J Membrane Biol (2010) 233:63–72
DOI 10.1007/s00232-010-9225-x

The Evolution of Fatty Acid Desaturases and Cytochrome *b5* in Eukaryotes

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Received: 9 July 2009 / Accepted: 8 January 2010 / Published online: 10 February 2010

Abstract Desaturases that introduce double bonds into the fatty acids are involved in the adaptation of membrane fluidity to changes in the environment. Besides, polyunsaturated fatty acids (PUFAs) are increasingly recognized as important pharmaceutical and nutraceutical compounds. To successfully engineer organisms with increased stress tolerance or the ability to synthesize valuable PUFAs, detailed knowledge about the complexity of the desaturase family as well as understanding of the coevolution of desaturases and their cytochrome *b5* electron donors is needed. We have constructed phylogenies of several hundred desaturase sequences from animals, plants, fungi and bacteria and of the cytochrome *b5* domains that are fused to some of these enzymes. The analysis demonstrates the existence of three major desaturase acyl-CoA groups that share few similarities. Our results indicate that the fusion of Δ^6-desaturase-like enzymes with their cytochrome *b5* electron donor was a single event that took place in the common ancestor of all eukaryotes. We also propose the Δ^6-desaturase-like enzymes as the most probable donor of the cytochrome *b5* domain found in fungal Δ^9-desaturases and argue that the recombination most likely happened soon after the separation of the animal and fungal ancestors. These findings answer some of the previously unresolved questions and contribute to the quickly expanding field of research on desaturases.

Keywords Fatty acid · Desaturase · Evolution · Cytochrome *b5* · Polyunsaturated fatty acid · Eukaryote · Fungus · Bioinformatics

Introduction

Membranes form a framework that provides the compartmentalization of the different biochemical processes in the cell (Lösel 1990). To maintain their function, these membranes have to preserve a suitable dynamic state of the bilayer even in a changing environment, which can alter their fluidity (Hazel and Williams 1990). This is achieved by active restructuring of the composition of the membrane lipids. Through the synthesis of the correct combination of acyl chains and polar head groups, the organism can maintain membrane lipids in a lamellar liquid crystalline phase and avoid the formation of a lamellar gel phase (Rilfors and Lindblom 2002). One of the most important adaptations to variations in temperature, water activity, pH, hydrostatic pressure, oxygen concentrations and other factors is the amount of unsaturation of the cellular fatty acids (reviewed by Gostinčar et al. 2009a).

Most organisms (with the exception of some bacteria) have a fatty acid synthetase that can produce only saturated fatty acids. Unsaturated fatty acids are produced by desaturases, which convert a single bond between two carbon atoms in an existing fatty acyl chain to a double bond (Aguilar and de Mendoza 2006; Hazel and Williams 1990; Los and Murata 1998), usually in the (more effective) *cis* configuration (Los and Murata 1998). The present study focuses on the most common acyl-coenzyme A

Electronic supplementary material The online version of this article (doi:10.1007/s00232-010-9225-x) contains supplementary material, which is available to authorized users.

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(acyl-CoA) desaturases that introduce unsaturated bonds into fatty acids bound to CoA (Los and Murata 1998). Typically, these desaturases consist of 300–350 amino acid residues (Los and Murata 1998; Meesters et al. 1997; Nakashima et al. 1996), and they are hydrophobic and span the membrane-lipid bilayer (Shanklin and Cahoon 1998; Shanklin et al. 1994). They can be grouped in several evolutionarily distant subfamilies with different substrate specificity (Hashimoto et al. 2008).

Cytochrome *b5* is needed for the successful desaturation of fatty acids as well as for many other oxidative reactions in the cell. It is a small heme-binding protein that appeared very early in evolution and is usually associated with the endoplasmic reticulum of higher plants, fungi and animals (Napier et al. 1997; Schenkman and Jansson 2003). Cytochrome *b5* acts as an electron-transfer component in the desaturation reaction (Schenkman and Jansson 2003), with two modes of action being possible. First, desaturation can be carried out by a multienzyme system, which is composed of a desaturase, NADH cytochrome *b5* reductase and cytochrome *b5*. In the process of double-bond formation, the membrane-bound cytochrome *b5* transfers electrons by lateral diffusion, from NADH cytochrome *b5* reductase to the desaturase (Thiede et al. 1986). Second, many desaturases are modular proteins that are composed of desaturase and cytochrome *b5* modules either on the N terminus in the case of Δ^6-desaturase-like enzymes (Napier et al. 1999) or on the C terminus in the case of fungal Δ^9-desaturases (Mitchell and Martin 1995; Napier et al. 1997). Apart from the desaturases, cytochrome *b5* domains or folds have been found in a number of unrelated proteins, such as nitrate reductases, sulfite oxidases and L-lactate dehydrogenases (Napier et al. 1997). It has been proposed that fusion of the desaturase and cytochrome *b5* domains has occurred more than once (Napier et al. 2003). These cytochrome *b5*-fusion desaturases have a central role in polyunsaturated fatty acid (PUFA) synthesis (Napier et al. 2003). The fusion enables the NADH cytochrome *b5* reductase to directly transfer electrons to the catalytic site of the cytochrome *b5* fusion desaturases via the cytochrome *b5*-like domain without the requirement for an independent cytochrome *b5* (Guillou et al. 2004; Mitchell and Martin 1995). This can potentially speed up electron transfer by presenting a correctly oriented heme group with respect to the dioxo-iron cluster, eliminating the need for diffusion and reorientation of the reduced cytochrome *b5* (Mitchell and Martin 1995). However, other studies have shown that both the Δ^6-desaturase cytochrome *b5*-like domain and microsomal cytochrome *b5* are important in the process of Δ^6 desaturation and that microsomal cytochrome *b5* cannot compensate for the essential role of the highly conserved HPGG motif in the rat Δ^6-desaturase cytochrome *b5*-like domain (Guillou et al. 2004).

