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Phylogenetic Analysis of Vascular Endothelial Growth Factor Diversity

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Abstract: The secreted glycoprotein vascular endothelial growth factor (VEGF) is a potent and specific mitogen for vascular endothelial cells, capable of stimulating angiogenesis during embryonic development and tumor formation. Despite intensive research, the functions of several VEGF family members remain a mystery. Insight into their evolutionary relationships could profoundly improve our understanding of why there are so many VEGFs and why we have not been able to dissect their function to our satisfaction. It appears from the presence of several structurally related VEGF proteins that VEGF proteins do have a long divergence history. We investigated the evolution and phylogenetic relationships among VEGF proteins by using the information from the molecular data. Consistent phylogenetic trees were generated with character-based and distance-based methods. Several gene duplication events were detected that led to the formation of today's VEGF diversity.

Key Words: Vascular endothelial growth factor, phylogeny, evolution

Vaskülar Endotelial Büyüme Faktor Çeşitliliğinin Filogenetik İncelenmesi

Özet: Salgılanan glikoprotein vaskülar endotelial büyüme faktörü (VEGF) vaskülar endotelial hücreleri için özgül ve güçlü bir mitojen olup embriyo gelişimi ve tümor oluşumu sırasında anjiyogenesisi uyarabilme kapasitesine sahiptir. Bu konuda fazla miktarda araştırma yapılmasına rağmen, bazı VEGF üyelerinin fonksiyonları bilinmemektedir. VEGF proteinleri arasındaki filogenetik ilişkinin ortaya çıkarılması neden bu kadar fazla sayida VEGF proteininin olduğu ve neden bizlerin hala VEGF proteinlerinin fonksiyonları hakkında yeterince bilgi sahibi olamadığımız gerçeğinin ortaya çıkmasına yardım edecektir. Yapısal ilişkisi olan beş büyüme faktörünün varlığı (VEGF-A, VEGF-B, VEGF-C, VEGF-D ve PlGF) VEGF proteinlerinin uzun bir değişim tarihine sahip olduğunu göstermektedir. Biz bu yüzden moleküler dataları kullanarak VEGF proteinlerinin evrimini ve aralarındaki filogenetik ilişkiyi inceledik. Karakter-temelli ve mesafe-temelli metodlar kullanılarak kararlı filogenetik ağaçlar oluşturduk. Bugünkü VEGF çeşitliliğinin gen duplikasyonları sonucu ortaya çıktığı belirlendi.

Anahtar Sözcükler: Vasküler endotelial büyüme faktörü, filogenetik, evrim

Introduction

Vascular endothelial growth factor (VEGF) is a mitogen primarily active on vascular endothelial cells and is essential for the formation of new blood vessels during embryogenesis and in many pathological conditions (1). The human VEGF gene was first cloned and characterized from cultured vascular smooth muscle cells and shown to encode a polypeptide of 45,000 molecular mass that forms a homodimeric glycoprotein (2).

Since its characterization, VEGF has been shown to play important roles in pathogenesis. Intervention in the VEGF system may provide promising approaches to cancer therapy (3). VEGF is shown to be a candidate hormone for facilitating glucose passage across the blood-brain barrier under critical conditions (4). It can stimulate the elongation, network formation, and branching of non-proliferating endothelial cells in cultures that are deprived of oxygen and nutrients (5). Aqueous levels of VEGF correlate well with the severity of mascular edema (6). Two major VEGF isoforms can induce leukocyte stasis (leukostasis) within the retinal vasculature and blood-retinal barrier (BRB) (7). VEGF is directly involved in the pathogenesis of proliferative diabetic retinopathy (8). It can also play a major role in human corpus luteum regulation by paracrine actions (9). VEGF is found to be a potent stimulator of endothelial cell proliferation that has been implicated in tumor growth of thyroid carcinomas (10). VEGF may explain the microangiopathy, neovascularization, and accelerated

vasopermeability that occur in Crow-Fukase syndrome (11).

Despite the importance of the VEGF family of proteins in pathogenesis and cell proliferation, we do not know much about their phylogeny or evolution. The main cause limiting the study of VEGF phylogeny is the presence of short and hard to align fragmented sequences. Phylogenetic trees with such data generate unlinked tree topologies with discrepancies and no phylogenetic resolution. In this study, an approach different than the conventional one was used to elucidate the phylogenetic relationships among VEGF sequences from various taxa. Rather than using the whole VEGF sequences, only the most conserved region was utilized.

