Two Separate Genes Encode the Catalytic 70 kDa V-ATPase Subunit in *Psilotum* and *Equisetum*

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Vacuolar type ATPases have been found on various endomembranes of eukaryotic cells, *e.g.* lysosomes, chromaffin granules, vesicles derived from the Golgi apparatus, endosomes and vacuoles. Although this ATPase type is targeted to different compartments in one cell, only one gene for each subunit had been found per genome.

Using PCR across intron-exon boundaries we show that two different genes encode the catalytic subunit of the V-ATPase in *Psilotum nudum* and *Equisetum arvense*. The substitution rates for the three codon positions and the intervening sequences show that in *Psilotum* both genes are transcribed and are under selection pressure, however, this seems not to be the case for *Equisetum*. The relatively high similarity between the two genes found in each species as compared to the interspecies similarities suggest that for some time after the gene duplication had occurred the two copies were subject to gene conversion mechanisms. An unexpected degree of conservation of the intervening sequences themselves is noted and statistically verified, however, no structural constraints that could explain these findings were detected.

Introduction

Ion pumping ATPases/ATP synthases, especially proton-pumping ATPases, play a central role in bioenergetics. Their importance is evident from the fact no known life form exists without them. Based on inhibitors and subunit compositions these transport ATPases have been divided into three categories: P(or E1E2)-, F(or F_1F_0)-, and V(or vacuolar)-type ATPases.

P-type ATPases are characterized by their sensitivity to vanadate and the formation of an acyl phosphate intermediate during ATP hydrolysis.

F-type ATPases are inhibited by oligomycin, venturicidin and azide. Their physiological function is ATP synthesis in mitochondria and chloroplasts and the energization of the plasma membrane in eubacteria. F-ATPases are multi subunit enzymes. They can be dissociated into a water-soluble, ATP-hydrolyzing F_1 part and a membrane component (F_0) involved in H^+ translocation. The subunit composition of the F_1 part is $\alpha_3\beta_3\gamma_1\delta_1\varepsilon_1$. The molecular masses of α and β are about 50–60 kDa, the γ is about 30–36 kDa and the δ and ε subunits are below 20 kDa (for further discussion see [1] and [2]).

V-type ATPases are inhibited by nitrate, but insensitive to vanadate, azide and oligomycin [3]. They are found in the vacuolar membrane of plants [4], lysosomes [5], endosomes [6], clathrin coated vesicles [7], secretory granules [8], Golgi vesicles [9–11], and the plasma membrane of some specialized animal cells [12–14]. Like the F-ATPases they can be dissociated into a water-soluble and a membrane portion [15, 16]. Their function is the acidification of the interior of the organelles, coupling the hydrolysis of ATP to the translocation of protons. In addition to several single copy subunits the water-soluble part contains three copies each of the two major polypeptides [17], a catalytic subunit of about 70 kDa binding the ATP that is hydrolyzed during the catalytic cycle [18] and a noncatalytic subunit of about 60 kDa in size. These two subunits have been shown to be homologous to the beta and alpha subunits of the F-ATPases respectively [19, 20].

It has not only been shown that the catalytic and noncatalytic subunits of ATPases present in

Abbreviations: PCR, polymerase chain reaction; V-ATPase, H^+-pumping ATPase of the vacuolar type. The sequences reported in this paper have the following EMBL database accession numbers: X 5683, *E. arvense,* small intron; X 5684, *E. arvense,* large intron; X 5685, *P. nudum,* small intron; X 5686, *P. nudum,* large intron.

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archaebacteria are more similar to the corresponding V-type ATPase subunits than they are to F-type ones but also that the catalytic (β and 70 kDa) and noncatalytic (α and 60 kDa) subunits are homologous to each other. These two subunit types (catalytic and noncatalytic) presumably arose by gene duplication before the divergence of archaebacteria, eubacteria and eukaryotes [20, 21].

Extending the use of proton-pumping ATPases as molecular markers for the evolution of organisms to the evolution of early land plants, we present data on two early branching land plants *Psilotum* and *Equisetum*. Employing two oligonucleotide primers directed to part of the core region of the catalytic subunit of the V-type ATPase from *Psilotum* and *Equisetum*, we were able to demonstrate that this subunit is encoded by at least two different genes in these plants. Implications for the evolution of the V-type ATPase in early land plants are discussed.

