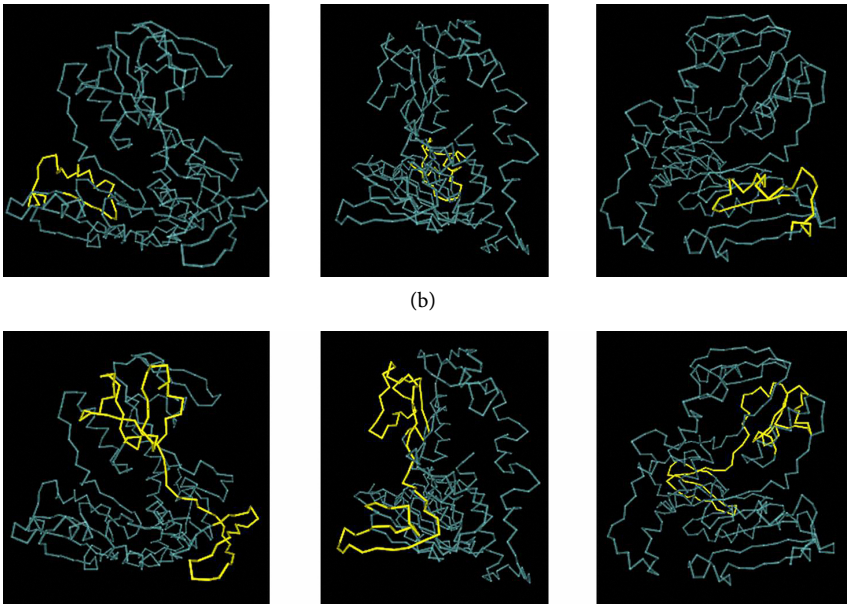
Altogether calculations resulted in 27.4 alpha helices, 24.8% beta strand structures, 11.9% beta loops and 35.9% other windings SWISS-Model [32] [33] [34] [35].

Table 6. Number of nucleotides and number of amino acids, molecular weights and isoelectric points of the enzyme dihydrolipoyl dehydrogenase of *Xerocomus pruinatus* and *X. badius.*

Species	Nucleotides (bp)		Amino acids	Molecular weight (Da)	calculated isoelectric point
	DNA	cDNA			
Xerocomus pruinatus	1721	1460	486	55537.6	9.99
Xerocomus badius	1631	1370	454	52163.5	9.68



(a)



(b)

(c)

Figure 6. (a) 3D structure of the enzyme NADH-diaphorase from the ectomycorrhiza fungi *Xerocomus badius* and *Xerocomus pruinatus*. One and the same 3D structure is represented from different angles. (grey background = large β-sheet areas, blue = α-helix, red = β-sheet, turquoise = random coil). The 3D schemes were displayed and colored in YASARA View (http://www.yasara.org/); (b) Dimerization site at the C-terminal end of the enzyme NADH-diaphorase from the ectomycorrhiza fungi *Xerocomus badius* and *Xerocomus pruinatus*; (c) NADH-binding site of the enzyme NADH-diaphorase from the ectomycorrhiza fungi *Xerocomus badius* and *Xerocomus pruinatus*.