In the analysis presented here, 5 VEGF sequences were utilized (VEGF-A to E). Despite its non-canonical name, PlGF (Placental Growth Factors), a classical member of the VEGF family (12), was also included in our analysis. We extended our analysis to the PDGF (platelet derived growth factors) family since these are also cysteine-knot ligands. VEGF/PlGF/PDGF belong to the group of the cysteine-knot superfamily of proteins, which are all characterized by the presence of 8 conserved cysteine residues. While our prime concern was the phylogeny of the group of the cysteine-knot superfamily, we included the viral VEGFs in our analysis. We wish to know whether the viral VEGF sequences reflect a true phylogenetic relationship. In addition, some non-mammalian sequences, e.g., *Drosophila* VEGFs (PVFs) and snake venom VEGF (VEGF-F) were included to know which other VEGFs these sequences group with. As we were trying to decipher the ancient history of the VEGF family of ligands, *Drosophila* sequences helped us to outgroup the phylogentic trees and present the direction of the evolutionary process.

Materials and Methods

VEGF sequences were retrieved from the NCBI database (www.ncbi.nih.gov) or Swiss-Prot database (htpp://us.expasy.org/sprot/) and the accession numbers are summarized in Table 1. Before obtaining a multiple alignment, dot blot analysis was performed in BioEdit (freely available at www.mbio.ncsu.edu/BioEdit) to examine whether the sequences are similar enough to be aligned unambiguously. Only sequences that are part of a diagonal in dot plots are used for subsequent analysis. The selected sequences were aligned in Clustal X using default parameters (11) and low scoring segments on the overall alignment were calculated. There were obvious alignment errors, major gaps and sections of dubious quality due to differences in sequence lengths. Therefore, the sequences were used to find the most conserved regions (MCRs) to be able to generate a uniform and meaningful sequence alignment. MCRs were searched in the unaligned sequences with MEME (Multiple Elicitation of Motifs by Expectation-maximization) (14) and MAST (Motif Alignment and Search Tool) (15) using the default parameter settings from the web interface. A 50 amino acid long stretch of amino acid sequence covering the cysteine-knot structure was found in all studied sequences. Finally, an alignment in Clustal X was created with the MCRs and edited manually in Seaview (16). Gaps were minimized without disturbing the overall alignment and to maintain the positional homology that is needed for further phylogenetic inference (17). Once a multiple sequence alignment was prepared, the alignment was used for further evolutionary analysis. The sequence data explorer of MEGA2 was used to obtain the statistical information (18).

The phylogenetic analysis was conducted using PHYLIP (19). Two main tree-building methods were used: distance and character-based methods. Based on the matrix containing pairwise distance values that were calculated using PROTDIST, a neighbor-joining tree was built (20). To choose an out-group to infer the root of a tree, dot plots were compared in BioEdit as suggested in Salemi and Vandamme (21). The potential out-group was determined based on the knowledge that out-group the chosen should belong to a clearly distinct lineage with respect to the in-group sequences and it should not be so divergent and should be aligned unambiguously. As the representative of the character -based methods, trees were built with the maximum parsimony method using the program PROTPARS. The input order of the sequences was randomized with a jumble number of 10.

To evaluate the reliability of the inferred trees, bootstrap analysis was used (22). The sequence data were bootstrapped 1000 times by randomly choosing columns from the original alignment by using the program SEQBOOT. The majority rule consensus trees were created by CONSENSUS and trees were drawn with DRAWTREE and edited in Adobe Illustrator 10.

Table 1. NCBI accession numbers of VEGF sequences used in phylogenetic analysis.