Materials and Methods

All chemicals are from Pharmacia, BRL and Merck. [α-^{35}S]dATP (specific activity 1000 μCi/mmol) from DU PONT NEN Products. Plant material (*Psilotum nudum* and *Equisetum arvense*) was obtained from the University of Connecticut Biological Sciences greenhouse.

Isolation of genomic plant DNA

4 g of fresh plant tissue were ground in a precooled mortar in the presence of liquid nitrogen. 10 ml of extraction buffer (100 mM Tris-HCl, pH = 8; 100 mM EDTA; 250 mM NaCl and 100 μg/ml Proteinase K) was added, incubated at 65 °C for 2 h and the cell debris was removed by centrifugation at 10,000 × *g* for 15 min. The supernatant was extracted once with phenol and once with chloroform, 100 μl/ml ethidium bromide (10 mg/ml) was added, the solution was adjusted to a density of 1.55 g/ml with CsCl and spun to equilibrium in a VTi 65.2 rotor (Beckman) at 65,000 rpm for 16 h at 20 °C. The DNA band visualized under UV light was harvested and the ethidium bromide was removed by extraction with SSC buffer (0.15 M NaCl; 15 mM Na_3 citrate) equilibrated isopropanol and the DNA was precipitated with isopropanol at −20 °C for 12 h.

The vacuum-dried pellet was resuspended in 25 μl dH_2O and 2 μl was used for the subsequent PCR reaction.

Polymerase chain reaction

A portion of the gene coding for the V-type ATPase was amplified by the Polymerase Chain Reaction using two primers (32 bp and 33 bp long) constructed after conserved regions in known cDNA sequences (see Results). The optimum conditions of the reaction with respect to yield were: *Taq* Polymerase buffer (70 mM Tris-HCl, pH = 8.8, with 2 mM $MgCl_2$ and 0.1% (v/v) Triton X-100); 0.1% (v/v) Tween 20; 100 μg/ml BSA; 50 pmol of each primer; dATP, dCTP, dGTP and dTTP (200 μm each) 10–30 ng of genomic plant DNA and 1.25 U of *Taq* Polymerase (New England Biolabs) in a total volume of 50 μl. The sample was overlaid with 100 μl of mineral oil to prevent evaporation. The samples were run in a Thermal Cycler (Cetus Corp.) for 30 cycles, each cycle consisted of 1.5 min denaturation at 94 °C, 1.5 min of annealing at 45 °C and 2 min of polymerization at 72 °C. After the last cycle the polymerization at 72 °C was extended to 20 min. The reaction products were analyzed by electrophoresis on an 0.8% agarose gel, revealing two bands with a length of approximately 290 bp and 250 bp for *Psilotum* and 280 bp and 250 bp for *Equisetum* respectively. A piece of agarose containing the amplified DNA band was cut out from the gel, placed in an Eppendorf vial and soaked for 12 h at 4 °C in 50 μl dH_2O.

Sequencing of PCR amplified DNA

After 12 h of soaking, when sufficient DNA had diffused out of the agarose, 1 μl of the solution was used for a second PCR. The conditions for the reaction were the same as described above, but only 25 cycles were run with a rise in the annealing temperature to 65 °C and a reduction in the polymerization time at 72 °C to 1 min. 40 μl out of a 50 μl reaction were loaded on a 0.8% agarose gel an run for 2 h with 10 V/cm. The band was localized under UV light and a scalpel used to make an incision in the gel, directly in front of the band. Using wide edged forceps a piece of activated DEAE cellulose (soaked 5 min in 10 mM EDTA, 5 min in 0.5 M NaOH, rinsed with 10 ml of dH_2O