Despite previous attempts (Sperling et al. 2003), several questions concerning the source of the cytochrome *b5* domain in desaturases, time of the fusion and reason for the fusion have so far remained unanswered. Using the increasing amount of genomic data now available and a large-scale analysis of protein data, we propose answers to these questions. Due to the role of desaturases in membrane adaptation to environmental conditions, detailed knowledge about the complexity of desaturase diversity and evolution is useful for genetically engineering industrially important eukaryotes that will be able to endure stressful conditions encountered in biotechnological and agronomic processes. Furthermore, understanding the evolution of the interaction of desaturases and associated electron-transfer components such as cytochrome *b5* may contribute to optimizing the production of economically valuable PUFAs such as pharmaceutical and nutraceutical compounds with recombinant desaturases that have to integrate with the cell machinery of the recipient organism and interact with foreign electron donors.

Materials and Methods

Gene Phylogeny Reconstruction

Homologues of the Δ^6-desaturases, Δ^9-desaturases, Δ^{12}-desaturases and cytochrome *b5* proteins were identified by protein–protein BLAST searches (Altschul et al. 1997) against a GenBank nonredundant protein database with *E* values < 0.01. Truncated sequences and sequences without the characteristic conserved histidine motif were discarded. Protein sequences were aligned using the L-INS-i method in MAFFT software (Katoh et al. 2005; Katoh and Toh 2008). In all cases of fusion desaturase–cytochrome *b5* proteins, the desaturase and cytochrome modules were separated according to Hsiao et al. (2007) and Sperling et al. (1995), and the subsequent analyses were carried out for each module separately. Gene trees were generated with the PhyML software (Guindon and Gascuel 2003) with aLRT implementation for the calculation of branch supports as a minimum of SH-like and Chi2-based support (Anisimova and Gascuel 2006). The analysis was run using a WAG model of amino acid substitution, the optimized proportion of invariable sites and the six substitution rate categories with an optimized gamma distribution parameter. Separate sets of trees was generated by the neighbor-joining method, implemented in MEGA4 (Tamura et al. 2007) (data not shown) and on a representative subset of sequences with MrBayes software, applying Bayesian inference (Huelsenbeck and Ronquist 2001; Ronquist and Huelsenbeck 2003) (data not shown). Separate trees were constructed for each desaturase group and each eukaryotic kingdom, to increase

the accuracy of the phylogeny inference in search of gene duplications. To save space, the duplications found are illustrated on trees containing all of the kingdoms.

Cytochrome *b5* Domain Sequence Analysis

MEGA4 (Tamura et al. 2007) was used to calculate the proportion of acidic amino acids in the cytochrome *b5* domains. Sequence logos and graphical representations of the frequencies of amino acid residues were drawn using the WebLogo 3 service (Crooks et al. 2004). The numbers of changes per site and the reconstruction of the ancestral sequences were estimated using the PAML software (Yang 1997, 2007).

Results

Our search detected three major groups of desaturases: Δ^6-like, Δ^9-like and Δ^{12}-like. While they shared some similarities, these were not sufficient for reliable alignment; and the resulting tree showed three extremely distant groups (data not shown). Therefore, separate phylogenetic trees were constructed for each of these groups.

Δ^6-Desaturase-like enzymes were found in all of the major eukaryotic kingdoms: animals, plants and fungi (Fig. 1). The Δ^6-desaturases and Δ^8-sphingolipid desaturases in plants do not cluster separately despite their different functions. The desaturases from *Mucoromycotina* are found in the group together with other fungal desaturases, as well as in two separate clusters, one containing enzymes from several genera and the other represented solely by enzymes from *Mortierella alpina*. Several other enzymes form separate lineages, from green algae (*Mantoniella* sp., *Ostreococcus* sp.) and the liverwort *Marchantia polymorpha* to the sea anemone *Nematostella vectensis*.

Numerous Δ^9-desaturase-like proteins were found in animals and fungi, as well as their homologous enzymes in bacteria (Fig. 2). A few of the homologues found in plants presumably originated in plastids and form a cluster together with other bacterial desaturases.

Δ^{12}-Desaturase-like enzymes were found in plants and fungi, as well as in bacteria (Fig. 3). These plant desaturases form two distinct clusters, one as Δ^{12}/ω^6-desaturases and the other as Δ^{15}/ω^3-desaturases. The second is closer to the bacterial sequences, especially to the cluster containing cyanobacteria, while the cluster with the other bacterial and plastidial enzymes is fairly distant from other groups. The only animal homologues are proteins from the nematodes *Caenorhabditis* sp. and *Brugia* sp. and (forming a separate lineage, as seen for the Δ^6-desaturase-like enzymes) the anemone *N. vectensis*.

