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# Comprehensive Analysis of Orthologous Protein Domains Using the HOPS Database

Christian E.V. Storm and Erik L.L. Sonnhammer<sup>1</sup>

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One of the most reliable methods for protein function annotation is to transfer experimentally known functions from orthologous proteins in other organisms. Most methods for identifying orthologs operate on a subset of organisms with a completely sequenced genome, and treat proteins as single-domain units. However, it is well known that proteins are often made up of several independent domains, and there is a wealth of protein sequences from genomes that are not completely sequenced. A comprehensive set of protein domain families is found in the Pfam database. We wanted to apply orthology detection to Pfam families, but first some issues needed to be addressed. First, orthology detection becomes impractical and unreliable when too many species are included. Second, shorter domains contain less information. It is therefore important to assess the quality of the orthology assignment and avoid very short domains altogether. We present a database of orthologous protein domains in Pfam called HOPS: Hierarchical grouping of Orthologous and Paralogous Sequences. Orthology is inferred in a hierarchic system of phylogenetic subgroups using ortholog bootstrapping. To avoid the frequent errors stemming from horizontally transferred genes in bacteria, the analysis is presently limited to eukaryotic genes. The results are accessible in the graphical browser NIFAS, a Java tool originally developed for analyzing phylogenetic relations within Pfam families. The method was tested on a set of curated orthologs with experimentally verified function. In comparison to tree reconciliation with a complete species tree, our approach finds significantly more orthologs in the test set. Examples for investigating gene fusions and domain recombination using HOPS are given.

[The NIFAS viewer is integrated in the Stockholm Pfam site (<http://Pfam.cgb.ki.se>) and the test set of putative orthologs and detailed results are available at [ftp://ftp.cgb.ki.se/pub/HOPS/.](ftp://ftp.cgb.ki.se/pub/HOPS/)]

The concepts of orthology and paralogy (Fitch 1970) are widely used. A search in PubMed reveals an increase of the use of the regular expression "ortholog\*" in abstracts from 28 in 1990, 68 in 1994, 302 in 1998, to 840 in 2001. An in-depth explanation of orthology and paralogy can be found in recent publications (Fitch 2000; Sonnhammer and Koonin 2002). Numerous applications and analyses rely on the use of orthologous sequences, for instance, transferring functional annotation (Stein 2001), phylogenetic footprinting (Blanchette et al. 2002), and evolutionary and comparative studies (Makalowski et al. 1996; Mushegian et al. 1998; Xie and Ding 2000).

A standard approach for assigning orthology in a phylogenetic tree is tree reconciliation (Goodman et al. 1979; Page 1994). Here a given species tree is compared with a gene tree. This works by postulating the minimum number of duplication and gene-loss events in the gene tree necessary to reconcile it with the species tree. Orthologous assignments can then be made from this reconciled tree. Given a correct species and gene tree, this method can reliably distinguish between orthologs and paralogs. In theory, tree reconciliation is superior to BLAST-based (Altschul et al. 1997) approaches for finding orthologs (Tatusov et al. 1997; Remm et al. 2001). Such methods neither use the information provided by a species tree, nor take unequal rates of evolution into account.

However, one drawback of tree reconciliation is that it uses a given, fixed species tree: For some species the evolutionary history is still controversial, for example, the phylogenetic relationship of *Homo sapiens*, *Caenorhabditis elegans*, and *Drosophila melanogaster* (Mushegian et al. 1998; Xie and Ding 2000; Blair et al. 2002). In addition, a reconstructed phylogenetic tree, espe-

cially for short sequences, might not reflect the species tree because of random effects. Simplifications in the phylogenetic model used can also lead to an incorrect sequence tree. For such cases tree reconciliation might not find the correct orthologous sequences.

Here we present an approach to resolve these problems by organizing the sequences into evolutionarily distinct subgroups. Orthology is then inferred between these subgroups using ortholog bootstrapping (Storm and Sonnhammer 2002). The results are saved in a database named HOPS (Hierarchical analysis of Orthologous and Paralogous Sequences). The HOPS data can be analyzed and displayed graphically with a tree in an extended version of the NIFAS browser (Storm and Sonnhammer 2001).

Recent studies indicate a high rate of horizontal transfer for bacteria (Doolittle 1999; Koonin et al. 2001; Snel et al. 2002). The present algorithms for tree reconciliation do not account for horizontal transfer of genes. If a gene has been horizontally transferred, tree reconciliation might fail to find its orthologous genes (Gogarten and Olendzenski 1999). Therefore, bacterial sequences are not included in the analysis.

## METHODS

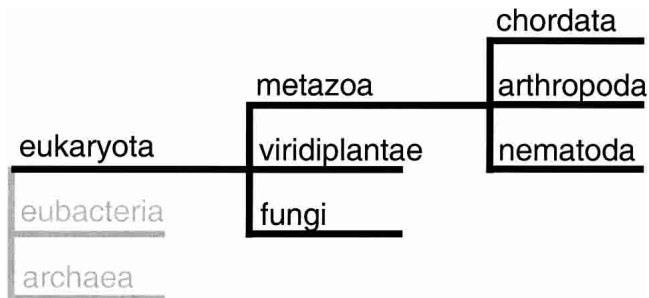
### Data

This paper is based on the 3735 protein families in Pfam 7.2 (Bateman et al. 2002). The sequences in each alignment are clustered following a hierarchical scheme derived from the species tree (see Fig. 1). Clades that can be considered equidistant to each other from an evolutionary perspective are assigned to the same level. At present, the grouping scheme consists of two levels: eukaryotes and metazoans. A higher level containing the top level of all three kingdoms could be used as well, but is presently not implemented because of frequent misassignments that would be caused by horizontal transfer in prokaryotes. Therefore, the bacterial and archaeal kingdoms were excluded from HOPS.

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**Figure 1** The HOPS hierarchy of species groups. Only the levels marked in black are used for orthology analysis in this paper because of the high levels of horizontal transfer among eubacteria and archaea.

Each level is divided into evolutionarily distinct species groups built around completely sequenced genomes. This minimizes the chance that paralogs could be mistaken for orthologs,

because the true orthologous sequence might not be known yet. These species groups, or lineages, are treated as one “pseudospecies” in the orthology analysis, which is carried out only between species groups at the same level. Orthologous relations within a species group are not analyzed.

The eukaryotic level is split up into the clades of Metazoa, Viridiplantae, and Fungi. The metazoan level is divided into Chordata, Nematoda, and Arthropoda. Sequences from species that do not belong to any of those clades are not analyzed. For instance, a sequence from an Echinodermata species (sea urchins, starfish, etc.) would not be taken into account on the metazoan level, because it doesn’t belong to any of the species in the Chordata, Nematoda, or Arthropoda groups. But it would be analyzed on the eukaryotic level, because it is part of the metazoan group. Therefore, orthologs to this sequence may be assigned in the Viridiplantae and Fungi groups, but not on the metazoan level.

