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Letter to the editor

New insight into the evolution of aquaporins from flowering plants and vertebrates: Orthologous identification and functional transfer is possible

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ABSTRACT

Aquaporins (AQPs) represent a family of channel proteins that transport water and/or small solutes across cell membranes in the three domains of life. In all previous phylogenetic analysis of aquaporin, trees constructed using proteins with very low amino acid identity (<15%) were incongruent with rRNA data. In this work, restricting the evolutionary study of aquaporins to proteins with high amino acid identity (>25%), we showed congruence between AQPs and organismal trees. On the basis of this analysis, we defined 19 orthologous gene clusters in flowering plant species (3 PIP-like, 7 TIP-like, 6 NIP-like and 3 SIP-like). We described specific conserved motifs for each subfamily and each cluster, which were used to develop a method for automatic classification. Analysis of amino acid identity between orthologous monocotyledon and dicotyledon AQPs from each cluster, suggested that PIPs are under high evolutionary constraint. The phylogenetic analysis allowed us the assignment of orthologous aquaporins for very distant animal lineages (tetrapods-fishes). We also demonstrated that the location of all vertebrate AQPs in the ortholog clusters could be predicted by comparing their amino acid identity with human AQPs. We defined four AQP subfamilies in animals: AQP1-like, AQP8-like, AQP3-like and AQP11-like. Phylogenetic analysis showed that the four animal AQPs subfamilies are related with PIP-like, TIP-like, NIP-like and SIP-like subfamilies, respectively. Thus, this analysis would allow the prediction of individual AQPs function on the basis of orthologous genes from *Arabidopsis thaliana* and *Homo sapiens*.

1. Introduction

Aquaporins (AQPs) comprise a diverse family of channel proteins, which transport water and small solutes across cell membranes in Bacteria, Eukarya and Archaea. Since their discovery in 1992 (Preston et al., 1992), much progress has been made in understanding the phylogenetic relationships between aquaporins and their classification (Johanson et al., 2001; Zardoya and Villalba, 2001; Zardoya, 2005). The unveiling of AQP structure has contributed to a deep understanding of water and solute transport (Walz and Fujiyoshi, 2009). Aquaporins have six transmembrane helices and two additional membrane embedded domains. The amino- and carboxy-terminal halves show sequence similarity to each other and are arranged as tandem repeats, apparently originated from the duplication of a half-sized gene (Quigley et al., 2002; Reizer et al., 1993). Each half of the molecule bears one hydrophobic loop, which includes two highly conserved NPA motives (Asn–Pro–Ala) (Johanson et al., 2001; Park and Saier, 1996). A third motif was found to be common to all family members: AEF (Ala–Glu–Phe) (Zardoya and Villalba, 2001). These three motifs have been used for AQP classification.

Independently of the kingdom, the entire aquaporin field shares the interest in clarifying biological roles, understanding mechanisms of action and studying their subcellular localization. Interestingly, the greatest AQP family diversification occurred in vertebrates and plants (Zardoya, 2005). Considering the increasing availability of sequenced genomes, integration of the animal and plant information now becomes an interesting challenge.

For the last ten years many studies have discussed the nomenclature of plant aquaporins and many efforts have been focused on proposing a consistent nomenclature (Johanson et al., 2001). As a result of these attempts plant AQPs were classified into seven subfamilies: PIPs (plasma membrane intrinsic proteins), TIPs (tonoplast intrinsic proteins), NIPs (NOD26-like intrinsic proteins), SIPs (small basic intrinsic proteins), XIPs (x intrinsic proteins), HIPs (hybrid intrinsic proteins) and GIPs

Abbreviations: AQPS, aquaporins; PIPs, plasma membrane intrinsic proteins; TIPs, tonoplast intrinsic proteins; NIPs, NOD26-like intrinsic proteins; SIPs, small basic intrinsic proteins; XIPs, x intrinsic proteins; HIPs, hybrid intrinsic proteins; GIPs, GlpF-like intrinsic proteins; PhaA, thiolase II; PhaB, reductase; PhaC, polyhydroxybutyrate polymerase; AACT, acetoacetyl-CoA thiolase; NJ, neighbor-joining; ME, minimum evolution; MP, maximum parsimony; ER, endoplasmic reticulum; PM, plasma membrane.