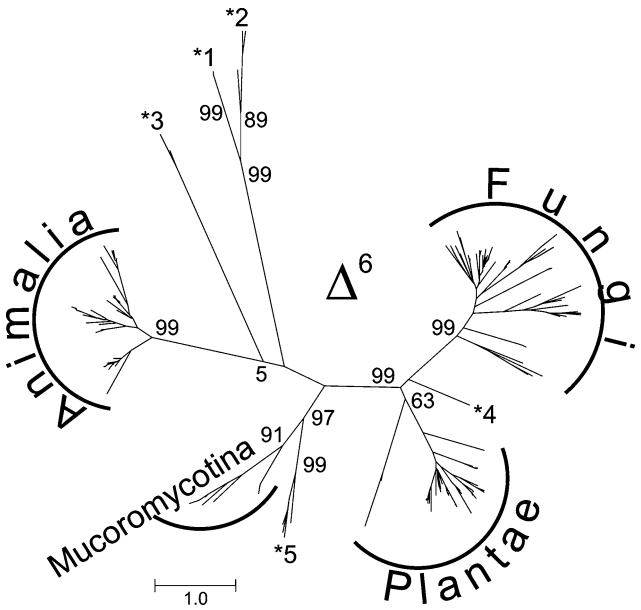

Fig. 1 Phylogeny of the Δ^6-desaturase-like enzymes. The cytochrome *b5* module was removed from the sequences prior to analysis. The tree was constructed with the PhyML software; numbers at major nodes represent branch supports calculated with the aLRT method as a minimum of SH-like and Chi2 values. GenBank accession numbers of proteins used for the construction of the phylogeny are included as supplemental information (Table 1). The numbered groups contain sequences from the following organisms (with accession numbers): **1*, *Mortierella alpina* (O74212, AAC39508, AAC72755, BAD95486, AAR28035); **2*, *Marchantia polymorpha* (AAT85663), *Mantoniella squamata* (CAQ30478), *Ostreococcus tauri* (CAL57370); **3*, *Mantoniella squamata* (CAQ30479), *Ostreococcus tauri* (AAW70159); **4*, *Nematostella vectensis* (XP_001640617); **5*, *Ceratodon purpureus* (CAB94992, CAB94993), *Marchantia polymorpha* (AAT85661), *Physcomitrella patens patens* (XP_001763930)

Cytochrome *b5* domains were found in all of the Δ^6-desaturase-like and fungal Δ^9-desaturase-like enzymes, fatty-acid desaturases from mycobacteria and Δ^9-desaturase-like enzymes from *Dictyostelium discoideum* (XP_644535, XP_636528), *Trypanosoma* sp. (XP_847430, XP_802304) and *Leishmania* sp. (XP_001683743, XP_001466025, XP_001565496, XP_001687650, XP_001464234) but not from *Paramecium tetraurelia* (XP_001430497, XP_001427073), *Perkinsus marinus* (EER12488), *Phytophthora infestans* (EEY56644) and *Tetrahymena* sp. (XP_001018825, BAA20463, XP_001014958). Soluble cytochrome *b5* homologues in bacteria (cytochromes *b558*) were found only in a few species (see below). Comparison of their phylogenies predicted by neighbor-joining (data not shown) and maximum likelihood methods using different amino acid substitution models showed that the deep-branch phylogenetic positions of the following groups are not stable: (1) soluble bacterial cytochromes *b558* (*Rhodopseudomonas palustris* [YP_531806], *Ectothiorhodospira shaposhnikovii* [P82291], *Ectothiorhodospira*

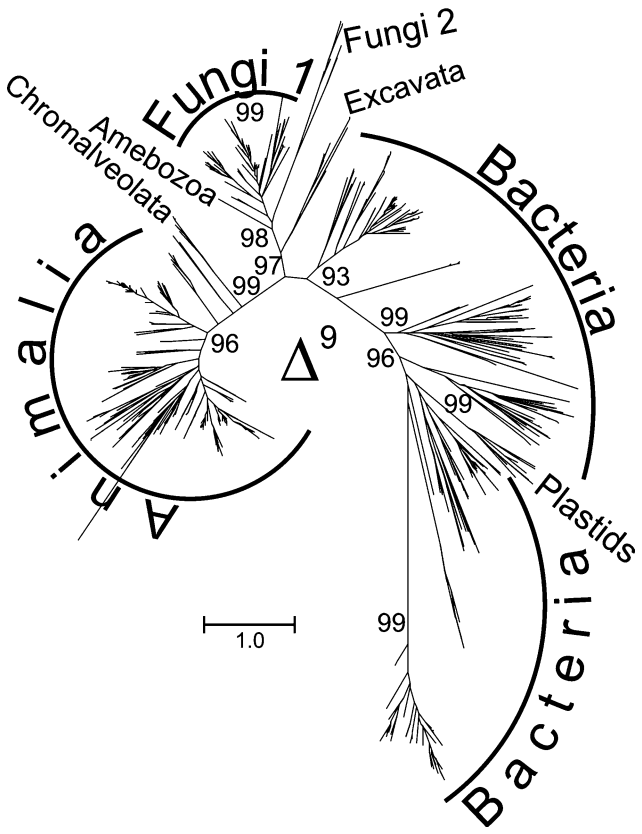

Fig. 2 Phylogeny of the Δ^9-desaturase-like enzymes. The cytochrome *b5* module was removed from the fungal sequences prior to analysis. The tree was constructed with the PhyML software; numbers at major nodes represent branch supports calculated with the aLRT method as a minimum of SH-like and Chi2 values. GenBank accession numbers of proteins used for the construction of the phylogeny are included as supplemental information (Table 2)