TAXA	NCBI (or Swiss-Prot) accession numbers
VEGF-A	
Bos taurus (Bovine)	P15691
Bitis gabonica (Gaboon viper)	P83906
Canis familiaris (Dog)	Q9MYV3
Gallus gallus (Chicken)	P67964
Coturnix coturnix japonica (Japanese quail)	P67965
Equus caballus (Horse)	Q9GKR0
Homo sapiens (Human)	P15692
Mesocricetus auratus (Golden hamster)	Q99PS1
Mus musculus (Mouse)	Q00731
Sus scrofa (Pig)	P49151
Rattus norvegicus (Rat)	P16612
Ovis aries (Sheep)	P50412
Trimeresurus flavoviridis (Habu)	P67860
Spalax leucodon ehrenbergi (Ehrenberg's mole rat)	Q9QX39
Felis silvestris catus (Cat)	BAB68520
Capreolus capreolus (Roe deer)	AAF73233
Brachydanio rerio (Zebrafish)	Q5RHW5
Oncorhynchus mykiss (Rainbow trout)	Q6H8S7
VEGF-B	
Bos taurus (Bovine)	NP_776913
Mus musculus (Mouse)	P49766
Rattus norvegicus (Rat)	O35485
Homo sapiens (Human)	P49765
VEGF-C	
Homo sapiens (Human)	P49767
Mus musculus (Mouse)	P97953
Bos taurus (Bovine)	Q9SX50
Brachydanio rerio (Zebrafish)	Q7T3I6
Coturnix coturnix (Common quail)	O57352
VEGF-D	
Homo sapiens (Human)	O43915
Mus musculus (Mouse)	P97946
Rattus norvegicus (Rat)	O35251
Gallus gallus (Chicken)	Q8QGD7
Viral VEGF proteins (VEGF-E)	
Orf virus (strain NZ7)	P52585
Orf virus strain D1701	Q772M8
Orf virus (strain NZ2)	P52584
Pseudocowpox virus	Q8B571
Viral VEGF proteins (VEGF-E)	
Orf virus (strain NZ7)	P52585
Orf virus strain D1701	Q772M8
Orf virus (strain NZ2)	P52584
Pseudocowpox virus	Q8B571
VEGF-F	
Snake venom	1WQ9A
PIGF	
Ovis aries (Sheep)	AAN77495
Canis familiaris (Dog)	XP_547910
Brachydanio rerio (Zebrafish)	XP_685640
Homo sapiens (Human)	P49763
PDGF	
Caenorhabditis elegans	AAF60517
Caenorhabditis briggsae	CBG15137
Gallus gallus (Chicken)	NP_989637
Mesocricetus auratus (Golden hamster)	BAA78768
Mus musculus (Mouse)	NP_032834
Canis familiaris (Dog)	AAW47931
Brachydanio rerio (Zebrafish)	AAH78289
Xenopus leaves	P13698
PVF	
Drosophilia melanogaster-PVF1	NP_523407
Drosophilia melanogaster-PVF2	NP_523499
Drosophilia melanogaster-PVF3	NP_523500
VEGF	
Podocoryne carnea	AAS79435

Results

Clustal X was used to align the sequences with default parameters. The alignment showed large gaps with dubious quality. Increasing the gap-opening penalty did not help to improve the overall alignment. Subsequent efforts to edit failed because there were too many gaps at both the N and C termini, mainly due to the differences in lengths of VEGF sequences. We then chose to search highly conserved regions and performed a phylogenetic analysis accordingly. MEME and MUST are freely available tools that allow the detection of highly conserved regions in groups of related protein (or nucleotide) sequences. The search results revealed a conserved region of 50 amino acids present in all available VEGF sequences. The conserved sequences were extracted from the original sequence data, aligned and edited before any phylogenetic analysis was carried out (Figure 1).