and stored in dH_2O at 4 °C) cut to appropriate size was inserted and the electrophoresis continued for 5 min with 10 V/cm until the band of the DNA had completely migrated into the paper. After removal from the gel the paper was rinsed with 10 ml of a low salt elution buffer (50 mM Tris-HCl, pH = 8; 0.15 M NaCl and 10 mM EDTA, pH 8) to remove pieces of agarose, transferred to an Eppendorf vial and the DNA eluted at 65 °C for 30 min in the presence of 100 µl of a high salt elution buffer (50 mM Tris-HCl, pH = 8; 1 M NaCl and 10 mM EDTA, pH = 8). The eluate was transferred to a new tube, 100 µl of fresh high salt elution buffer were added to the paper and the incubation continued for 15 min. The combined eluates were extracted with phenol, precipitated with ethanol at −20 °C for 12 h, and the pellet was vaccum-dried and resuspended in 25 µl dH_2O.

The nucleotide sequence was determined using the ^{T7}Sequencing Kit (Pharmacia LKB Biotechnology) and [α-^{35}S]dATP (1000 µCi/mmol). The primers used for sequencing were two shorter versions (18 bp) of the PCR primers, lacking the redundant region (Fig. 1). Annealing of the primers to the template was achieved by denaturing 11.5 µl of the purified double stranded DNA at 95 °C for 3 min in the presence of 2 µl annealing buffer and 0.5 µl (50 pmol) of the primer and immediate freezing in an ethanol/dry ice bath. After thawing at room temperature the sequencing procedure was continued as described in the kit manual. The samples were electrophoresed in a 8% denaturing polyacrylamide gel containing 8 M urea and analyzed by autoradiography. Using the left and right PCR primer for sequencing the nucleotide sequence of the amplified DNA fragments could be obtained from both directions without any subcloning.

Results

For the specific amplification of part of the catalytic subunit of the V-type ATPase from genomic DNA from primitive land plants two oligonucleotide primers were constructed using sequences from *Daucus carota* and *Neurospora crassa* (Fig. 1). The primers span a 156 bp segment in *Daucus carota* and a 153 bp fragment in *Neurospora crassa,* including the primers. Using these primers to amplify genomic DNA from *Psilotum nudum*

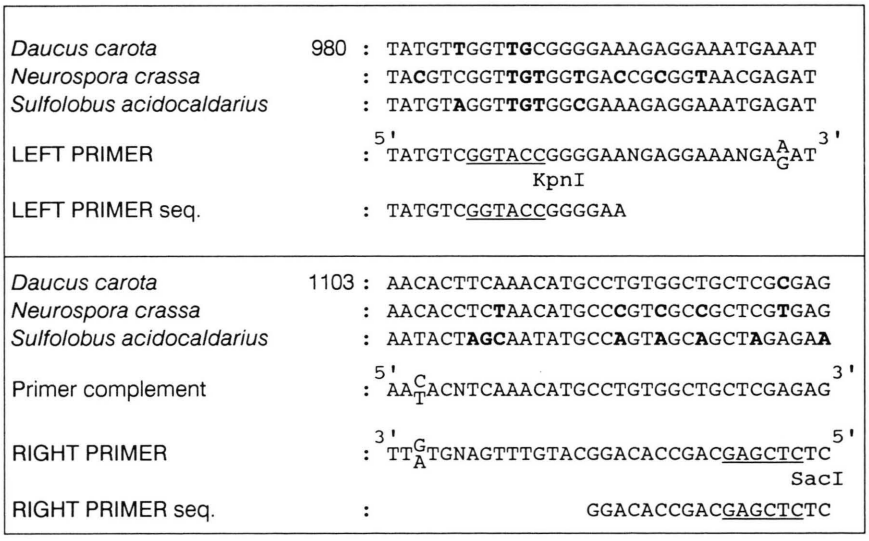

Fig. 1. Left and right primers that were used to amplify part of the coding region of the V-type ATPase from genomic plant DNA. The strands are shown aligned to the sequences that were used to construct the primers. Numbers correspond to the cDNA sequence given in [19]. Mismatches between the sequences and the primers are emphasized in bold print. The shorter primers used to sequence the amplified DNA are depicted below the the primers used for the PCR. In case of the right primer the complementary sequences (bottom lines) were used to prime the synthesis of the strand in the direction of the left primer.

and *Equisetum arvense* yielded two fragments of different size termed small (S) and large (L) for each species. Both small fragments have the same length (252 bp including primers), the large fragments being 294 bp and 276 bp long in *Psilotum* and *Equisetum* respectively.