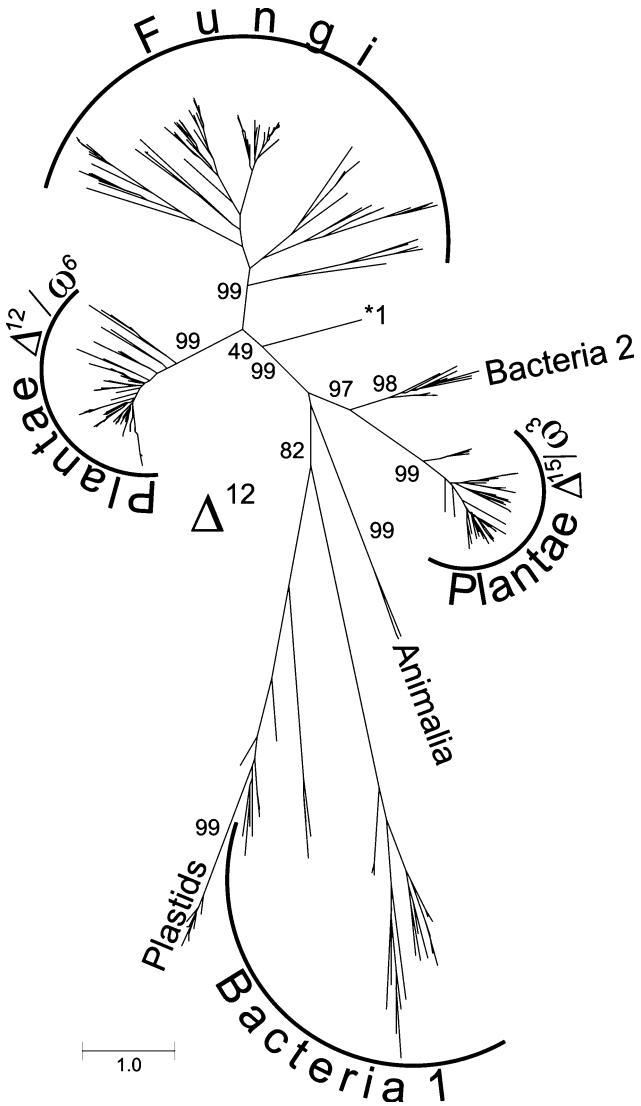

Fig. 3 Phylogeny of the Δ^{12}-desaturase-like enzymes. The tree was constructed with the PhyML software; numbers at major nodes represent branch supports calculated with the aLRT method as a minimum of SH-like and Chi2 values. GenBank accession numbers of proteins used for the construction of the phylogeny are included as supplemental information (Table 3). Sequence numbered with **1* is a predicted protein from *Nematostella vectensis* (XP_001632452)

vacuolata [1CXY]); (2) cytochrome domains from fatty-acid desaturase proteins (or similar hypothetical proteins) from bacteria (*Mycobacterium tuberculosis* [NP_335866, NP_215887], *Mycobacterium ulcerans* [YP 905723], *Mycobacterium marinum* [YP_001850493], *Nocardia farcinica* [YP_121146]). These sequences were excluded from the subsequent analyses, which significantly improved the quality of the alignment.

The phylogenetic analysis of cytochrome domains (Fig. 4) shows that they are highly conserved and they group according to their source. Soluble cytochromes *b5* cluster together, and the group is further divided into animal, plant and fungal clusters. Cytochrome domains from animal and plant Δ^6-desaturase-like enzymes form a poorly supported cluster, while the support for the cluster of fungal Δ^6-desaturase-like and Δ^9-desaturase-like enzymes is somewhat higher. While the Δ^6-desaturase-like enzymes and Δ^9-desaturase-like enzymes share few similarities, their cytochrome *b5* domains show high degrees of identity and are easy to align. In addition to these groups, cytochrome *b5* domains from Δ^6-desaturase-like enzymes from *Mucoromycotina* and *M. alpina* also form separate lineages, corresponding to the separate desaturase lineages seen in Fig. 1.