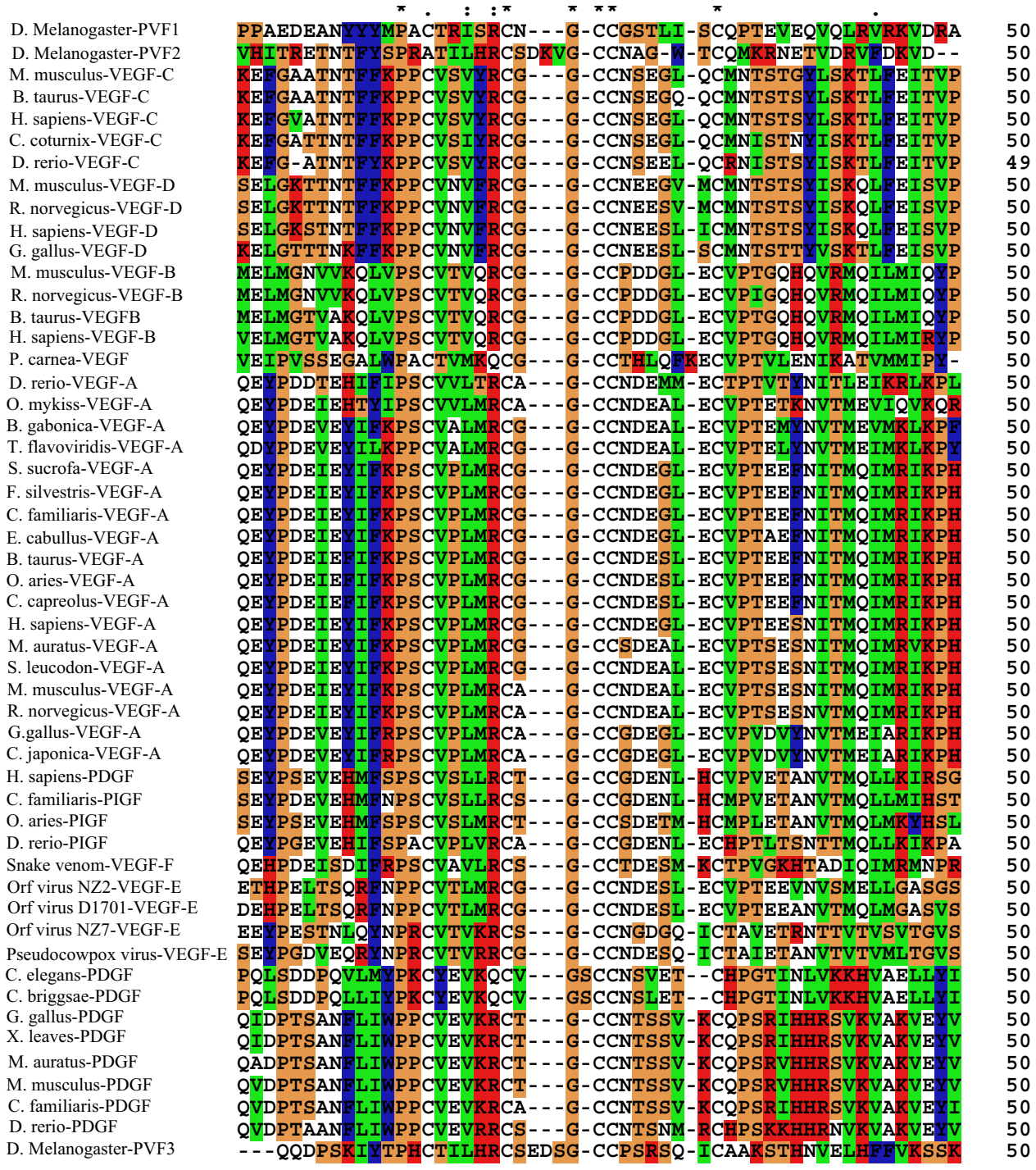


Figure 1. Multiple sequence alignment of the most consensus region (MCR) of VEGF proteins. MCRs were searched in the unaligned sequences with MEME (Multiple Elicitation of Motifs by Expectation-maximization) and MAST (Motif Alignment and Search Tool) using the default parameter settings from the web interface. Sequences were aligned in Clustal X and edited in Seaview.

Character-based phylogenetic analysis

We used the MP method to carry out a character-based phylogenetic analysis (23, 24). The amino acid data matrix contained 50 amino acids with 41 variable sites, of which 40 were potentially parsimony informative. For 52 polypeptide sequences, a fully bifurcating unrooted tree containing 52 terminal nodes, 50 internal nodes, and 101 branches (edges) was obtained. While several nodes are not well-supported, some of the nodes do have relatively high bootstrap values. The unrooted tree shown in Figure 2 represents the phylogenetic relationships among various VEGF