To improve the quality of ortholog assignments, we can use an outgroup criterion at the metazoan level. All sequences from the eukaryotic groups that are not part of the metazoan group are treated as outgroup sequences. If an outgroup sequence is found

**Table 1.** Results of HOPS and RIO for the Test Set of Putative Orthologs for *H. sapiens* and *D. melanogaster*

<i>H. sapiens</i>	<i>D. melanogaster</i>	Domain	HOPS (%)	RIO (%)
143E_HUMAN	143E_DROME	14-3-3	100	98
ADHX_HUMAN	ADHX_DROME	adh_zinc	16.5	0
APE1_HUMAN	RRP1_DROME	Exo_endo_phos	100	85
ARF1_HUMAN	ARF1_DROME	arf	89.5	92
ARI1_HUMAN	ARI1_DROME	IBR	100	96
ARL1_HUMAN	ARL1_DROME	arf	100	100
ATPG_HUMAN	ATPG_DROME	ATP-synt	100	100
ATPO_HUMAN	ATPO_DROME	OSCP	100	63
CAPB_HUMAN	CAPB_DROME	F_actin_cap_B	100	95
CG1C_HUMAN	CG1C_DROME	Cyclin	100	80
CLAT_HUMAN	CLAT_DROME	Carn_acyltransf	94	89
COXA_HUMAN	COXA_DROME	COX5A	99	78
DYNA_HUMAN	DYNA_DROME	CAP_GLY	50.5	29
ERH_HUMAN	ER_DROME	ER	100	98
FAC2_HUMAN	FAC2_DROME	Abi	87	62
H2AZ_HUMAN	H2AV_DROME	Histone	76	21
H4_HUMAN	H4_DROME	Histone	62	1
IF2A_HUMAN	IF2A_DROME	S1	78	62
KC2B_HUMAN	KC2B_DROME	CK_II_beta	18.5	36
MCM2_HUMAN	MCM2_DROME	MCM	100	97
MCM4_HUMAN	MCM4_DROME	MCM	100	100
PP1B_HUMAN	PP1B_DROME	Metallophos	76	43
PRS8_HUMAN	PRS8_DROME	AAA	84.5	—
PSA2_HUMAN	PSA2_DROME	Proteasome	99.5	98
PSA3_HUMAN	PSA3_DROME	Proteasome	74.5	13
PSA4_HUMAN	PSA4_DROME	Proteasome	100	100
PSA5_HUMAN	PSA5_DROME	Proteasome	79	95
PSA6_HUMAN	PSA6_DROME	Proteasome	77	44
PSB1_HUMAN	PSB1_DROME	Proteasome	50.5	37
PSB3_HUMAN	PSB3_DROME	Proteasome	72.5	22
PURA_HUMAN	PURA_DROME	Adenylsucc_synt	100	100
RAS1_HUMAN	RAS1_DROME	HHH	0	0
RBJK_HUMAN	RBJK_DROME	TIG	97	48
RLA1_HUMAN	RLA1_DROME	60s_ribosomal	35	12
RLA2_HUMAN	RLA2_DROME	60s_ribosomal	45.5	21
RPB2_HUMAN	RPB2_DROME	RNA_pol_B	100	96
RPB6_HUMAN	RPB6_DROME	RNA_pol_Rpb6	90	62
RS18_HUMAN	RS18_DROME	Ribosomal_S13	24	3
RTC1_HUMAN	RTC1_DROME	RTC	100	6
SSB_HUMAN	SSB_DROME	SSB	97.5	92
T2DA_HUMAN	T2DA_DROME	TFIID_A	82.5	87
T2FB_HUMAN	T2FB_DROME	TFIIF_beta	100	98
TCPA_HUMAN	TCPA_DROME	cpn60_TCP1	99.5	60
TCPG_HUMAN	TCPG_DROME	cpn60_TCP1	97	57
UBCI_HUMAN	O62622	UQ_con	100	96
VATF_HUMAN	VAF1_DROME	ATP-synt_F	97	54
XPA_HUMAN	XPA_DROME	XPA	100	67

A “—” indicates no results reported.

**Figure 2** New commands in NIFAS for analyzing orthology. The three checkboxes to the left are for applying cutoffs. If the “collapse” checkbox is marked, all proteins that have an orthology bootstrap support below the cutoff are hidden. The “Orthology” checkbox groups orthologs based on an average-linkage algorithm described in Hollich et al. (2002). If the orthologous groups are ambiguous, “auto adjust” tries to minimize the number of overlapping orthologous groups by going through all cluster cutoffs within the range of the corresponding value, with a mean of the original cutoff. In the box to the middle, the user can choose the HOPS level for which ortholog assignments are shown. Presently these are the eukaryotic and metazoan levels. The checkboxes to the right allow choosing what species of the present level should be included in the clustering.

between two candidate orthologs in the tree, this means that they are probably not true orthologs but were clustered together because the true ortholog was lost in one of the species. The outgroup species did not lose the ortholog and is hence more closely related than the false ortholog. We have not used the outgroup criterion at the eukaryotic level, however, because of the frequent horizontal transfers from eukaryotes to prokaryotes.

### Orthostrapper

Orthology assignments are calculated by a program called Orthostrapper (Storm and Sonnhammer 2002) that performs “ortholog bootstrapping,” or “orthostrapping.” Orthostrapper analyzes a set of bootstrap trees instead of the optimal tree for orthologs. The algorithm detects orthologous relations between two (groups of) species. The frequency of orthology assignments in the bootstrap trees can be interpreted as a confidence value for the possible orthology of two proteins.

Here Orthostrapper is run on pairs of species groups at the same level. Each Pfam alignment is split according to the phylogenetic groups mentioned above, and six pairwise comparisons are carried out: on the eukaryotic level, Metazoa–Viridiplantae, Metazoa–Fungi, and Viridiplantae–Fungi; on the metazoan level, Chordata–Nematoda, Chordata–Arthropoda, and Nematoda–Arthropoda.

Ortholog groups between more than two species groups are generated by merging the pairwise results. This is only allowed

between species groups at the same HOPS level. If there are incongruencies between the pairwise results, this needs to be flagged. Partial sequences/fragments shorter than 50% of the alignment length were removed to improve the tree quality. Ortholog bootstrapping with 200 pseudosamples was then used to analyze the alignments. Alignments with more than 1200 sequences were not analyzed. Because of their arbitrary length, Pfam families of type “repeats” were excluded from the analysis.

### Test Set

To evaluate the accuracy of ortholog assignments, we compiled a set of curated orthologs for *H. sapiens*–*Saccharomyces cerevisiae*, *H. sapiens*–*C. elegans*, and *H. sapiens*–*D. melanogaster*.