Using the shorter versions of the PCR primers (Fig. 1) the sequence of the large and small fragments was determined from both directions resulting in a 80% overlap in the nucleotide sequence read from the autoradiographed gels.

Both fragments, the large and small ones, exhibited a 156 bp coding region which is interrupted by an intron close to 5′ end of the sequence. The longest intervening sequence is 138 bp in *Psilotum*, the corresponding *Equisetum* intron being only 120 bp long. Both introns present in the small fragments are 96 bp in length (Fig. 2).

An alignment of the sequences depicted in Fig. 3 shows the same exon-intron junctions in all four sequences, the last nucleotides of the 5′ exon being 5′-GCAGA, and the first of the 3′ exon GGT-3′. The intron begins with 5′-GTG and ends on CCA-3′. A closer comparison of the coding regions shows a very high homology (86–95%) between the *Psilotum* and *Equisetum* fragments (Fig. 4). The highest degree of similarity is found between the large and small *Psilotum* fragment (95%). Both *Psilotum* fragments show an equal homology in the coding region to the small *Equisetum* fragment but are lesser related to the large *Equisetum* fragment (87%). Furthermore, the similarity between the coding regions of the *Equisetum* fragments themselves is only 86%. As expected from phylogeny the coding regions from the plant species are about 65% and 45% identical to the corresponding sequences in *Neurospora crassa* (20) and *Methanococcus thermolithotrophicus* (18), respectively.

The divergence between the two fragments of *Psilotum* and the small fragment of *Equisetum* is mainly derived from a change in the third codon positions (Fig. 4). In contrast, the change of nucleotides between the two *Equisetum* fragments is equally distributed, being 86%, 86% and 85% for the first, second and third codon position respectively.

Looking at the intron sequences, the highest degree of similarity (80%) is found between the two small fragments. The *Equisetum* fragments are 76% identical in their introns, in contrast the *Psilotum* fragments are only 56% identical. In addition, the similarity between the small fragment of *Psilotum* and the large *Equisetum* fragments is significantly higher (71%) than the large fragments compared together (59%).

Whereas in the case of the exons the alignment is aided by the encoded amino acid sequence, the alignment of the intron sequences is more ambiguous. In order to test whether the intron regions indeed show more similarity than expected from two random sequences that are aligned using gaps, we analyzed pairwise comparisons of the intron sequences (Table I). The results clearly demonstrate

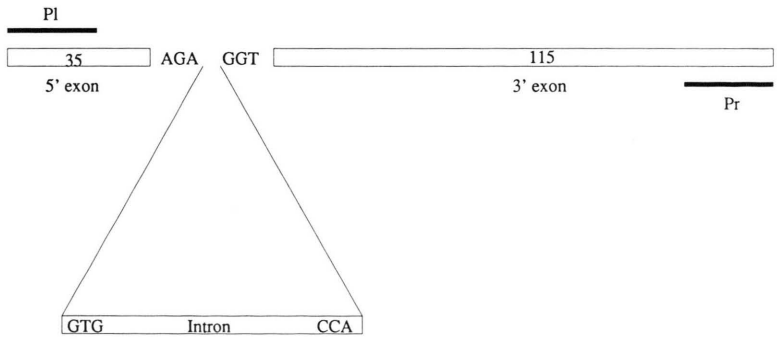

Fig. 2. Schematic drawing of part of the DNA fragment amplified from genomic plant DNA of *Psilotum nudum* and *Equisetum arvense* using to oligonucleotid primers, Primer left (Pl) and Primer right (Pr). The 156 nucleotides long coding region is interrupted close to the 5′ end of the sequence by an intron of variable length. All numbers are in nucleotides. Note the common sequence at the junctions of the exons and intron found in all four amplified DNA fragments.