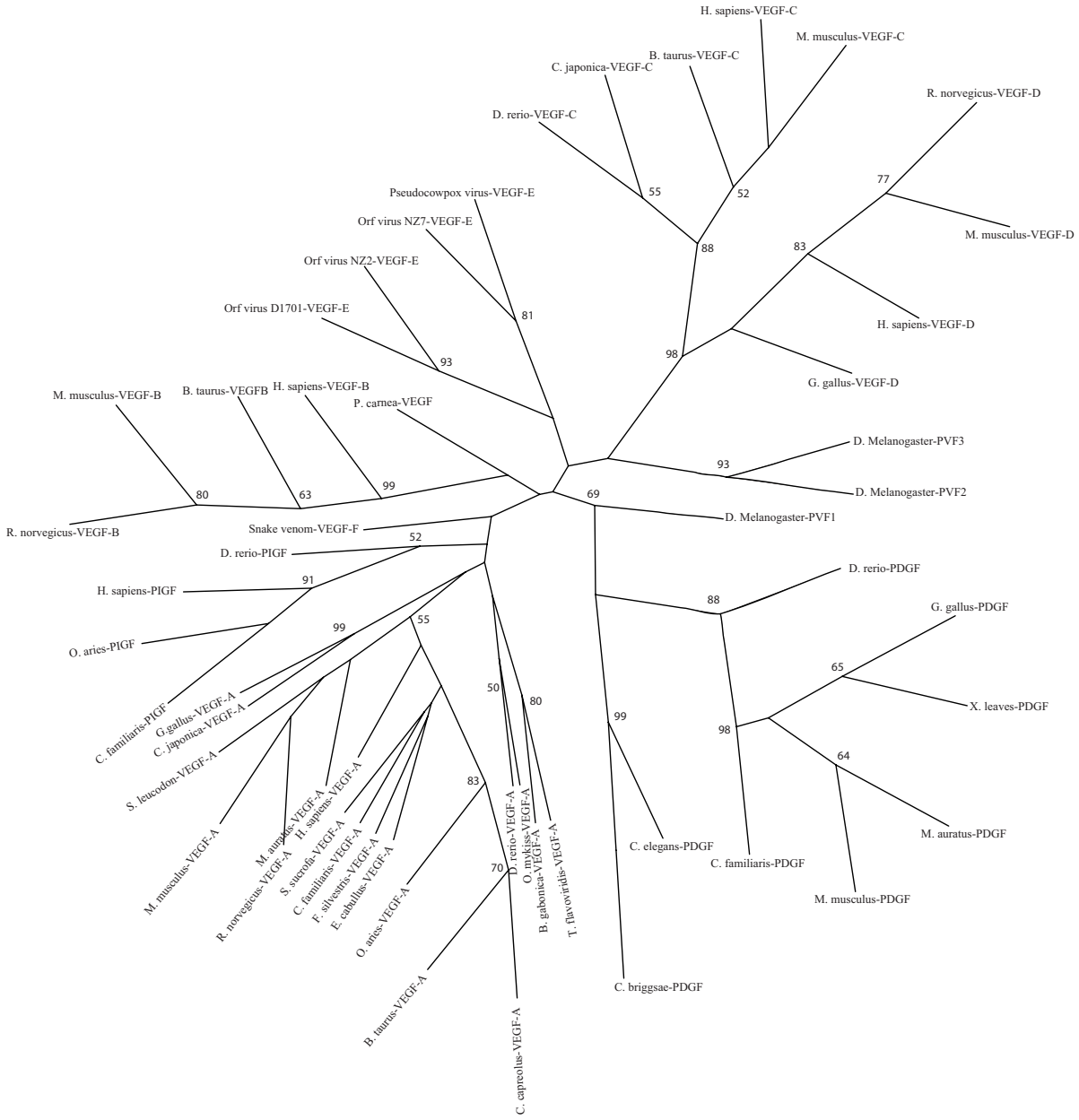


Figure 2. Unrooted maximum parsimony tree of VEGF proteins showing the phylogenetic relationships. The most parsimonious tree shows the relation between VEGF isoforms of different organisms as well as different isoforms of the same organism. Bootstrap values in percentage were calculated from 1000 datasets. Values less than 50% are not shown.

sequences. By this analysis, VEGF sequences were placed in their expected locations and formed relatively well supported groups. There is clear congruity between the VEGF parsimony phylogeny and the expected relationships of VEGF sequences. Since the groups did not form based on the properties of each taxon, the tree appears to reflect the history of the VEGF protein family rather then the evolutionary history of the corresponding taxa. According to the MP tree, VEGF-A and -B form independent branches, whereas VEGF-C and –D form 2 branches that separated from each other relatively recently. The fact that a gene duplication event separates the VEGF-C and –D subfamily from the rest is expected since these are the only VEGF sequences having long cysteine rich C-terminal propeptides.

Phylogenetic analysis of PVF sequences from *Drosophila* indicated that PVF-1 is homologous to the PDGF family, whereas PVF-2 and -3 are more closely related to the VEGF-C and –D sequences. It is known that all 3 PVFs act through a single receptor, PVR, which makes this finding more interesting (25). We therefore suggest that the receptor binding regions of PVFs are outside the most conserved region. *Podocoryne carnae* is the most basic phylum of the animal kingdom to have tissue organization and a nervous system even though it lacks a vascular system. Although we expected the VEGF of *P. carnea* to group with a possible homologue, *Drosophila* PVF-1, it grouped as a sister to the VEGF-Bs.

PIGF binds the VEGF-R1 receptor and is predominantly expressed in the placenta, heart, and lungs (26). PIGF is grouped as a sister to the VEGF-As, indicating a similar evolutionary history. Within the human VEGF family, VEGF-A was reported to be the most closely related placental growth factor (53% amino acid identity) to the PIGF family. The virus encoded VEGF-Es form a single group with VEGF-E (D1701) and VEGF-E (NZ2) most closely related and VEGF-E (NZ7) and VEGF-E from *Pseudocowpox* virus most closely related. Due to their enormously accelerated generation cycle and mutability, viral VEGF sequences might be the result of divergent evolution rather than reflecting true phylogenetic relationships.