The inclusion in the analysis of cytochrome *b5* domains from other (also unrelated) enzymes changes the relationships between the major groups (Supplemental Fig. S1). Cytochrome domains from Δ^6-desaturase-like enzymes form a (poorly supported) separate cluster in the phylogram constructed by ML. However in the phylogram constructed by MrBayes (data not shown) the domains from fungal

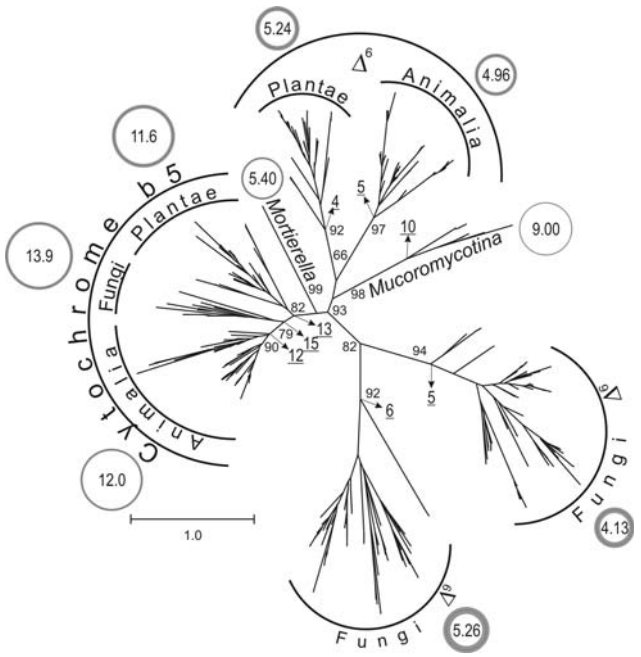

Fig. 4 Phylogeny of cytochrome *b5* proteins and homologous domains from desaturases and number of acidic amino acid residues in the vicinity of the conserved HPPG motif of the cytochrome *b5* domain. The tree was constructed with the PhyML software; numbers at major nodes represent branch supports calculated with the aLRT method as a minimum of SH-like and Chi2 values. GenBank accession numbers of proteins used for the construction of the phylogeny are included as supplemental information (Tables 1, 2 and 4). The number of acidic residues is represented as the area of the circles enclosed by the *black line*, while the thickness of the *gray line* represents the standard deviation. The (average) numerical value is in the center of the circles for each group and marked with *arrows* and *underlined* for the ancestor sequences, as reconstructed with the PAML software

Δ^6-desaturase-like and Δ^9-desaturase-like enzymes still cluster together.

Analysis of acidic amino acid residues (Fig. 4) shows that the greatest reductions in these residues (almost 65%) are present in the cytochrome *b5* domain from fungal Δ^9-desaturase-like enzymes compared to fungal independent cytochromes *b5*. Other desaturase groups also show low proportions of acidic residues, with the exception of cytochrome *b5* domains from a separate lineage of *Mucoromycotina* Δ^6-desaturase-like enzymes, where the reduction is only 25%.

Detailed analysis of the amino acid sequences in the vicinity of the conserved HPGG cytochrome *b5* motif shows several highly conserved amino acids (Fig. 5), among which there are also acidic ones (positions 10, 17 and 32 in the case of Δ^9-desaturase-like enzymes and 32 and 35 in the case of Δ^6-desaturase-like enzymes). The predicted substitutions show a preference toward maintaining the acidic residues in positions 16 and 17.

Phylogenetic positioning of the homologous enzymes from the same species reveals several duplication events in all three major eukaryotic kingdoms (Supplemental Figs. S2–S4).

Discussion

Phylogenetic analysis of the known desaturases reveals a complex evolution and diversity of these enzymes and their cytochrome *b5* modules. The existence and expression of different desaturase genes, resulting in a rich array of fatty acid species, provide the molecular basis for the acclimation of cells to various environmental factors (Gostinčar et al. 2009a; Hashimoto et al. 2008; Kayukawa et al. 2007).

The Diversity of the Acyl-CoA Desaturase Family

Our results confirm the existence of the following major groups of acyl-CoA desaturases: Δ^6-like, Δ^9-like and Δ^{12}-like. In most cases, the grouping of enzymes was as expected and corresponds to the phylogenetic relationships of the organisms they were isolated from. The notable exceptions are discussed below.

Δ^6-Desaturase-like enzymes were the only group that was widely distributed in all three major eukaryotic kingdoms: animals, plants and fungi (Fig. 1). Several separate lineages are seen, in addition to groups representing these kingdoms. The most prominent are two fungal groups, one with enzymes from various *Mucoromycotina* genera and the other containing only enzymes from *M. alpina* (group 1). *M. alpina* is also represented by enzymes in the other two fungal groups (labeled as *Mucoromycotina* and fungi). Separate clustering of *Mucoromycotina* desaturases has been reported before (Hsiao et al. 2007; Laoteng et al. 2000; Michinaka et al. 2003). We observed two such lineages, one clustering with the enzymes from liverworts and mosses (group 5) and the second with liverworts and green algae (group 2). A separate lineage with two proteins from stramenopiles (group 3) and another with a predicted protein from the sea anemone *N. vectensis* (lineage 4) positioned far away from the other animal desaturases is also seen.