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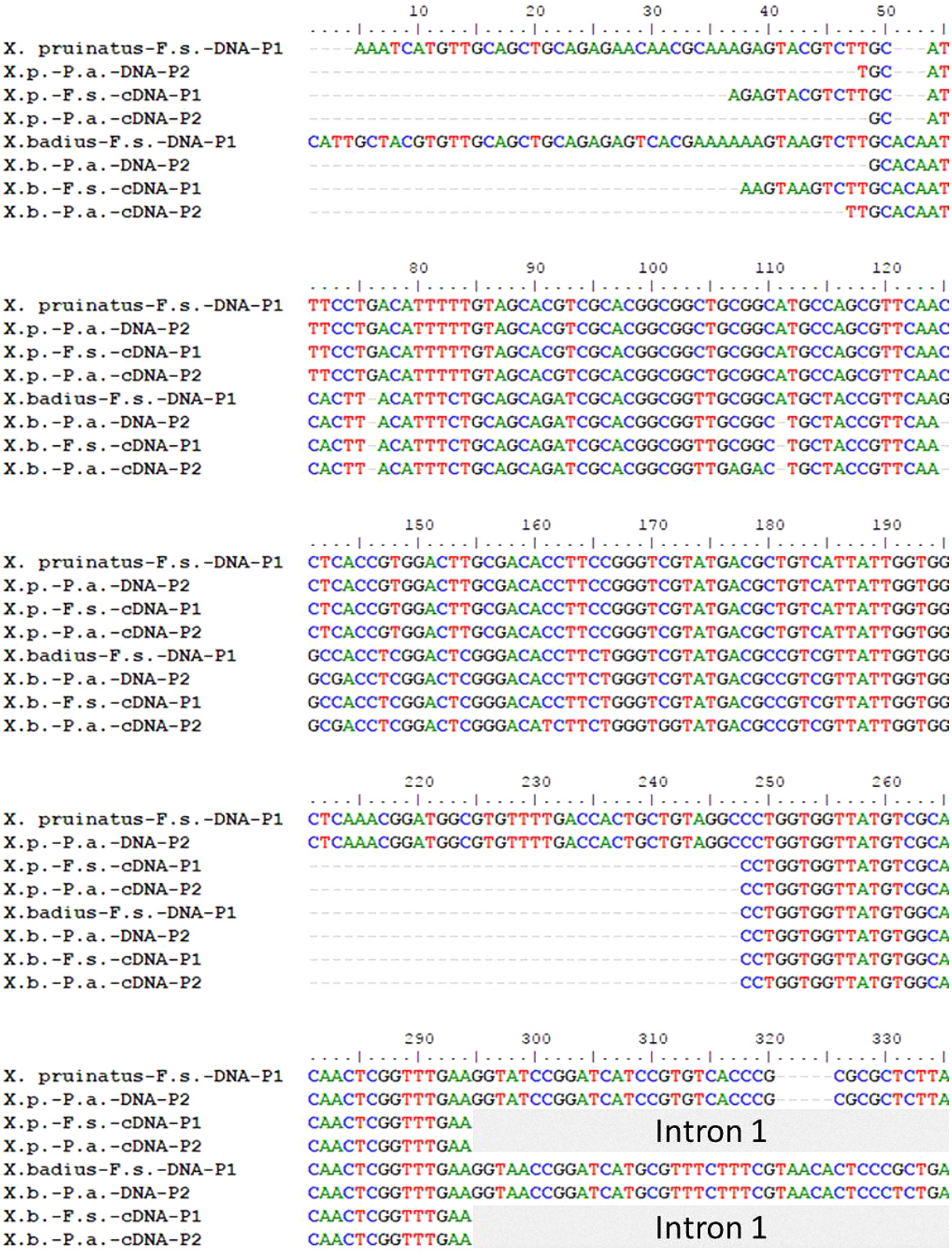
Appendix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