Because we are dealing with multiple forms of the VEGF protein in our analysis, the history of the protein can reflect the history of gene duplications. However, an unrooted tree only places the individual taxa relative to each other without presenting the direction of the evolutionary process. Dot plot analysis and midpoint rooting indicated that PVF-2 of *Drosophila* is the best candidate to become an out-group, because the protein is not very distantly or closely related to the other VEGFs (the 8 cysteine residues of the cysteine-knot structure are highly conserved, except in PVF-2, which lacks cysteine 2) and also present in a relatively more primitive organism. We therefore rooted the maximum parsimony tree by choosing PVF-2 of *Drosophila* as the out-group and determined the direction of the evolutionary process. Examination of the rooted tree indicated the presence of several major gene duplication events that ultimately led to the formation of VEGF-A, -B, -C and -D proteins (Figure 3).

Distance-based phylogenetic analysis

The methods used in phylogenetic analysis are based on assumptions about how the evolutionary process works. These assumptions can be implicit, as in parsimony methods, or explicit, as in distance methods. Therefore, it is essential to apply different methods to a single molecular data set to obtain robust results. We thus carried out a distance-based phylogenetic analysis. For this purpose, the neighbor-joining method was used (20). To infer a tree with the program Neighbor.exe of the PHYLIP package for the VEGF alignment, pairwise evolutionary distances were calculated with the program PROTDIST, employing a Jones-Taylor-Thornton matrix. The built cladogram overall showed similarities to the MP tree with higher bootstrap values (Figure 4). In both instances (MP and NJ), large clusters containing VEGF-A, –B, –C and –D proteins formed (Figure 5). However, within the groups there were minor differences in topology. Otherwise, the general topology is stable, whatever algorithm is used. One characteristic remaining constant is that the VEGF-A sequences are always grouped together as well as the VEGF-B, -C, –D, PIGF and PDGF sequences. Neighbor-joining analysis of the sequences revealed that the VEGF/PDGF/PIGF family tree is essentially composed of 4 major branches evolved from a common ancestor, a VEGF-A branch comprising VEGF-As, -Es, -F and PIGFs; a VEGF-B branch comprising VEGF-Bs and *P. carnea* VEGF; a VEGF-C branch comprising VEGF-Cs, -Ds and –Es; and a PDGF branch comprising PDGF, PVF-1, -2 and -3.