To avoid any ambiguity, the sequences in the test set should fulfill the following criteria:

- Experimentally verified function
- Same substrate and activity for putative orthologs
- One-to-one orthology
- Single-domain proteins

To narrow the set of proteins, all single-domain proteins (according to Pfam-A) in one species were scored with BLAST to all single-domain proteins in the second species. From the alignments, best–best hit pairs (sequences that rank each other as best match when searching both ways) were extracted. To remove many-to-many orthologous relations, each sequence of the best–best hit pairs was then aligned to all sequences within its species. If any sequence within the same species had a lower *e*-value than the corresponding ortholog, the pair was excluded from the test set. From the remaining pairs, sequences with functional annotation by homology were excluded. The functional descriptions of each sequence pair were then compared. Only if both proteins use the same substrate and show the same activity were they included in the final test set.

This procedure resulted in a set of 102 human–yeast, 19 human–worm, and 47 human–fly putative orthologs, shown in Tables 1–3. It should be pointed out that the main criteria for the test set are ‘experimentally verified function’ and ‘same substrate and activity,’ which is why the test set is relatively small. The reciprocal BLAST scoring was done to limit the necessary number of (manual) comparisons of sequence annotations.

### Phylogenetic Analysis

To analyze why HOPS fails in some cases to find the correct ortholog, we used MrBayes (Huelsenbeck and Ronquist 2001) to reconstruct trees from the sequences. The substitution matrix

**Table 2.** Results of HOPS and RIO for the Test Set of Putative Orthologs for *H. sapiens* and *C. elegans*

<i>H. sapiens</i>	<i>C. elegans</i>	Domain	NIFAS (%)	RIO (%)
ACBP_HUMAN	ACBP_CAEL	ACBP	4.5	0
CAPB_HUMAN	CAPB_CAEL	F_actin_cap_B	100	0
CRTC_HUMAN	CRTC_CAEL	Calreticulin	72	0
CUL1_HUMAN	CUL1_CAEL	Cullin	12.5	0
DAD1_HUMAN	DAD1_CAEL	DAD	93.5	49
EAA2_HUMAN	EAA1_CAEL	SDF	62.5	70
EF2K_HUMAN	EF2K_CAEL	MHCK EF2 kinase	100	100
ERH_HUMAN	ERH_CAEL	ER	81.5	0
GRE1_HUMAN	GRPE_CAEL	GrpE	45	32
KC2B_HUMAN	KC2B_CAEL	CK_II_beta	100	20
O43447	CYPB_CAEL	pro_isomerase	8	10
O60573	IFE4_CAEL	IF4E	95.5	89
OM20_HUMAN	OM20_CAEL	MAS20	86.5	51
RNHL_HUMAN	RNHL_CAEL	RNase_HII	91	22
RPB2_HUMAN	RPB2_CAEL	RNA_pol_B	100	31
S61G_HUMAN	S61G_CAEL	SccE	99.5	58
SAHH_HUMAN	SAHH_CAEL	AdoHcyase	80.5	25
TCPA_HUMAN	TCPA_CAEL	cpn60_TCP1	91.5	33
TCPD_HUMAN	TCPD_CAEL	cpn60_TCP1	36	2

**Table 3.** Results of HOPS and RIO for the Test Set of Putative Orthologs for *H. sapiens* and *S. cerevisiae*

<i>H. sapiens</i>	<i>S. cerevisiae</i>	Domain	HOPS (%)	RIO (%)
LSM6_HUMAN	LSM6_YEAST	Sm	0	1
SODC_HUMAN	SODC_YEAST	sodcu	0	0
LSM4_HUMAN	LSM4_YEAST	Sm	0	0
SMD1_HUMAN	SMD1_YEAST	Sm	0	0
SMD2_HUMAN	SMD2_YEAST	Sm	0.5	3
LSM5_HUMAN	LSM5_YEAST	Sm	1	0
LSM3_HUMAN	LSM3_YEAST	Sm	2.5	0
ACBP_HUMAN	ACBP_YEAST	ACBP	4	2
UBCJ_HUMAN	UBC7_YEAST	UQ_con	5	4
RUXF_HUMAN	RUXF_YEAST	Sm	8.5	0
H4_HUMAN	H4_YEAST	Histone	10	0
FAC2_HUMAN	RCE1_YEAST	Abi	12	24
RPCX_HUMAN	RPCX_YEAST	DNA_RNApol_7kD	17	65
CAPB_HUMAN	CAPB_YEAST	F_actin_cap_B	24	36
CCHL_HUMAN	CCHL_YEAST	Cyto_heme_lyase	27	80
CDD_HUMAN	CDD_YEAST	dCMP_cyt_deam	29.5	1
UCR6_HUMAN	UCR7_YEAST	UCR_14kD	31	56
PRSA_HUMAN	PRSA_YEAST	AAA	36.5	—
RS18_HUMAN	RS18_YEAST	Ribosomal_S13	37	2
NOS6_HUMAN	SIK1_YEAST	Nop	40	13
COXG_HUMAN	COXG_YEAST	COX6B	46.5	60
OM20_HUMAN	OM20_YEAST	MAS20	47.5	24
KIME_HUMAN	KIME_YEAST	GHMP_kinases	50	24
RA51_HUMAN	RA51_YEAST	HHH	50.5	14
NOP5_HUMAN	NOP5_YEAST	Nop	52	1
T2D5_HUMAN	T2D5_YEAST	TAF	55	74
TBCA_HUMAN	TBCA_YEAST	TBCA	56.5	16
SNX8_HUMAN	MVP1_YEAST	PX	57.5	84
CP51_HUMAN	CP51_YEAST	p450	59	—
S61G_HUMAN	S61G_YEAST	SecE	63	1
GPT_HUMAN	GPT_YEAST	Glycos_transf_4	64.5	31
OXA1_HUMAN	OXA1_YEAST	60KD_IMP	69	30
LSM2_HUMAN	LSM2_YEAST	Sm	71	2
PHB_HUMAN	PHB_YEAST	Band_7	72.5	11
PSB2_HUMAN	PSB2_YEAST	Proteasome	73	11
RPBX_HUMAN	RPBX_YEAST	RNA_pol_N	75.5	23
MPPA_HUMAN	MPPA_YEAST	Peptidase_M16	77.5	84
IF2A_HUMAN	IF2A_YEAST	S1	77.5	49
RPB9_HUMAN	RPB9_YEAST	RNA_POL_M_15KD	78	96
FOLC_HUMAN	FOLE_YEAST	Mur_ligase_C	78	65
PSA1_HUMAN	PSA1_YEAST	Proteasome	78	45
PRS4_HUMAN	PRS4_YEAST	AAA	78.5	—
PR11_HUMAN	PR11_YEAST	DNA_primase_5	78.5	1
DHYS_HUMAN	DHYS_YEAST	DS	79	18
GRE1_HUMAN	GRPE_YEAST	GrpE	82.5	24
FAC1_HUMAN	ST24_YEAST	Peptidase_M48	83	2
ORN_HUMAN	ORN_YEAST	Exonuclease	83	1
HEMZ_HUMAN	HEMZ_YEAST	Ferrochelatase	84	97
RNHL_HUMAN	RNHL_YEAST	RNase_Hll	84	7
PSB1_HUMAN	PSB1_YEAST	Proteasome	85.5	9
G CSP_HUMAN	G CSP_YEAST	GDC-P	86	0
PSA6_HUMAN	PSA6_YEAST	Proteasome	86.5	53
PSA4_HUMAN	PSA4_YEAST	Proteasome	86.5	23
DNLI_HUMAN	DNLI_YEAST	DNA_ligase	86.5	0
IF4E_HUMAN	IF4E_YEAST	IF4E	87	2
CY1_HUMAN	CY1_YEAST	Cytochrome_C1	88	21
NTF2_HUMAN	NTF2_YEAST	NTF2	88.5	39
TCPB_HUMAN	TCPB_YEAST	cpn60_TCP1	88.5	0
PRS8_HUMAN	PRS8_YEAST	AAA	89	—
PSB3_HUMAN	PSB3_YEAST	Proteasome	89	38
T2EB_HUMAN	T2EB_YEAST	TFIIE_beta	90	100
VATF_HUMAN	VATF_YEAST	ATP_synt_F	90	18
PSA7_HUMAN	PSA7_YEAST	Proteasome	90.5	20
UBCH_HUMAN	UBC8_YEAST	UQ_con	91	19
KAD2_HUMAN	KAD1_YEAST	Adenylatekinase	91.5	80
MD21_HUMAN	MAD2_YEAST	HORMA	91.5	7
T2DB_HUMAN	T2DB_YEAST	TFIID-18	92	83
RANG_HUMAN	YRB1_YEAST	Ran_BP1	92	82
MSRA_HUMAN	MSRA_YEAST	PMSR	92	0
UFD1_HUMAN	UFD1_YEAST	UFD1	93	67