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                ----------Primer 2----------> Exon  ------Intron----------
Psilotum L      TATGTTGGTTGCGGGGAAAGAGGAAATGAGATG GCAGA GTGCAAGTGTAAACCTCTACTT 60
Equisetum L     TATGTTGGTTGCGGGGAGCGAGGAAAGGAAATG GCAGA GTG-AGATTACTCTTGCTTAAT 59
Equisetum S     TATGTTGGTTGCGGGGAGAGAAAGAATGAAATG GCAGA GTGT-GATTAT-TTT-GTCCTT 57
Psilotum S      TATGTTGGTTGCGGGGAAAGAGGAAATGAGATG GCAGA GTGTGGAGTCCCTCT-CTGCTT 59
Neurospora      TACGTCGGTTGTGGTGAGCGCGGTAACGAGATG GCTGA ---------------------- 38
M.thermol.      TACATCGGATGTGGGGAAAGAGGAAACGAGATG ACTGA ---------------------- 38

                ------------------------------Intron---------------------------
Psilotum L      AATTGC-TTATGCAGAGTCAGGCTCCT-ATATGTATTTTTTTGGCACAGAAGGTTCCCTGTA 120
Equisetum L     GAGTG--TTGTG-AGAATAACACATGTGAGGTCTTGTTTTTTGAATCTGATGAAT-AATATG 117
Equisetum S     ATCTCAGTTGTG-AGATAC---CATGTGAGGTCTTGTTT--TGCAAATTGTGA---AATG-- 108
Psilotum S      AAG--AGTTGTG-AGATAC---CATGTGAGGT--ATTTTTTTGCATACTTAGA---AATG-- 108
Neurospora      ------------------------------------------------------------
M.thermol.      ------------------------------------------------------------

                ------------------------------Intron---------------------- Exon
Psilotum L      GACAAGAGGATGCCATATGGGATTGATATAATTTACTTTGTACGTTTTTCATT--CCA GGT 179
Equisetum L     GGT--GACG-TCCGTTATGGAATCT-TC--GATCACTT-----------CATTATCCA GGT 161
Equisetum S     --CAAGA-----------------TTC-GGATCACTT-----------CATTATCCA GGT 137
Psilotum S      --TTAAA-----------------TTT-GGATCACTT-----------CATTATCCA GGT 137
Neurospora      ------------------------------------------------------------ AGT 41
M.thermol.      ------------------------------------------------------------ AGT 41

                ------------------------------Exon----------------------------
Psilotum L      ACTTATGGATTTCCCCCAACTGACAATGACTTTACCCGATGGCCGTGAAGAATCTGTCATGA 241
Equisetum L     TCCGATGCCCCTTAGGCAACTGACAATGACTTTACCCGATGGCCGTGAAGAATCTGTCATGG 223
Equisetum S     CCTTATGGATTTTCCTCAACTGACAATGACTTTACCCGATGGCCGTGAAGAATCTGTCATGA 199
Psilotum S      TCTTATGGATTTCCCCCAACTGACAATGACTTTACCCGATGGCCGTGAAGAATCTGTCATGA 199
Neurospora      CTTGAAGGATTTCCCCGAGCTGTCTATCGAGGTC---GACGGCCGCAAGGAGCCCATCATGA 100
M.thermol.      TATTGAAGAATTCCCACACTTGGAAGACATTAAA---------ACAGTAAACAAGTTAATGG 94

                -------Exon--------- <----------Primer 3--------------
Psilotum L      AGCGTACCACACTTGTAGCC AACACATTAAACATGCCTGTGGCTGCTCGCGAG          294
Equisetum L     AGCGTACCACACTTGTAGCC AAGACTCTAAACATGCCTGTGGCTGCTCGCGAG          276
Equisetum S     AGCTGACCACACTTGTAGCC AAGACTTCAAACATGCCTGTGGCTGCTCGCGAG          252
Psilotum S      AGCGTACCACACTTGTAGCC AAGACATTAAACATGCCTGTGGCTGCTCGCGAG          252
Neurospora      AGCGCACGACCCTCATCGCC AACACCTCTAACATGCCCGTCGCCGCTCGTGAG          153
M.thermol.      ATAGGACTGTTTTAATTGCA AACACCTCAAACATGCCGGTTGCTGCGAGGGAA          147
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Fig. 3. Alignment of the sequences of the large (L) and small (S) fragments from *Psilotum nudum* and *Equisetum arvense* and the corresponding sequences of the V-type ATPases from *Neurospora crassa* and *Methanococcus thermolithotrophicus*. The alignment was done by eye utilizing several computer generated pairwise alignments. Gaps were introduced in order to maintain the alignment. Both *Neurospora* and *Methanococcus* lack the intron present in the sequenced *Psilotum* and *Equisetum* fragments. The coding region of *Neurospora* and *Methanococcus* is shorter than in the plant sequence (3 and 9 nucleotides respectively). For a closer evaluation of the alignment see Fig. 4.