Contrary to observations made by Sperling et al. (2003) and López Alonso et al. (2003), our phylograms based on significantly larger data sets do not indicate a single separation of Δ^6-desaturase and Δ^8-sphingolipid desaturase systems, either in plants or in fungi. Instead, the enzymes from each kingdom form a single homogenous group with desaturases of both specificities from the closely related species clustering together. This suggests that the duplications and specializations of these Δ^6/Δ^8 pairs have happened more than once (Supplemental Fig. S2); these would

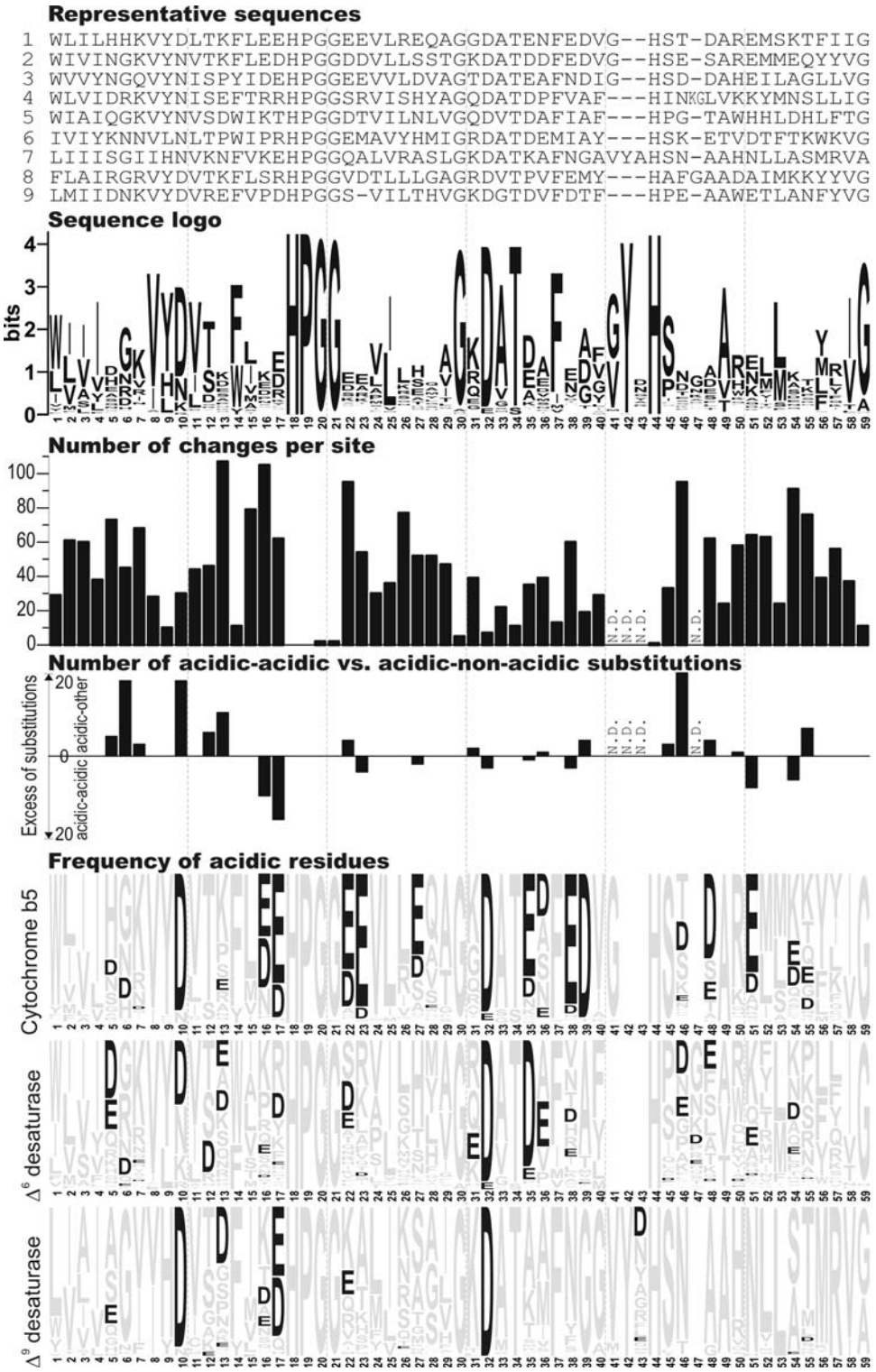

Fig. 5 Analysis of the vicinity of the conserved HPPG motif of the cytochrome *b5* domain. Representative sequences are as follows: *1*, *Homo sapiens* cytochrome *b5* (NP_683725); *2*, *Arabidopsis thaliana* cytochrome *b5* (NP_180831); *3*, *Pichia stipitis* cytochrome *b5* (XP_001383573); *4*, *Homo sapiens* Δ^5-desaturase (AAF29378); *5*, *Arabidopsis thaliana* Δ^8-sphingolipid desaturase (AAM64895); *6*, *Pichia stipitis* Δ^6-desaturase (XP_001384602); *7*, *Pichia stipitis* Δ^9-desaturase (XP_001383212); *8*, *Mortierella alpina* Δ^5-desaturase (AAC39508); *9*, *Mortierella alpina* Δ^6-desaturase (ABN69090). Number of changes per site and substitutions were calculated with the PAML software for all of the sequences shown in Fig. 4. GenBank accession numbers of proteins are included as supplemental information (Tables 1, 2 and 4)

have possibly been facilitated by a common regiospecificity shared by both Δ^6-desaturase and Δ^8-sphingolipid desaturases, as proposed by Sperling et al. (1998). The phylograms point to other major duplications, among which there is one in mammals and one in *Pezizomycotina* fungi (Supplemental Fig. S2).