(continued)

**Table 3.** Continued

<i>H. sapiens</i>	<i>S. cerevisiae</i>	Domain	HOPS (%)	RIO (%)
KCY_HUMAN	UMP_K_YEAST	Adenylatekinase	94	20
VAOD_HUMAN	VAOD_YEAST	vATP-synt_AC39	94.5	—
ATPO_HUMAN	ATPO_YEAST	OSCP	94.5	6
RPB6_HUMAN	RPB6_YEAST	RNA_pol_Rpb6	94.5	6
XPB_HUMAN	RA25_YEAST	Helicase_C	95	—
RCL1_HUMAN	RCL1_YEAST	RTC	96.5	17
PRS7_HUMAN	PRS7_YEAST	AAA	97	—
COX_X_HUMAN	COX_X_YEAST	UbiA	97	45
PSA3_HUMAN	PSA3_YEAST	Proteasome	97	1
PRS6_HUMAN	PRS6_YEAST	AAA	98	—
T2EA_HUMAN	T2EA_YEAST	1FII_alpha	98	66
TCPA_HUMAN	TCPA_YEAST	cpn60_TCP1	98	52
RUXG_HUMAN	RUXG_YEAST	Sm	98	41
PSB4_HUMAN	PSB4_YEAST	Proteasome	98	13
PSA5_HUMAN	PSA5_YEAST	Proteasome	98	1
COPB_HUMAN	COPB_YEAST	Adaptin_N	98.5	1
COX_A_HUMAN	COX_A_YEAST	COX5A	99	40
TCPD_HUMAN	TCPD_YEAST	cpn60_TCP1	99	29
ATPG_HUMAN	ATPG_YEAST	ATP-synt	99.5	96
TCPH_HUMAN	TCPH_YEAST	cpn60_TCP1	99.5	20
PSA2_HUMAN	PSA2_YEAST	Proteasome	99.5	8
TCPG_HUMAN	TCPG_YEAST	cpn60_TCP1	99.5	7
T2FB_HUMAN	T2FB_YEAST	TFIIF_beta	100	100
BLMH_HUMAN	BLH1_YEAST	Pept_C1-like	100	100
PURA_HUMAN	PURA_YEAST	Adenylsucc_synt	100	98
XPA_HUMAN	RA14_YEAST	XPA	100	80
NFS1_HUMAN	NFS1_YEAST	Aminotran_5	100	68
E2BA_HUMAN	E2BA_YEAST	IF-2B	100	61
MCM4_HUMAN	CC54_YEAST	MCM	100	60
MCM2_HUMAN	MCM2_YEAST	MCM	100	53
RPB2_HUMAN	RPB2_YEAST	RNA_pol_B	100	51
TCPQ_HUMAN	TCPQ_YEAST	cpn60_TCP1	100	4

A “—” indicates no results reported.

used was Jones–Taylor–Thornton. The trees were built both with homogeneous and heterogeneous evolution among sides, using a  $\gamma$  distribution in the latter case.

### Access to the Data

All data are available at <ftp://ftp.cgb.ki.se/pub/HOPS/>. The file “manually removed” lists protein pairs that show a reciprocal best BLAST score and have experimentally verified function, but were excluded from the test set based on differences in the catalytic activity or the substrate used.

### Comparison With Tree Reconciliation

RIO version 0.3 (Zmasek and Eddy 2002) was chosen for the comparison with a tree reconciliation method. RIO searches Pfam families for orthologs by reconciling the sequences tree with the complete tree of all species. We submitted all human proteins in the test set through the Web interface at <http://www.rio.wustl.edu>, and the resulting ortholog support values for the corresponding ortholog in the test set were taken for the comparison. If a sequence other than the ortholog from the test set showed a higher orthology score, this was marked.