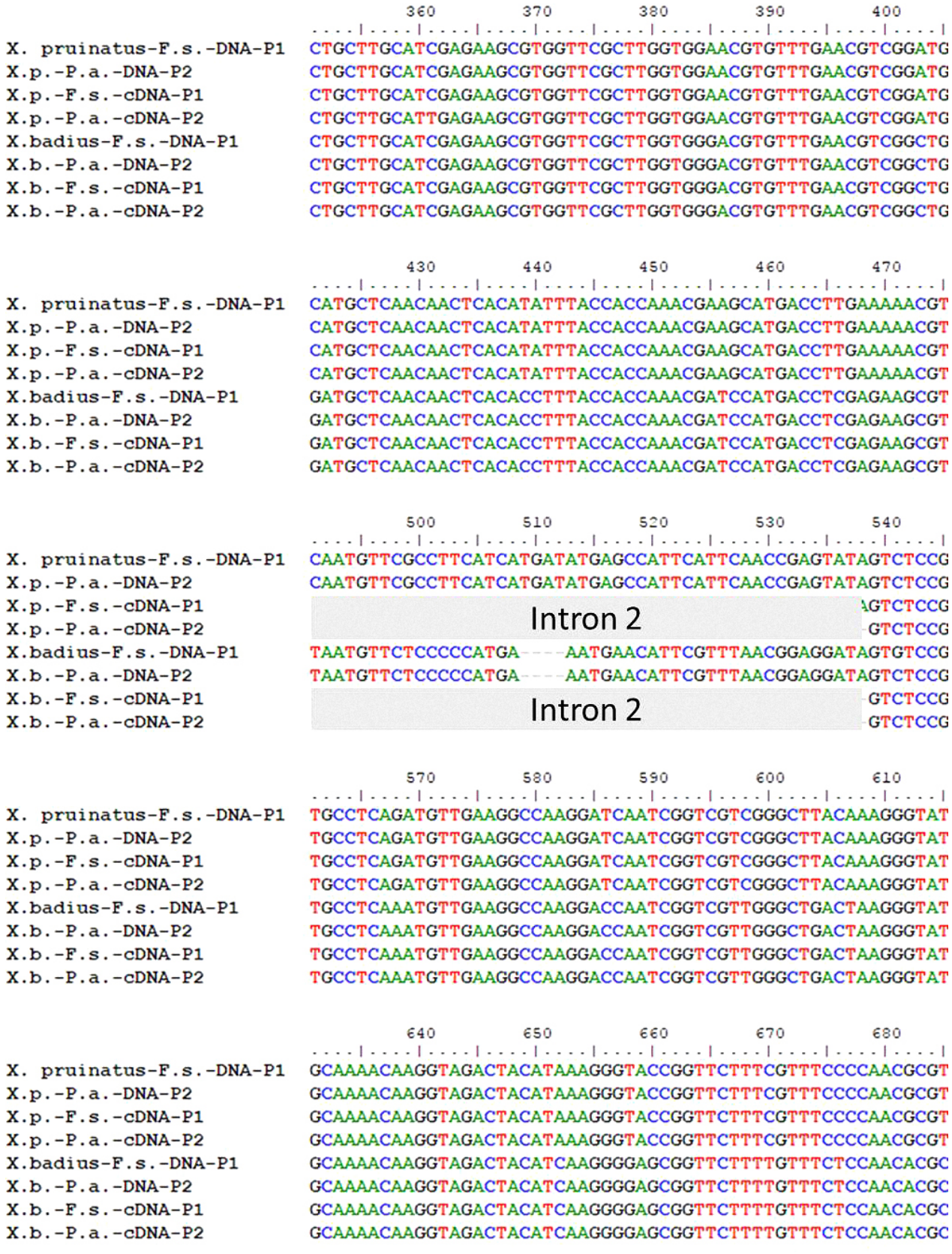
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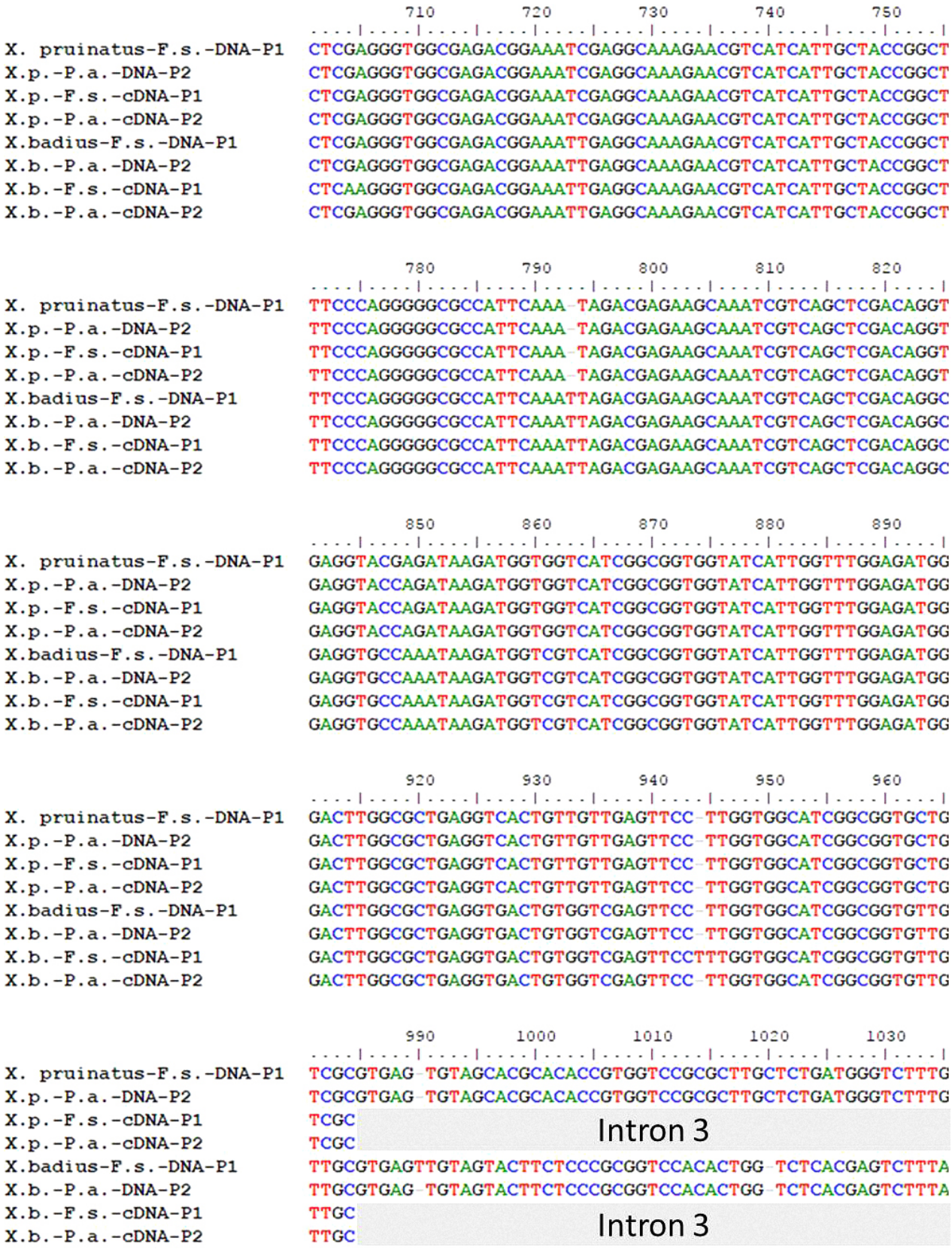
(b)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