the homology between the introns of the small fragments and the homology of the introns in the small fragment to the intron in the large fragment from *Equisetum*. However, this analysis fails to detect any significant similarity between the intron in the large *Psilotum* fragment and the other three intron sequences.

	Psil. L [%]		*Equis.* L [%]		*Equis.* S [%]		*Psil.* S [%]		*Neurosp.* [%]		*M. thermol.* [%]	
Psilotum L			86.2	30	100.0	30	100.0	30	69.0	29	40.7	27
			90.0	30	96.7	30	100.0	30	93.1	29	66.7	27
			85.7	30	86.7	30	96.7	30	34.5	29	29.6	27
Equisetum L	87.4	90			86.2	30	86.2	30	53.6	29	34.6	27
	58.7	108			86.7	30	90.0	30	82.8	29	55.6	27
					85.7	30	85.7	30	25.9	29	24.0	27
Equisetum S	94.4	90	86.2	90			100.0	30	69.0	29	40.7	27
	55.1	92	80.0	92			96.7	30	89.7	29	63.0	27
							86.7	30	31.0	29	29.6	27
Psilotum S	98.9	90	87.4	90	94.4	90			69.0	29	40.7	27
	56.2	92	74.1	92	80.4	92			93.1	29	66.7	27
									34.5	29	33.3	27
Neurospora	65.5	87	54.8	87	63.2	87	65.5	87			33.3	27
											70.4	27
											22.2	27
M. thermol.	45.7	81	38.5	81	44.4	81	46.9	81	42.0	81		

Fig. 4. Comparison of the % identities found at the 1st, 2nd, 3rd codon and intron positions of the large (L) and small (S) DNA fragments from *Psilotum nudum* and *Equisetum arvense* and the sequences of *Neurospora crassa* and *Methanococcus thermolithotrophicus*. The alignment used to calculate the values is depicted in Fig. 3. The first, second and third entries in the fields above the diagonal correspond to the first, second and third base positions respectively. The numbers below the diagonal are the % identities for the coding (average for the three codon positions) and intervening sequences. The first and second entries correspond to the exons and introns respectively. The numbers given to the right of the % values are the actual numbers of positions used to calculate the tabulated values. When compared to the plant samples, the *Methanococcus* sequence as expected shows a lower identity than *Neurospora*. Note the high degree of similarity in the base composition between the *Psilotum* and *Equisetum* fragments. For an extended discussion see Text.

Table I. *z*-Values for the pairwise comparison of the intervening sequences. Only nucleotide positions within the introns were used, positions outside the introns and the four nucleotides at the ends of the introns that define the splice sites were not included in this analysis. For each pairwise comparison first the optimum alignment score was calculated according to the method described by Needleman and Wunsch [30], using a score of +1 per match and a gap penalty of −2. Then the two sequences were randomized and the optimal alignment score for the randomized sequences was calculated. The latter process (randomization and alignment) was repeated 50 times and the mean and standard deviation for the alignment scores obtained for the randomized sequences were calculated. The tabulated *z*-values give the distance between the alignment score of the actual sequences and the mean of the randomized sequences expressed as multiples of the standard deviations. Usually *z*-values >3 are considered to prove homology (*i.e.* values of this size are very unlikely to be due to convergent evolution or chance; in the above tabulation these are printed in bold typeface; [31]). The values above and below the diagonal were calculated using different randomizations, therefore, the entry in column a, row b is not necessarily identical to the value in column b, row a.