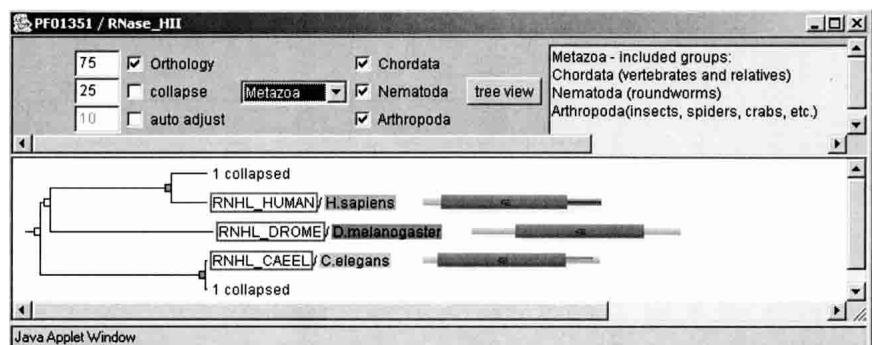
## RESULTS

### Accessing the HOPS Database

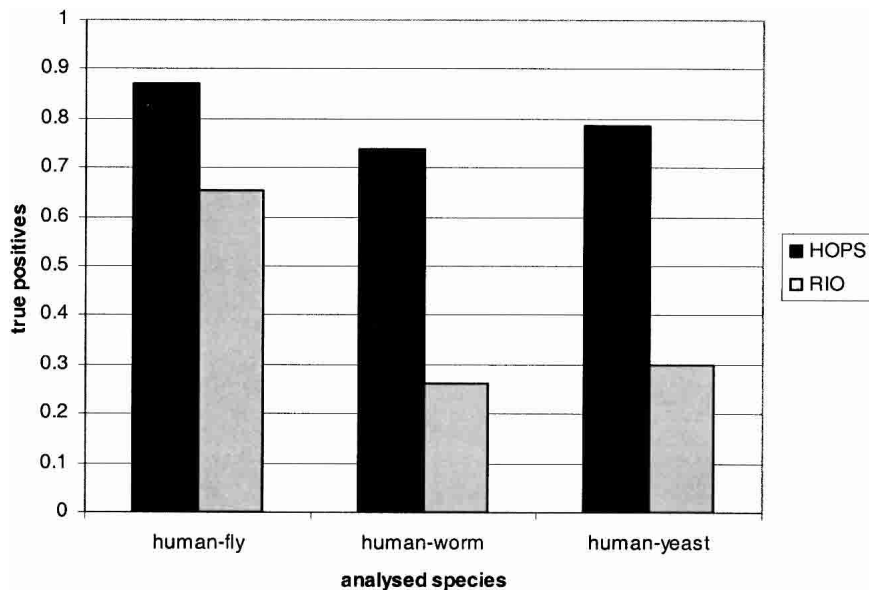
The HOPS database can be accessed either via the NIFAS browser or a standard Internet

browser. NIFAS is a Java applet for viewing phylogenetic trees of domains, connected to schematic graphical representations of the proteins’ domain structure (Storm and Sonnhammer 2001).

The increase of computer power over the last years makes it possible to now provide precalculated neighbor-joining trees with bootstrap support for families of up to 250 sequences and up to 1500 sequences with no bootstrap support. Although the information content of phylogenetic trees of this size with no bootstrap support is at best questionable, it allows viewing the ortholog bootstrap values calculated for the larger families within NIFAS.



**Figure 3** Orthologs for *RNHL\_HUMAN* in HOPS, based on the domain *RNase\_HII*. The orthology between *RNHL\_HUMAN* and *RNHL\_CAEEL* is not found by RIO, because the tree topology for *RNHL\_HUMAN*, *RNHL\_DROME*, and *RNHL\_CAEEL* does not reflect the Ecdysozoa hypothesis (see text for further explanation).



**Figure 4** Performance of HOPS and RIO on the putative orthologs from our test set. A 50% cutoff was used to assign orthology. Shown are the fractions of orthologs from the test set found with HOPS, respectively, RIO. See text for further information.

### NIFAS

The original functionality of NIFAS remains unchanged. A button is added to the navigation bar to change to orthology mode. If no orthology information for a given family is available (because it contains, e.g., only bacterial sequences), this button is deactivated. The new commands of the orthology mode can be seen in Figure 2.

While the orthology navigation panel is shown, a click with the left mouse button on any domain schematic will open a new window. In this window, all proteins are displayed that contain at least one domain with an orthology score above the Orthology value. A right click on a domain schematic will show the same information in the browser window in text format.

The species group of a sequence is shown by color-coding the background of the species name next to the sequence identifier.

Colored boxes around the sequence identifier highlight orthologous relations. Each group of orthologs and paralogs has a different color assigned (Fig. 3). Sometimes the results of the clustering can be ambiguous, especially if clustering sequences from more than two species. In these cases, the boxes for sequences that are grouped in more than one cluster are drawn with multiple colors, one for each cluster to which the sequence is assigned.

### Access Through an Internet Browser

An html page is available at <http://pfam.cgb.ki.se/HOPS/>. Here one can enter a SWISS-PROT/TrEMBL identifier and get a list of its HOPS orthologs. This page shows the same information as right clicking on a domain schematic in NIFAS does, but also includes domain diagrams and annotation.

### Accuracy of the Test Set

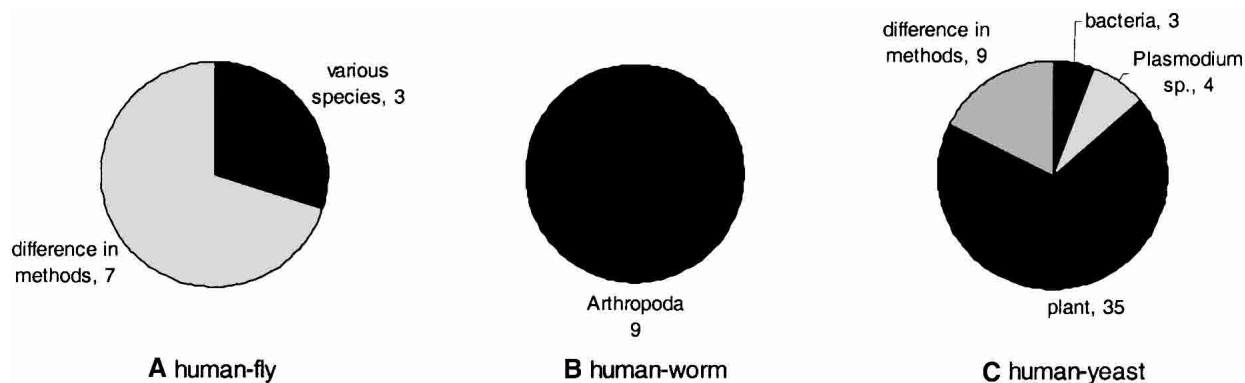
For some genes, both methods fail to find the correct orthologous sequence. An explanation would be that the sequences are not true orthologs. However, for none of these sequences does either HOPS or RIO find a sequence with a higher ortholog bootstrap support, indicating that the test set is correct but contains some very difficult cases.

### Comparing HOPS With RIO

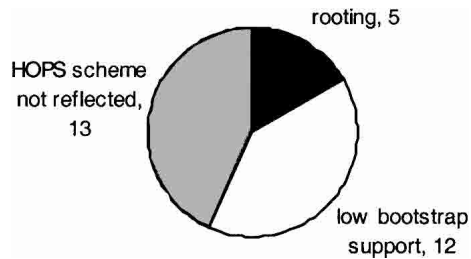
Figure 4 presents a summary of the HOPS–RIO comparison. A detailed list of the results is shown in Tables 1–3. For some families, RIO does not provide orthology scores. These families are not taken into account for calculating the percentage of true positives for RIO in Figure 4.