Δ^9-Desaturases exist in almost all organisms as the most important desaturases in unsaturated fatty acid biosynthesis (Abe et al. 2006). However, plant Δ^9-desaturases belong to a group of acyl-acyl carrier protein (acyl-ACP) desaturases that are apparently not related to the more common acyl-CoA desaturases (Shanklin and Cahoon 1998; Sperling

et al. 2003). The few existing plant acyl-CoA desaturases with Δ^9-like specificities apparently originated from plastids and are clustered together with bacterial homologues (Fig. 2). Several major duplication events have occurred in insects (one of these branching into separate Δ^9 and Δ^{11} clusters), mammals, *Saccharomycotina* and *Pezizomycotina* fungi (Supplemental Fig. S3).

Opposite to the Δ^9-desaturase-like enzymes, the Δ^{12}-desaturase-like enzymes were found in plants but not in animals (Fig. 3), with the exception of enzymes from nematodes and a sea anemone. These animal enzymes may be the descendants of an ancestor that was lost in all other animals or may originate from an ancient lateral gene-transfer event. In the case of plants, three groups are seen, as proposed by Sperling et al. (2003): Δ^{12}/ω^6-desaturases inherited from the ancestor of the eukaryotic cell and Δ^{12}/ω^6- and Δ^{15}/ω^3-desaturases, most likely acquired from cyanobacteria in the process of endosymbiosis and plastid formation. Additional (and possibly several) duplications are seen inside the Δ^{12}/ω^6 and Δ^{15}/ω^3 plant groups, as well as in fungal clusters of *Saccharomycotina*, *Pezizomycotina* and *Basidiomycota* (Supplemental Fig. S4).

The deep branching separate lineages of unique desaturase enzymes in some organisms indicate their complex evolution. Duplication events might have enabled the evolution of enzymes with novel functions or regulatory mechanisms. Duplications are especially numerous in insects, where desaturases are involved in pheromone synthesis (Fang et al. 2009; Hashimoto et al. 2008). They have also been documented in fungi (Gostinčar et al. 2009b; Meesapyodsuk et al. 2007). The frequent occurrence of duplications indicates an evolutionary advantage of having several different desaturases, but possibly only in certain conditions, since gene losses seem to be just as common (Fang et al. 2009). The phylograms show that duplications can be relatively recent, resulting in nearly identical proteins (Gostinčar et al. 2009b), or ancient, such as in *M. alpina* and *M. polymorpha*. These phylogenetically (and possibly functionally) distant enzymes may be of particular interest for their biotechnological potential. For example, the extensively studied *M. alpina* is known for its production of an unusually wide range of PUFAs (Michaelson et al. 1998) and is used for commercial production of several fatty acids (Sakuradani and Shimizu 2009).

The Evolution of the Desaturase–Cytochrome *b5* Interaction

Eukaryotic acyl-CoA desaturases accept electrons from an electron-transport system that is composed of cytochrome *b5* and NADH-dependent cytochrome *b5* reductase (Dailey and Strittmatter 1980; Mitchell and Martin 1995). Phylogenetic analysis of cytochrome *b5* domains from desaturases showed the same major groups as for the corresponding desaturase parts of the enzymes (Fig. 4).

The evolutionary origins of the cytochromes *b5* are not clear. Only a few examples of cytochrome *b558*, a prokaryotic homologue of cytochrome *b5*, have been found in bacteria (Kostanjevečki et al. 1999), while the cytochrome domain in desaturase-like enzymes has been found only in *Mycobacterium tuberculosis*, *M. ulcerans*, *M. marinum* and *Nocardia farcinica*. Despite the increasing number of sequenced bacterial genomes, we found no additional prokaryotic homologues. In the phylogram, both groups form separate clusters with uncertain locations and provide us with no reliable information about the origins of eukaryotic cytochrome *b5* (data not shown). Construction of the phylogram of cytochrome *b5* domains (Fig. 4 and Supplemental Fig. S1) from various unrelated enzymes shows that the major clusters correspond to the groups of enzymes that contain the cytochrome module. The poorly supported deep branches connecting the major groups do not allow detailed speculation on the origins of the desaturase cytochrome domain. The source might be the soluble cytochromes, any of the cytochrome *b5* domains in other enzymes or even proteins that were lost later in evolution.

The cytochrome domains from the Δ^6-desaturase-like enzymes form a single cluster, which points to a single ancient fusion event that took place in the common ancestor of all eukaryotes. Indeed, all of the cytochrome *b5* fusion events appear to have occurred early in evolution (Supplemental Fig. S1), which raises the questions if (and why) the conditions were more favorable for these events in the past. While the group of Δ^6-desaturase-like and fungal Δ^9-desaturase-like enzymes is not strongly supported, Δ^6-desaturase-like enzymes appear as the most probable donor of the domain. The recombination, which resulted in fungal Δ^9-desaturase-like enzymes with the cytochrome domain at the C terminus, could have happened after the separation of the animal and fungal common ancestors but before the radiation of the ancestors of present-day fungi. In the phylogram expanded with other found cytochrome *b5* domains, including domains in desaturases from heterotrophic protists (Supplemental Fig. S1), the cluster of cytochrome domains from Δ^6-desaturase-like enzymes from animals, plants and fungi does not contain cytochrome domains from Δ^9-desaturase-like enzymes; however, this cluster is poorly supported and not consistent: Fungal cytochrome domains from both groups of desaturases cluster together in the phylogram constructed by Bayesian inference. This would explain the complete absence of the cytochrome domain in animal Δ^9-desaturase-like enzymes and its presence in all of the fungal homologues but also means that fusions of cytochrome domains to Δ^9-like desaturases of Amebozoa