	Equi. S	*Psil.* S	*Equi.* L	*Psil.* L
Equisetum S	**28.7**	**12.1**	**8.6**	−1.4
Psilotum S	**13.1**	**28.4**	**7.0**	0.4
Equisetum L	**7.8**	**7.3**	**32.4**	−0.3
Psilotum L	−1.5	0.4	−0.4	**34.1**

Discussion

Using genomic DNA of plants representing basal branches of the land plants as target for PCR amplification, we were able to amplify two stretches of DNA from each species used that encoded a part of the V-type ATPase catalytic subunit. The sequences of the large and small products demonstrate the existence of two gene copies encoding the catalytic 70 kDa subunit in these plants. Both products contain intron and exon sequences. In all cases the intron was located at the same position.

The high degree of similarity between the coding regions as well as the intervening sequences between the two copies in each species (see Fig. 4) suggests that for some time after the split of the two lines giving rise to *Equisetum* and *Psilotum* occurred gene conversion mechanisms were acting to keep the two copies within each species similar.

Both *Psilotum* genes are highly conserved in the coding region at the nucleotide level, changes in the sequence being mainly due to substitutions in the third codon position, which results in only one altered amino acid on the protein level. This suggest either a recent gene conversion event or an ev-

olutionary pressure acting on both copies of the gene (meaning that both genes are translated). The latter explanation is further supported by the fact that the intervening sequence, though conserved, shows a much lower degree of identity, thus contradicting a recent gene conversion event that would have acted on both exon as well as intron sequences and supporting the hypothesis that both gene copies are transcribed and under selective pressure in *Psilotum*.

In contrast, the two amplified coding regions of the *Equisetum* genes show a lesser conservation with no preferred codon position for the change of nucleotides. Taking into account the high sequence similarity between the two small fragments (94% and 80% in the coding region and the intron respectively) one explanation would be that the large gene of *Equisetum* is not under immanent evolutionary pressure (not transcribed?) and the divergence to the gene with the smaller intron is therefore higher as compared to the *Psilotum* coding region.

So far, all sequences of the catalytic subunit of V-type ATPases derived from fungal genomic DNA (*Neurospora crassa, Saccharomyces cerevisiae*) have revealed only a single copy of the gene [16, 22, 24, 25]. Therefore using the phylogenetic tree of the catalytic subunit of the V-type ATPase [21] a gene duplication of the ancestral catalytic subunit can be pinpointed after the separation of the fungi from the eukaryotic branch but before the appearance of early land plants like *Psilotum*.

The location of the introns in identical positions of the gene testifies to the homology of the introns themselves (*i.e.* an intron was already present in the ancestral gene). However, the degree of similarity between the introns in the small fragments from *Psilotum* and *Equisetum* comes as a surprise. Equisetatae are well documented in the fossil record since the late Devonian; suggesting that the ancestor of the two introns in the small fragments dates back at least 300 million years. As the Equisetatae are not likely to have evolved directly from the *Psilotum* ancestor but from an older ancestral species [26], these two introns probably evolved in separate species since more than 400 million years ago. A non-functional sequence that is not under selection pressure would be expected to be nearly randomized over these evolutionary distances. Assuming a substitution rate of 3.3 per nucleotide and per 10^9 years (a value that is typical for pseudogenes, introns and fourfold degenerate sites [27]) and using Jukes and Cantor's model [28] only about 30% of the positions are expected to be identical (for random sequences without introduction of gaps a mean of 25% identical residues is expected). The observed, unexpected similarity of the intron sequences suggests that either parts of the genome experience much lower nucleotide substitution rates than previously observed, or it indicates the presence of functional constrains on the intron sequence. However, following the latter indication, and using the algorithm described in [29], we were not able to detect similarities in the predicted secondary structures for these intron sequences.

Clearly, the availability of more sequences of this (and other) introns will provide further insight into the evolution of V-type ATPases and into the evolution and function of introns. Intervening sequences will be a valuable tool in studying the evolution within the plant kingdom, especially, since the coding regions for this protein do not seem sufficiently different to evaluate the phylogenetic relationship of more closely related species.

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