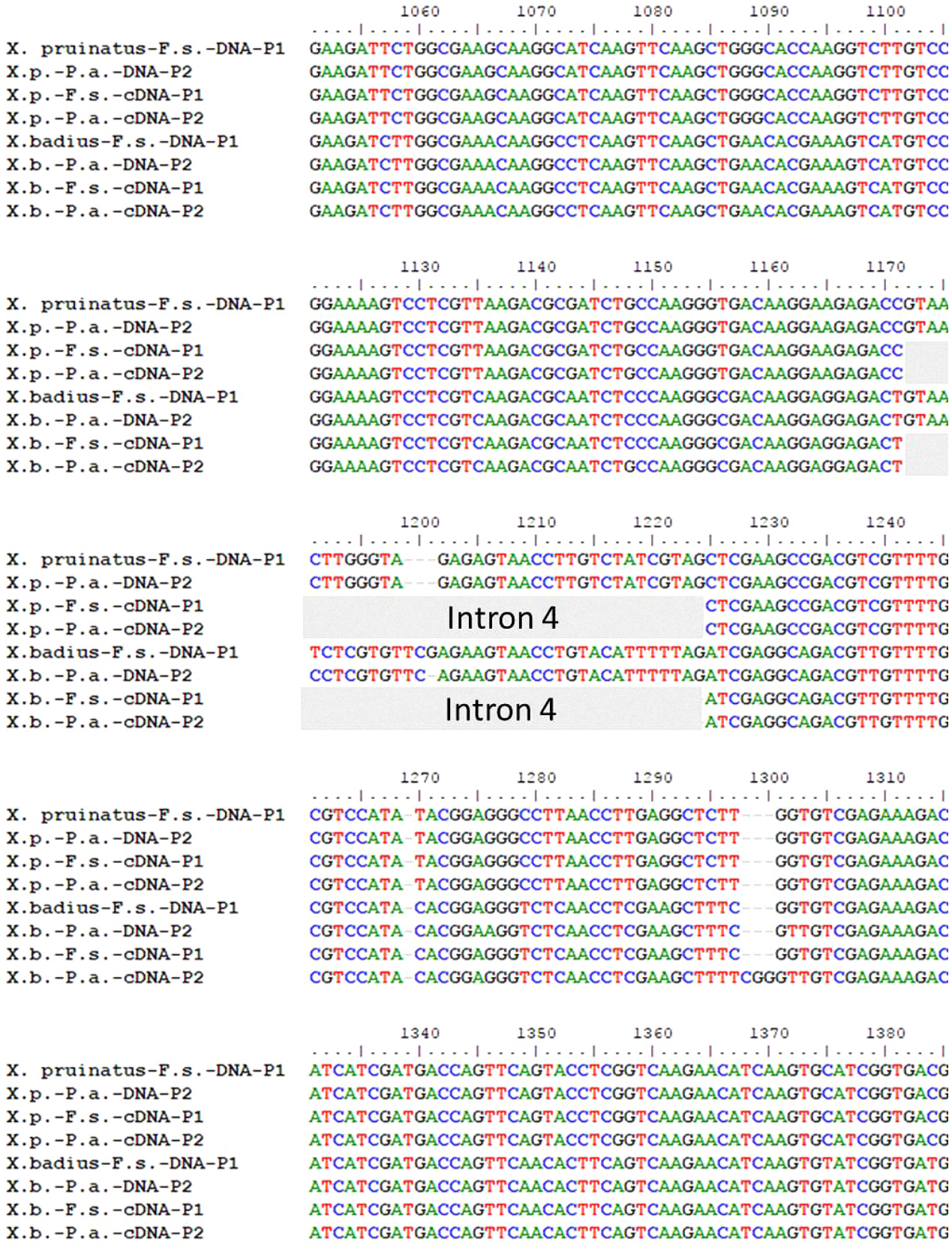
(c)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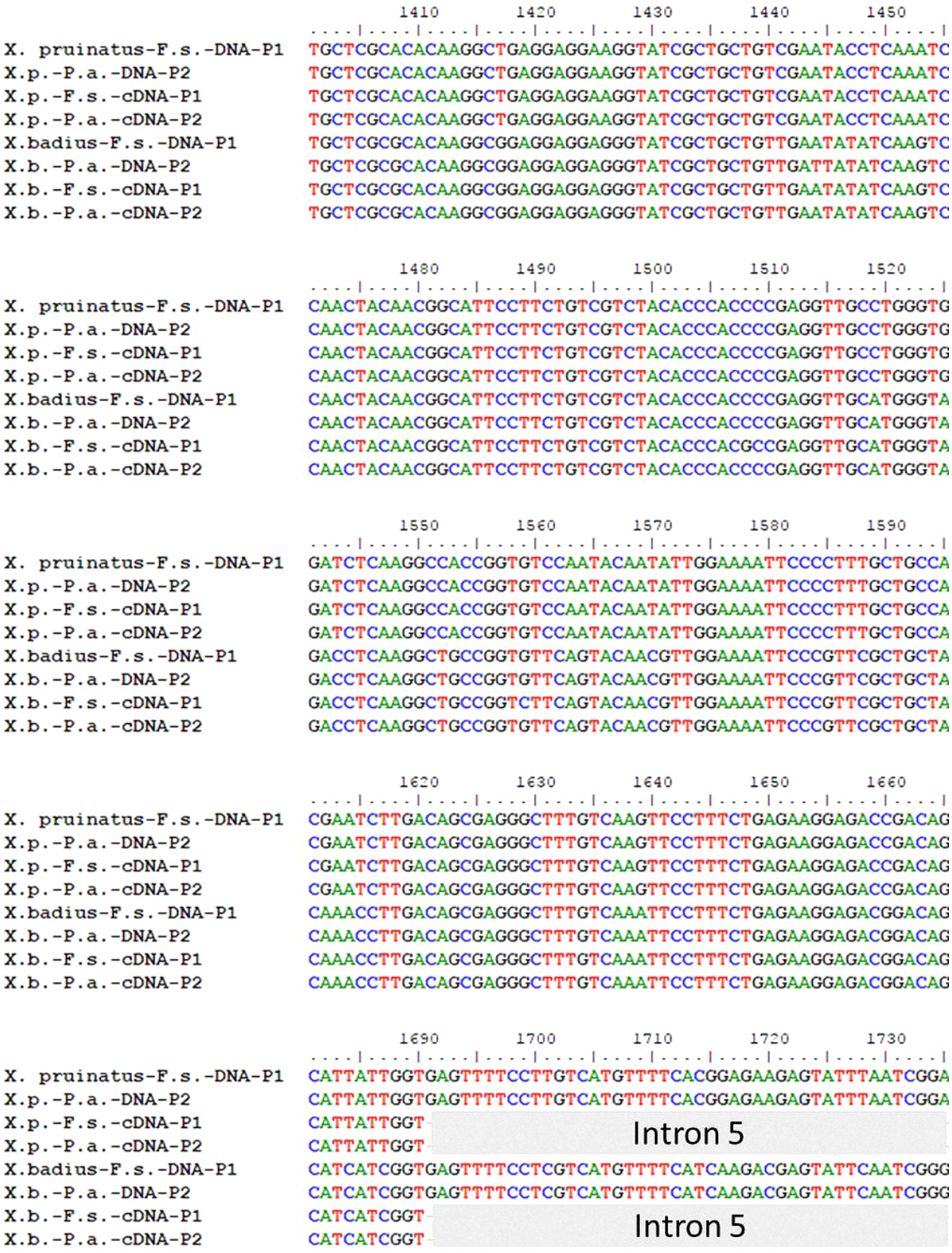