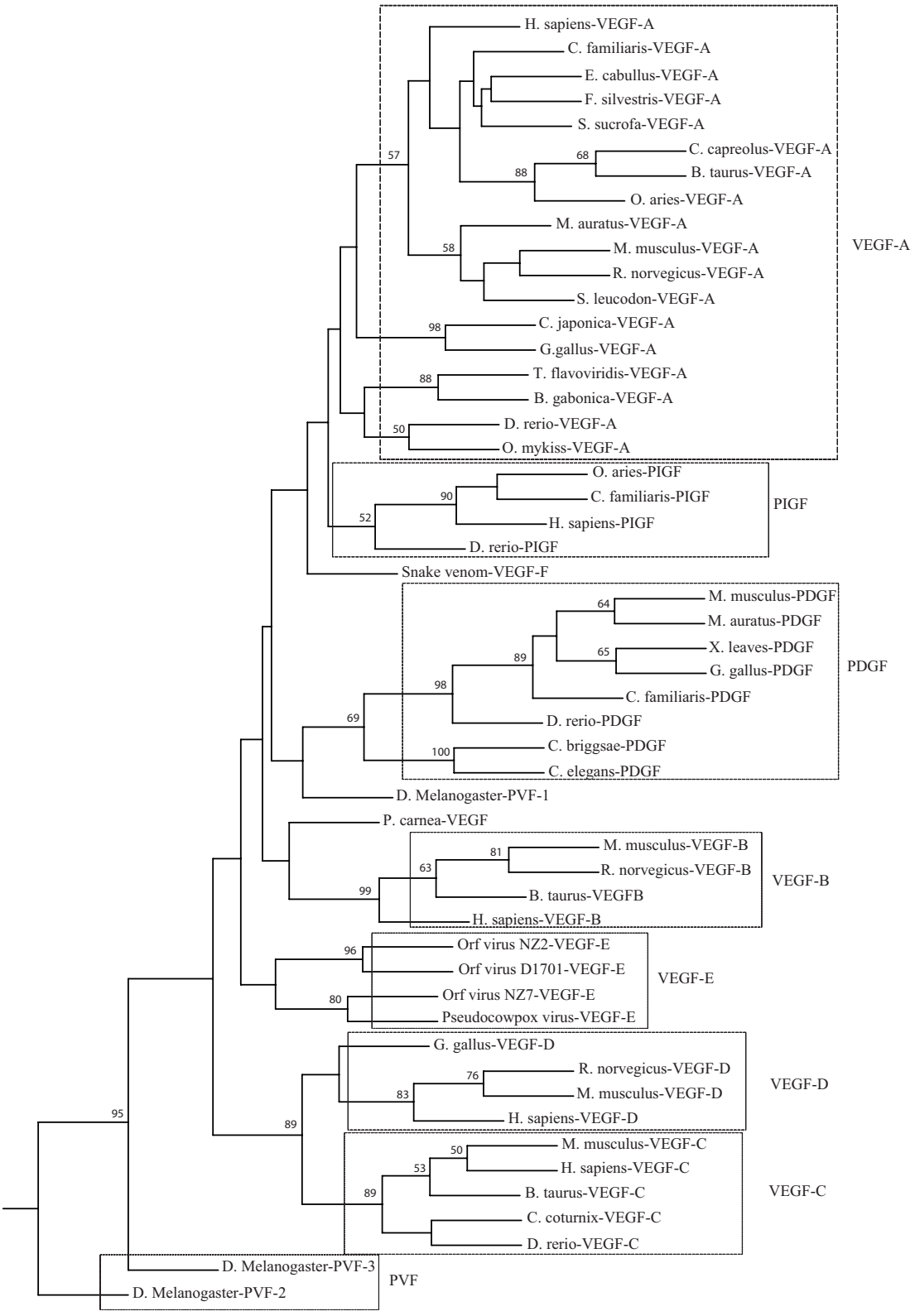
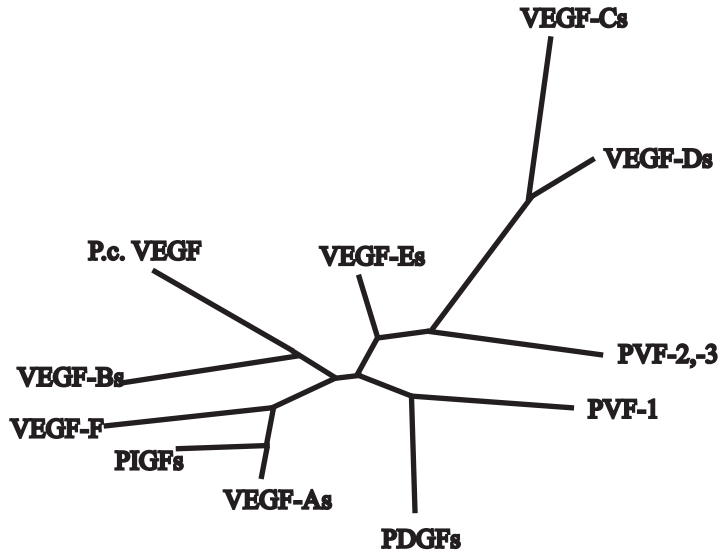


Figure 3. Rooted maximum parsimony tree of VEGF proteins showing the direction of the evolution and possible gene duplication events. Bootstrap values in percentage were calculated from 1000 datasets. Values less than 50% are not shown.

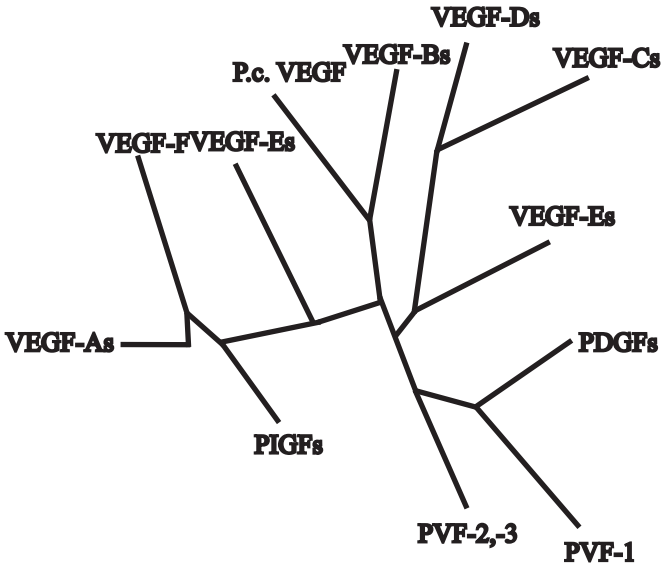
Figure 4. Unrooted neighbor-joining tree of VEGF proteins showing the phylogenetic relationships. To infer a tree with the program Neighbor.exe of the PHYLIP package for the VEGF alignment, pairwise evolutionary distances were calculated with the program PROTDIST, employing a Jones-Taylor-Thornton matrix. Bootstrap values in percentage were calculated from 1000 datasets. Values less than 50% are not shown.