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(GlpF-like intrinsic proteins) (Danielson and Johanson, 2008; Johanson and Gustavsson, 2002; Gupta and Sankararamakrishnan, 2009; Wallace and Roberts, 2004). However, to correctly understand the mechanisms underlying AQP diversification, their specialized functions and tissue distribution, a robust phylogenetic framework is mandatory. Moreover, due to the recent discovery of aquaporins, it is expected that modification in the phylogenetic analysis of AQPs be still on course. The growing databases have allowed an explosion of information in a very short period of time and the discovery of new aquaporins is still a growing list. In the case of plant aquaporins, gene redundancy has complicated the classification even more.

Orthologous proteins in different species are expected to have similar biochemical function and biological role. The robust method from finding orthologs is based on the analysis of phylogenetic trees. For orthologous assignment, the gene trees have to be congruent (same topology) with the species tree. Previous studies suggested that all proteins with MIP functional domain from Bacteria and Eukarya are truly homolog despite their very low amino acid identity (<15%). Under this assumption, AQPs from extremely distant taxa were included in the same phylogenetic tree, obtaining incongruence between AQPs and organismal trees (Zardoya and Villalba, 2001; Zardoya, 2005). Unfortunately, these incongruent patterns were interpreted as an intrinsic feature of the AQP family, and the possibility of non-homologous proteins or proteins with too much evolutionary distance was ruled out. However it is possible to reorganize the framework by selecting only sequence with high amino acid identity and moreover, contrast this new phylogenetic analysis with the available experimental information seeking for consistencies. As an example, we have been reported a shift from incongruence (Kadouri et al., 2005; Peretó et al., 2005; Rehm, 2003) to congruence (Ayub et al., 2007; Soto et al., 2011) when the phylogenetic analysis of PhaA, PhaB, PhaC and AACT was restricted to proteins with high amino acid identity (>25%). In addition, the equivalent activity and regulation of orthologous proteins described by phylogenetic analysis were experimentally demonstrated (Ayub et al., 2006, 2009; Soto et al., 2011). We also used this strict criterion to identify *At*TIP5;1 orthologous proteins from *Hordeum vulgate*, *Zea mays* and *Oryza sativa* (Soto et al., 2010). Interestingly, in this cluster of orthologous protein an export signaling to mitochondria and a motif sequence coding for pH regulation were shown, both confirmed experimentally (Soto et al., 2010).

In this paper we therefore explored the phylogeny of aquaporins restricting the analysis to proteins with high amino acid identity (>25%). By this strict criterion we showed for the first time congruence between AQPs and organismal trees. The advantage of this new perspective is that its congruence allows us to define clusters of orthologous genes for both flowering plants and vertebrates. Furthermore, we described specific conserved motifs for each orthologous cluster useful for automatic assignment of orthologs. It is interesting to highlight that with the new AQP phylogenetic framework for flowering plants and vertebrates, the putative function of individual AQPs could be predicted on the basis of orthologous genes from *Arabidopsis thaliana* and *Homo sapiens*.