(*Dictyostelium* sp.) and Excavata (*Leishmania* sp., *Trypanosoma* sp.) happened independently of this event (Fig. 2). If the fusion happened earlier, a subsequent loss of the cytochrome *b5* domain would have to have occurred in Δ^9-desaturase-like enzymes from animals and Chromalveolata (*Paramecium* sp., *Perkinsus* sp., *Plasmodium* sp., *Tetrahymena* sp., *Toxoplasma* sp., *Phaeodactylum* sp., *Phytophthora* sp., *Thalassiosira* sp.). A third possibility is the existence of two Δ^9-desaturase paralogues in early eukaryotes, one with the fusion and one without, of which one was (randomly) lost later in evolution, resulting in the unusual distribution of each desaturase type in present eukaryotes. This order of events is also supported by the position of protist sequences in the phylogram of desaturase parts of the enzymes (Fig. 2).

Other scenarios are possible too, such as a separate Δ^9-desaturase fusion with an independent cytochrome *b5* domain or a cytochrome module from other enzymes. In this case, convergent evolution (e.g., loss of acidic amino acids (Fig. 4)) might be the reason for the observed Δ^6/Δ^9 cluster. However, this order of events appears less likely.

Cytochrome *b5* acts as an electron-transfer component in a number of oxidative reactions (Schenkman and Jansson 2003), and it has to interact with many different proteins. One possible benefit of the duplication would be relaxed selection pressure and possible optimization of the interaction between the cytochrome and the (specific) desaturase, without impairing the performance of the cytochrome in other processes. An indicator of this optimization might be seen in the replacement of several acidic amino acid residues in the vicinity of the conserved heme-binding HPGG motif that followed the fusion of cytochrome *b5* to the desaturases. It has been proposed that this might stabilize a permanent and more hydrophobic interaction between the cytochrome and the desaturase parts (Sperling and Heinz 2001). Our data show that the reduction in the amount of acidic amino acids is most pronounced in the case of fungal Δ^9-desaturase-like enzymes (Fig. 4). Position 32 in Fig. 5 is the most conserved acidic site in all of the groups, while positions 38, 39, 48 and 51 show a reduction of acidic amino acids in all of the desaturases. The most interesting, however, are positions 10 and 17, which have high frequencies of acidic residues in cytochromes *b5* and fungal Δ^9-desaturase-like enzymes but not in Δ^6-desaturase-like enzymes, while position 5 shows just the opposite pattern (Fig. 4). These differences might result from a different arrangement of the domains in the primary protein structure in each of the two desaturase types. Alternatively, it might be a consequence of a different coevolution of the desaturase and cytochrome parts, leading to (as proposed by Napier et al. 2003) an optimized interaction between the two parts. The codependence of the two domains has been demonstrated by the exchange of the cytochrome *b5* domain of the Δ^6-desaturase and the Δ^8-sphingolipid desaturase from borage, which resulted in reduced activity of the chimeric enzyme (Libisch et al. 2000) even though both of the enzymes used in the study belong to the same large group of Δ^6-desaturase-like enzymes. Similarly, independent cytochrome *b5* cannot rescue the function of the rat Δ^6-desaturase with a mutated or deleted cytochrome *b5* domain (Guillou et al. 2004).

In the above scenario, the initial fusion would prove to be beneficial only in the long run. It has been proposed that the fusion conferred some evolutionarily selectable advantage to the carrier organism, possibly because of increased efficiency of the electron transport (Guillou et al. 2004; Sperling et al. 1995). However, the functioning of desaturases without the fusion with the cytochrome part (Δ^{12}-desaturase-like and animal Δ^9-desaturase-like enzymes) does not appear to be suboptimal compared to the fused enzymes (Sperling and Heinz 2001). In view of this fact we believe that the fusion was more likely an evolutionarily neutral event, which was later fixed by the increasing codependence of the cytochrome and desaturase domains and has led to a different but equally successful type of desaturases.

The diversity and complex evolution of desaturases underlines the importance of these enzymes in the cell machinery and shows that desaturases with different specificities can be beneficial for the survival of an organism, especially in changing environments. Additional knowledge about important or unusual desaturases, as well as their interactions with the cytochrome *b5* electron donor, will help us to successfully engineer organisms with novel characteristics and exploit them for the production or modification of industrially important lipids.

Acknowledgements We thank Prof. Aharon Oren from The Hebrew University of Jerusalem for critical reading of the manuscript and an anonymous reviewer of our previous paper (Gostinčar et al. 2009b) for focusing our attention on the questions regarding the cytochrome *b5* domain of desaturases. This study was supported by the Ministry of Higher Education, Science and Technology of the Republic of Slovenia, in the form of a Young Researcher grant (to C. G.) and grant no. J1-6715.

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