Discussion

The human VEGF/PlGF family of structurally related growth factors has at least 5 members, VEGF-A, VEGF-B, VEGF-C, VEGF-D and PlGF, all encoded by separate genes (27). These proteins are involved in orchestrating the complex physiological process of angiogenesis. Many physiological studies have been performed on determining the function and characterization of VEGF proteins. However, there is not much information with respect to the evolution and phylogeny of VEGF proteins from human and other organisms. We felt that such information may elegantly illustrate which members are



Maximum Parsimony tree



Neighbor-Joining tree

Figure 5. Overall comparison of unrooted VEGF trees showing the overall tree topologies.

more closely related and offer an overall picture of the evolutionary past of each VEGF member. From our analysis, VEGF-A seems to be the latest appearing form of the still existing VEGF proteins, and VEGF-C and –D are more closely related species to each other than the others. The trees we built represented the history of the VEGF proteins rather than the history of the taxa as indicated by the cases where one variant of human VEGF protein branched with a mouse VEGF protein.

The presence of VEGF-like proteins in several invertebrate species, e.g., in *P. carnea*, indicates that VEGF-like proteins must have emerged early in the evolutionary process. It is important to note that our tree routing was based on the assumption that PVF-2 of *Drosophila* is distantly related to the examined taxomic units. If we select a different out-group, e.g., VEGF of *P. carnea*, we end up having a different evolutionary lineage (data not shown). Therefore, it is important to point out that unless the out-group species is known with certainty, the rooted tree presented here only represents predictions.

It is difficult to establish biological correlates of the molecular evolution unless a specific function for each VEGF protein is known. Although it is already known that in general all of the vertebrate VEGFs and their cognate receptors studied so far are able to regulate angiogenesis and have key roles in the formation of vascular structures, each VEGF family member interacts with different receptor proteins and may have very specific functions. For example, VEGFR1 and VEGFR2 can bind to VEGF-A, but VEGFR3 cannot bind to VEGF-A (1). This may be explained by the fact that VEGF-A is an angiogenic growth factor rather than a lymphangiogenic growth factor (26). However, both VEGFR2 (an angiogenic growth factor receptor) and VEGFR3 (a lymphangiogenic growth factor receptor) can bind to VEGF-C and VEGF-D although VEGFR1 does not bind to both VEGF-C and VEGF-D. One obvious question, for instance, is whether the separation of the VEGF subtrees coincides with the evolution of the lymphatic system. We feel that such a link is weak since studies have demonstrated that VEGF-D is both angiogenic and lymphangiogenic (26-28). In addition, some of the VEGFs seem not to exist in certain vertebrates or have a different function. The phylogenic relationships we established here agree with the binding preferences of human VEGF proteins with their receptors.

To prevent biased sequence selection and avoid overrepresentation of the mammalian and especially human, mouse and rat sequences, we included homologous VEGF sequences of different taxa. Such diversity during analysis of VEGF sequences is expected to prevent tree distortions. Holmes and Zachary (2005) generated a NJ tree by using human VEGF sequences and several human VEGF analogues from *Drosphila* and Orf virus. Although the NJ tree they built in their study shares similarities to the tree built in this study, it is not directly comparable.

Phylogenetic trees built by using ribosomal RNA sequences, as initiated by Woese and his collaborators (29), tend to be more reliable than trees generated by using the other gene sequences because rRNA sequences have changed very little over time during evolution. Our phylogenetic analysis used the same logical approach and utilized only the most conserved region. Alignments produced in this way are shown to produce phylogeny that is closer to the true phylogeny than a similarly produced phylogeny from the complete sequence alignment (30). Although we were able to built similar trees consistently with different algorithms, it did not escape our attention that the bootstrap values were surprisingly low. This may be due to the presence of fewer alignment sites in MCR-only sequence alignment over the entire multiple sequence alignment. Furthermore, one might argue that by limiting the analysis to the easy stretch of VEGFs, where alignment is unambiguous, a significant part of the evolutionary information hidden in the molecular data is missed. It is, however, important that some sequences outside the VEGF homology domain defy alignment and information generated from such data is not reliable.

Although it is the DNA that contains all the information to create proteins, it is more appropriate to use protein sequences for phylogenetic analysis due to the codon-usage bias and phylogenetic noise reduction (21). For this reason, we also preferred to use the protein sequences in our analysis rather than the nucleotide sequences. In conclusion, to our knowledge this is the first detailed phylogenetic study describing the evolutionary relationships among the VEGF proteins.

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