### Results for the *H. sapiens*–*D. melanogaster* Test Set

The human–fly orthology inference gave the most similar result between HOPS and RIO. Applying a 50% orthology support cutoff for assigning orthology, HOPS found 87.5% of the orthologs in the test set. RIO assigned 65% of the orthologs correctly. In all



**Figure 5** Detailed analysis for the cases in which RIO fails to assign the correct ortholog contrary to HOPS. The labels show the reason, which is usually a sequence from a particular species that prevents RIO from assigning orthology correctly. The cases that could not be traced back to a sequence preventing correct orthology assignment are due to differences in the tree-building method or the inclusion of more sequences by RIO. (A) A total of 10 potential orthologous relations are not found by RIO in the human–fly test set, but are assigned correctly by HOPS. (B) Nine orthologous pairs in the human–worm test set assigned correctly by HOPS are not found by RIO. In all cases, the sequences reflect the Coelomata, not the Ecdysozoa model of evolution (see text for further explanation). (C) Results of the human–yeast comparison, in which 51 orthologous relations found with HOPS are missed by RIO. The majority of the cases (35) are not found by RIO because the human sequence is an outgroup to a plant/yeast clade. In three cases, bacterial sequences break the assignment of orthology by RIO, indicating possible horizontal gene transfers. Four of the orthologous relations are missed by RIO because of *Plasmodium sp.* sequences on the Chordata branch.



**Figure 6** Analysis of the 30 orthologous relations in the test set HOPS fails to find. The labels describe the reason for the misassignment. Of the 13 cases in which the HOPS clustering scheme is not reflected, eight go back to a single-domain family, the SM domain. Here the *S. cerevisiae* orthologs evolved much faster than the corresponding *S. pombe* ortholog. This leads to a tree in which only the *S. pombe* sequence is assigned as orthologous, but not the *S. cerevisiae* sequence. Other reasons are wrong rooting (five cases) of the tree and general low bootstrap support for the whole sequence family.

cases when only HOPS found the ortholog, RIO gave a score  $>0$ . As indicated in Figure 5A, most of these orthologs were given a lower bootstrap support by RIO because more sequences were included or because of other differences in the tree-building method. In general, larger trees tend to have lower bootstrap support than smaller trees (Zharkikh and Li 1995).

#### Results for the *H. sapiens*–*C. elegans* Test Set

The outcome for the human–worm analysis was quite different. Based on a 50% orthology support cutoff, HOPS assigns 74% of the orthologs correctly. RIO, on the other hand, found 26% of the orthologs in the test set. In 26% of the cases, RIO gave a score of 0 to a sequence pair that was orthologous by HOPS.

An analysis of the corresponding sequence trees shows why orthology inference with a complete species tree as done by RIO fails to find most of the orthologs (Fig. 5B). In these cases, the trees do not follow the “Ecdysozoa” phylogeny in the species tree used by RIO, which places *C. elegans* in a clade with arthropods. The fly sequences in these trees are in the same clade as the *H. sapiens* sequences, and the nematode sequences are basal to both—representing the “Coelomata” hypothesis (Fig. 3).

In this combination of gene and species tree, the tree reconciliation algorithm will assign the human and the worm sequence to be paralogous. In HOPS, the fly sequences are not included for the human–worm analysis; therefore, the orthologous sequence is correctly assigned.

#### Results for the *H. sapiens*–*S. cerevisiae* Test Set

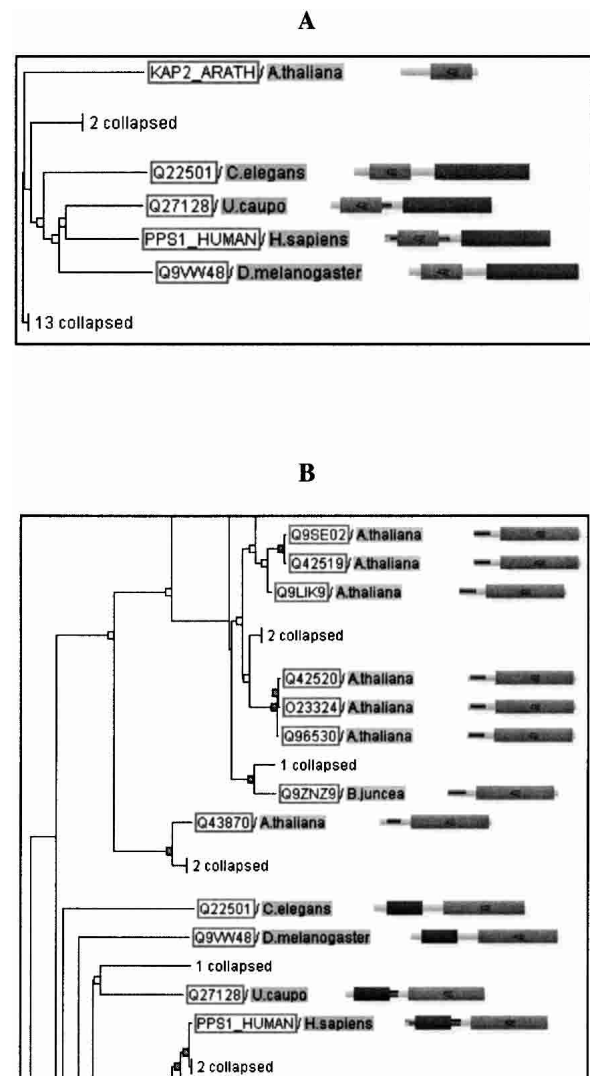
In the human–yeast analysis, HOPS assigned 78% of the orthologs correctly, RIO 31%. The reasons for this difference are summarized in Figure 5C. In 68% of these cases, the sequence trees did not reflect the species tree used by RIO, which places plants as an outgroup to a fungi/metazoa clade. For the human–yeast analysis, horizontal transfer of genes into intracellular parasites prevents tree reconciliation with a complete species tree from finding the correct ortholog. In 14% of the missed orthologous relations, bacterial or *Plasmodium sp.* sequences were on a branch with either yeast or human, thus breaking the orthology assignment.

#### False Negatives in HOPS

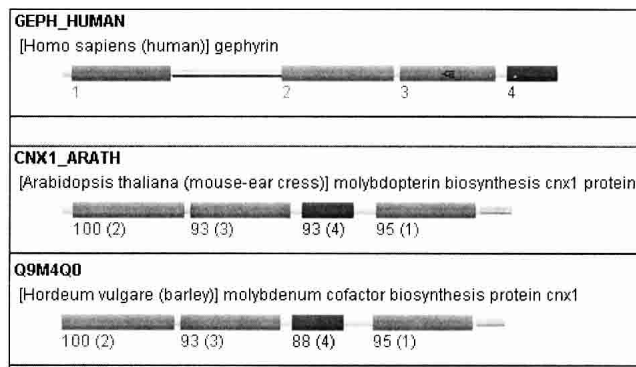
Figure 6 summarizes the cases in which HOPS failed to find the correct ortholog from the test set (all of these orthologous relations were also not found by RIO). In nearly one-fifth of the cases, this was caused by rooting problems. This can happen in small domain families with varying rates of evolution among the

lineages. Then the mid-tree rooting method used might place the root in the wrong branch.