(d)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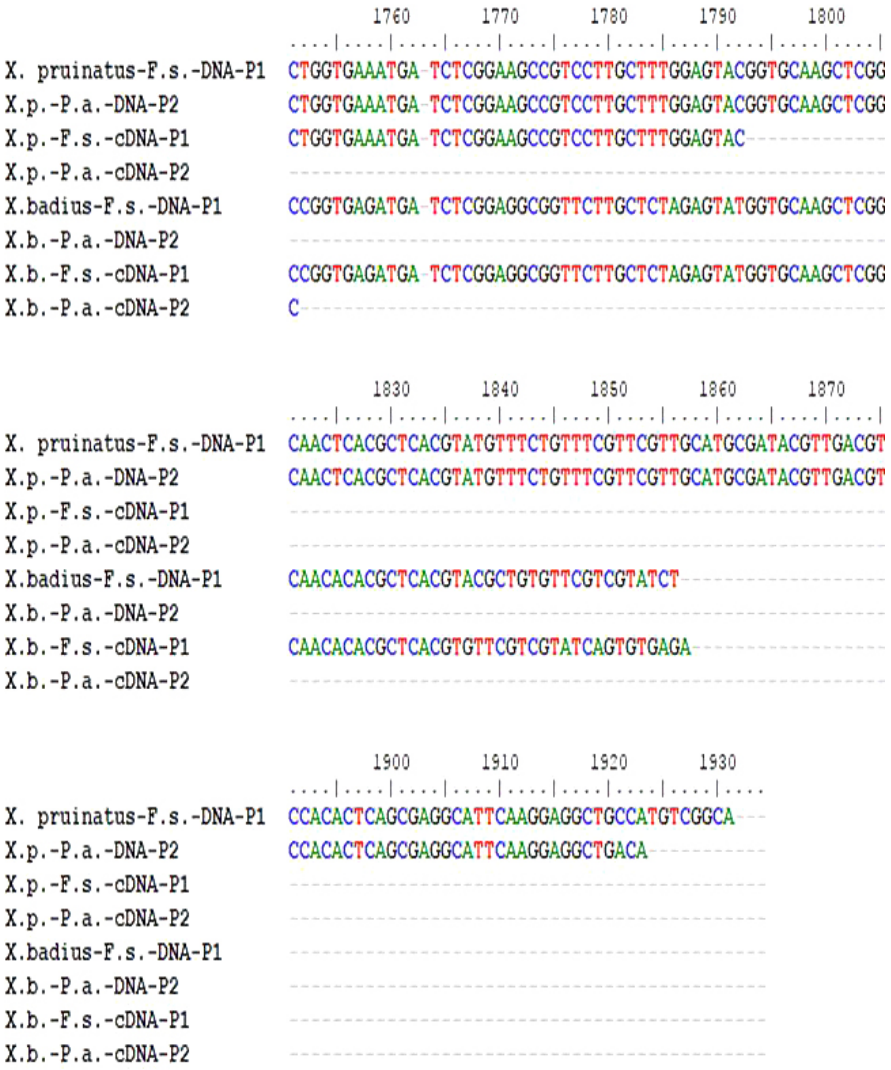
(e)



(f)

Figure A1. Comparison of the DNA and cDNA sequence of the enzyme NADH diaphorase in the two ectomycorrhiza fungi *Xerocomus badius* (Xb) and *Xerocomus pruinatus* (Xp) in symbiosis with beech (*Fagus sylvatica*, Fs) and spruce (*Picea abies*, Pa).