Nearly all (12 out of 13) of the cases in which the HOPS clustering scheme is not reflected by the gene tree come from a misplaced *Schizosaccharomyces pombe* sequence. More than 60% (eight out of 13) of these cases are observed in a single domain family (the SM domain). For instance, in the phylogenetic tree for the SM family, the *S. cerevisiae* LSM6\_YEAST sequence appears as an outgroup to an *S. pombe* + metazoan clade. Therefore, both methods assign orthology between the *S. pombe* and the human LSM6 but not between *S. cerevisiae* and human. Trees reconstructed with MrBayes (data not shown) give the same results. This indicates that this behavior does not originate from an error



**Figure 7** Detecting gene fusion. (A) Shown are selected members of the APS\_kinase family (seed alignment) with orthologous assignments between the Viridiplantae and the Metazoa group. The proteins of the two clades are orthologous to each other. The *A. thaliana* protein (KAP2\_ARATH) consists of one domain, APS\_KINASE. The proteins in the metazoan clade all have an additional domain, ATP\_Surylase. (B) The same metazoan proteins are shown as in A, but this time with the orthologous assignments displayed for the ATP\_Surylase domain (full alignment, here the ATP\_Surylase domain has a tree icon). Because both domains in the metazoan proteins have orthologous single-domain proteins in *A. thaliana*, this indicates a gene fusion in an early metazoan ancestor that lead to the bifunctional metazoan proteins.



**Figure 8** Analyzing orthology for multidomain proteins. Displayed are the two highest scoring orthologs for *GEPH\_HUMAN*: *CNX1\_ARATH* and *Q9M4Q0*. The numbers below the domains are the ortholog bootstrap values (%). The number in brackets after the score indicates the corresponding orthologous domain in *GEPH\_HUMAN*. Although the order of the domains is different, all domains show a high orthology score to the corresponding domain in *GEPH\_HUMAN*.

specific to the tree reconstruction method used in HOPS. For the SM family, the *S. cerevisiae* sequences show an elevated rate of evolution. In combination with the relative shortness of the alignment (111 residues), this might explain why none of the tree methods reconstructs the correct tree: a monophyletic *S. pombe* and *S. cerevisiae* branch basal to the metazoan clade.

In the remaining cases, orthology was not assigned because of generally low bootstrap support for the whole family. The alignment simply does not contain enough phylogenetic information to reconstruct a reliable phylogenetic tree. In other words, the ratio (informative sites)/(sequence number) is too small. This results in low ortholog bootstrap values.

### Investigating Gene Fusion

The HOPS database can be used to analyze evolution of domain structure between orthologs. Figure 7 shows an example of genes with different domain structures in plants and metazoa. The metazoan proteins consist of two domains and are bifunctional, with APS-kinase (Pfam: PF01583) and ATP-sulfurylase (Pfam: PF01747) activity. Both domains have orthologous single-domain proteins in *Arabidopsis thaliana*. This indicates that the metazoan proteins were created by a gene-fusion event. This happened most likely in an early metazoan ancestor, as all metazoan homologs have both domains, and no nonmetazoan proteins are known with this particular dual-domain architecture. However, a set of fungal proteins has the same domains in the inverted order. Only one domain (ATP-sulfurylase) is orthologous to the metazoan proteins in HOPS, however, indicating two independent domain accretion events in the metazoan and fungal lineages. Oddly, a bacterial protein from *Aquifex aeolicus* (SWISS-PROT: O67174/SATC\_AQUAE) has the same domain structure as the fungal proteins, and both domains are grouped with fungi in the NIFAS tree (data not shown). This implies a horizontal transfer from fungus to bacteria.

### Orthology Between Multidomain Proteins

Figure 8 shows an example of domain orthology between multidomain proteins. These plant and human molybdopterin biosynthesis proteins have the same domains, but in different order. Despite this, all domains are orthologous. A molybdopterin binding domain (Pfam: PF00994) is found N-terminally in chordates, whereas it is C-terminal in plants. The same domain is found N-terminally in arthropods as well, whereas in nematodes the four-domain combination does not appear. Instead, a single-

domain nematode protein is the closest relative to the additional PF00994 domain (see Fig. 9). It thus appears that this domain was inserted early in the metazoan lineage. What is unusual here is that the additional PF00994 domain in plants and chordates appears orthologous (ortholog bootstrap support = 95%), but the arthropod domain is placed as an outgroup and is thus not orthologous to either the plant or chordate domain.

There could be several reasons for this observation. Assuming that horizontal transfer between chordates and plants is ruled out, it can be explained by independent recombination events of paralogous copies of the PF00994 domain in each of the arthropod, chordate, and plant lineages. Given that the plant protein has a different domain order, it must have happened independently in the plant lineage. A scenario in which the arthropod recombination selected a paralogous copy of the PF00994 domain present early in the metazoan lineage, whereas the plant and chordate recombinations both selected the same (orthologous) copy, and the other copy was lost in all lineages, would be consistent with the present tree. On the other hand, if horizontal transfer of a single domain from chordates to plants were biologically feasible, this would be an alternative explanation. It would be interesting to know if the chordate and plant proteins have evolved into identical functions despite the different domain architecture.

## DISCUSSION

### Conclusion

The HOPS/RIO comparison shows that the simplified HOPS approach has a higher sensitivity for finding orthologs than classical tree reconciliation with a complete species tree.

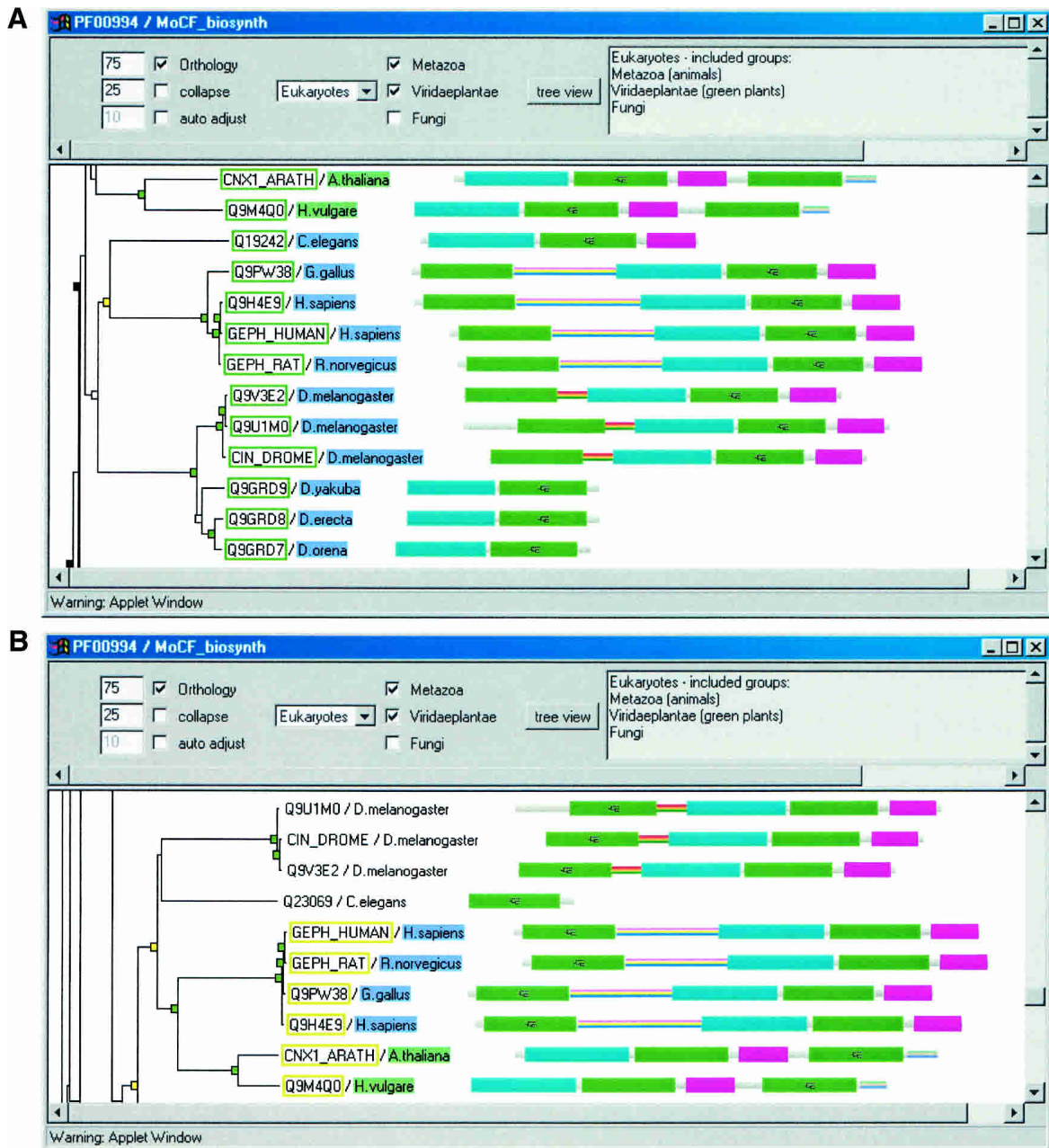
Orthology assignments are done on the domain level by HOPS. The orthologous relations can be viewed graphically in NIFAS. This combination provides a comprehensive and user-friendly analysis not only of orthologous relations but also of gene fusion and domain rearrangements. The two examples of orthology between genes with different domain architecture (Figs. 7–9) demonstrate the potential of HOPS to study the mechanism of domain rearrangements.

Additionally, these examples show that with increasing evolutionary distance, domain rearrangements and gene fusion can become an issue for assigning orthology. Any approach not taking into account the modular architecture of the proteins would fail to extract all orthologous information in the given examples.

The results of this study indicate that there is a limit to how much phylogenetic information can be included sensibly for finding orthologs. Up to a certain point, including additional information will improve the results. For instance, if one only analyzes sequences from two species for orthology, this can lead to a situation in which a paralog is incorrectly taken for an ortholog. This will happen if the true ortholog was lost or is not sequenced yet. Including sequences from additional species in the analysis would increase the chance that at least one true ortholog is present in the same clade. Tree-based methods like HOPS and RIO will then assign orthology and paralogy correctly.

But the lower success rate of RIO for finding orthologs between *H. sapiens* and *C. elegans* shows that the inclusion of additional sequences can have the opposite effect. Here the inclusion of all species in the analysis, especially arthropod sequences, prevents the tree-reconciling algorithm from finding the correct orthologs.

Even if the Coelomata hypothesis were used instead of the Ecdysozoa hypothesis in combination with tree reconciliation, this would not solve the problem. The investigated sequences are rather short, compared with full-length proteins. Therefore a tree reconstructed from them is more prone to “statistical fluxes” in



**Figure 9** Orthologous domains in multidomain proteins. Most molybdopterin biosynthesis proteins in metazoa and plants contain four domains (two molybdopterin-binding domains, PF00994 [green], and one PF03453 [blue] and PF03453 [red] domain). This domain arrangement appears to have been constructed by joining a common cassette of PF00994, PF03453, and PF03453 to another copy of PF00994. The three-domain cassette is intact, and all domains are orthologous in the same order. (A) Metazoa–plant orthologs of the central domain of the cassette. (B) Orthology of the additional PF00994 domain. Oddly, here only the chordate lineage of metazoa appears orthologous to the plant domain. The *Drosophila* protein CIN\_DROME cannot be considered orthologous to GEPH\_HUMAN in this domain despite having the same domain architecture. This indicates that the N-terminal domain in the arthropod and metazoan proteins derive from paralogous domains, which were added to the cassette independently in each lineage. The additional PF00994 domain was also added independently in the plant protein CNX1\_ARATH, but here the orthologous domain was used.

evolution. Assigning orthology based on speciation events that followed as close in time as for *D. melanogaster*, *H. sapiens*, and *C. elegans* is bound to fail for a high fraction of sequences. The only viable approach is to say that the exact grouping of these three species is unknown.

For the assignment of orthology in HOPS, we try to find the right balance between including too little and too much phylogenetic information. This is not always possible. If there is only one sequence for each lineage present in a family and no se-

quence from an outgroup species is available, HOPS would assign these sequences as orthologous. However, it is possible that these sequences are not orthologous, but paralogous. This would be the case if the true orthologs were lost.

We would like to point out that although RIO was used for the comparison with tree reconciliation, the program was not specifically designed to solve the problem of finding all orthologs between two species. Rather, the idea of RIO is to find orthologs in any species to a given query sequence. In most of the examples

in which RIO fails to find the ortholog pair on which we have focused, it typically does report an ortholog in some other species. We expect RIO to have a lower rate of false positives than HOPS. This expectation is based on RIO's stringent application of all available phylogenetic information from the species tree. However, estimating the false-positive rate is very hard, if not impossible. In the absence of the true ortholog, one cannot reliably say that two sequences that appear orthologous in a tree are not. In cases in which the true ortholog exists, both RIO and HOPS have very low false-positive rates, especially compared with cases in which many different species are clustered together (Remm et al. 2001), as in, for example, COGs (Tatusov et al. 1997).

Applying more advanced phylogenetic methods will allow including additional phylogenetic information in the analysis. The HOPS clustering scheme is set up in a way that it can handle ambiguity in the species tree and some errors encountered in neighbor-joining tree reconstruction. But the clustering scheme could easily be adjusted to include more phylogenetic information from the species tree in case a more advanced method for the tree reconstruction would be used.

At the moment, a large-scale analysis using, for instance, trees constructed in a Bayesian framework would not be practical. Calculating the tree for only a small protein family can easily take more than a day with MrBayes. In contrast, the computation of all trees and inference of orthology for all 3735 families done in this paper took less than a week on a 6 CPU UltraSPARC III system. With the increase of computational processing power, especially the increase due to parallel systems like Beowulf clusters, it will be possible to use advanced algorithms and methods for finding orthologs in the near future.

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