2. Methods

Aquaporin protein sequences of *A. thaliana* and *H. sapiens* were used as query to search against all available complete eukaryotic genomic databases in NCBI with protein annotation in GenBank with 15% amino acid identity as cut-off to get candidate homologs. Sequence search was performed by using BLASTP tool. Phylogenetic trees performed in this work were restricted to *Ricinus communis*, *Hevea brasiliensis*, *Populus trichocarpa*, *Glycine max*, *A. thaliana*, *Hordeum vulgare*, *O. sativa*, *Z. mays*, *Mus musculus*, *Rattus norvergicus*, *H. sapiens*, *Gallus gallus* and *Danio rerio* species belonging to plant and animal groups. Protein identity calculations were performed using MatGAT v2.02 (Campanella et al., 2003). In order to avoid pseudogenes or mutant aberrant alleles, protein sequences were aligned using ClustalW program and Bioedit Sequence Alignment Editor (Hall, 1999; Tamura et al., 2007). From an exhaustive analysis of the sequence differences, protein alignment was observed manually. Phylogenetic and molecular evolutionary analyses were conducted by using MEGA version 4.0 (Tamura et al., 2007). History reconstruction of plant AQPs was restricted to protein sequences with high amino acid identity (>25%). Phylogenetic trees were constructed using the neighbor-joining (NJ) method with genetic distances computed using Poisson correction model. This analysis was developed by setting the following parameters: substitutions to include = all, gaps/missing data = pair wise deletion, phylogeny test = bootstrap 500 replicates and root on midpoint. NJ method finds pairs of operational taxonomic units (called OTUs or neighbors) that minimize the total branch length at each stage of clustering of OTUs starting with a starlike tree (Saitou and Nei 1987). The main advantage of NJ method is that the branch lengths and the topology of a parsimonious tree can rapidly be obtained. Orthologous gene clusters identified by using NJ method were confirmed by using Minimum evolution (ME) and Maximum parsimony methods (MP). NJ trees were shown with bootstrap values for NJ analyses. In addition, we observed that bootstrap values for each cluster exceed 50% in ME and MP studies. We scanned subfamilies and clusters motifs by using Multiple Em for Elicitation 4.4.0 tool (Bailey and Elkan, 1994). Motif search for automatic classification of aquaporins, were performed by using Scanprosite program (Swiss Institute of Bioinformatics, http://www.expasy.org/tools/scanprosite). Analysis of evolutionary constraint of plant AQPs was performed by comparing three or four sequences of mono and dicots. Sequences selected from monocots and dicots belonging to *O. sativa* or *Z. mays* and *Ricinus communis*, *Hevea brasiliensis*, *Populus trichocarpa*, *Glycine max* or *A. thaliana*, respectively. These pairs of sequences analyzed were randomly selected, excluding products of the protein sequences belonging to TIPCLI, NIPCLII, NIPCLV and SIPCLII, which are not encoded by monocots sequences.

3. Results and discussion

3.1. Orthologous assignment in flowering plants and vertebrates AQPs

In order to perform a phylogenetic study of flowering plants and vertebrates AQPs, we restricted the analysis to well-characterized sequenced species belonging to flowering plant and vertebrate groups using proteins with high amino acid identity (>25%). Therefore we included all members of the subfamilies PIPs, TIPs, NIPs and SIPs from flowering plants and aquaporins and aquaglyceroporins from vertebrates but not members of the divergent subfamilies XIP, GIP and HIP from plants described recently (Danielson and Johanson, 2008; Gupta and Sankararamakrishnan, 2009) since they all failed to meet that requirement.

In flowering plants, we constructed one tree for each subfamily, except for PIPs where two trees were built. Based on their amino acidic identity and cluster organization in phylogenetic trees, PIPs are usually organized in two groups, PIP1 and PIP2 (Chaumont et al., 2001). Because alignment of the proteins of the classical PIP1 and PIP2 groups showed gaps and inserts that distorted the alignment (Fig. S1), we did not include all PIPs in the same tree despite the fact that the requirement of amino acid identity was satisfied (Phillips, 2006). Therefore, we constructed five phylogenetic trees for flowering plant AQPs: PIPs1, PIPs2, TIPs, NIPs and SIPs with 19 distinct ortholog clusters: 3 of PIPs, 7 of TIPs, 6 of NIPs and 3 of SIPs (Fig. 1). We consider correct to assign them as “ortholog clusters” as the topology of each AQP cluster (Figs. 1a, b, c and d) is congruent to the topology of the organismal tree (Fig. 1e). All orthologous gene clusters are completely novel, with no counterpart even in any previous phylogenetic analysis of AQPs. For better clarity, we compared orthologous gene clusters with the current nomenclature of Arabidopsis (Fig. 1).

Fig. 1a shows that the PIP Cluster I (PIPCLI) corresponded to the classical PIP1 group, while PIP Cluster II (PIPCLII) and PIP Cluster III (PIPCLIII) to the PIP2 group (Fig. 1b). Our analysis suggests that the common ancestor of mono and dicots have three types of PIPs that originated the clusters PIPCLI, PIPCLII and PIPCLIII.

Because an exhaustive search in protein NCBI database failed to find orthologous genes for AtPIP2;5, AtPIP2;6, OsPIP2;7 and OsPIP2;8 we included them in a separate cluster; this suggests that ancestral genes that gave rise to these genes evolved in a divergent form with respect to their orthologs. Interestingly, AtPIP2;5, AtPIP2;6, OsPIP2;7

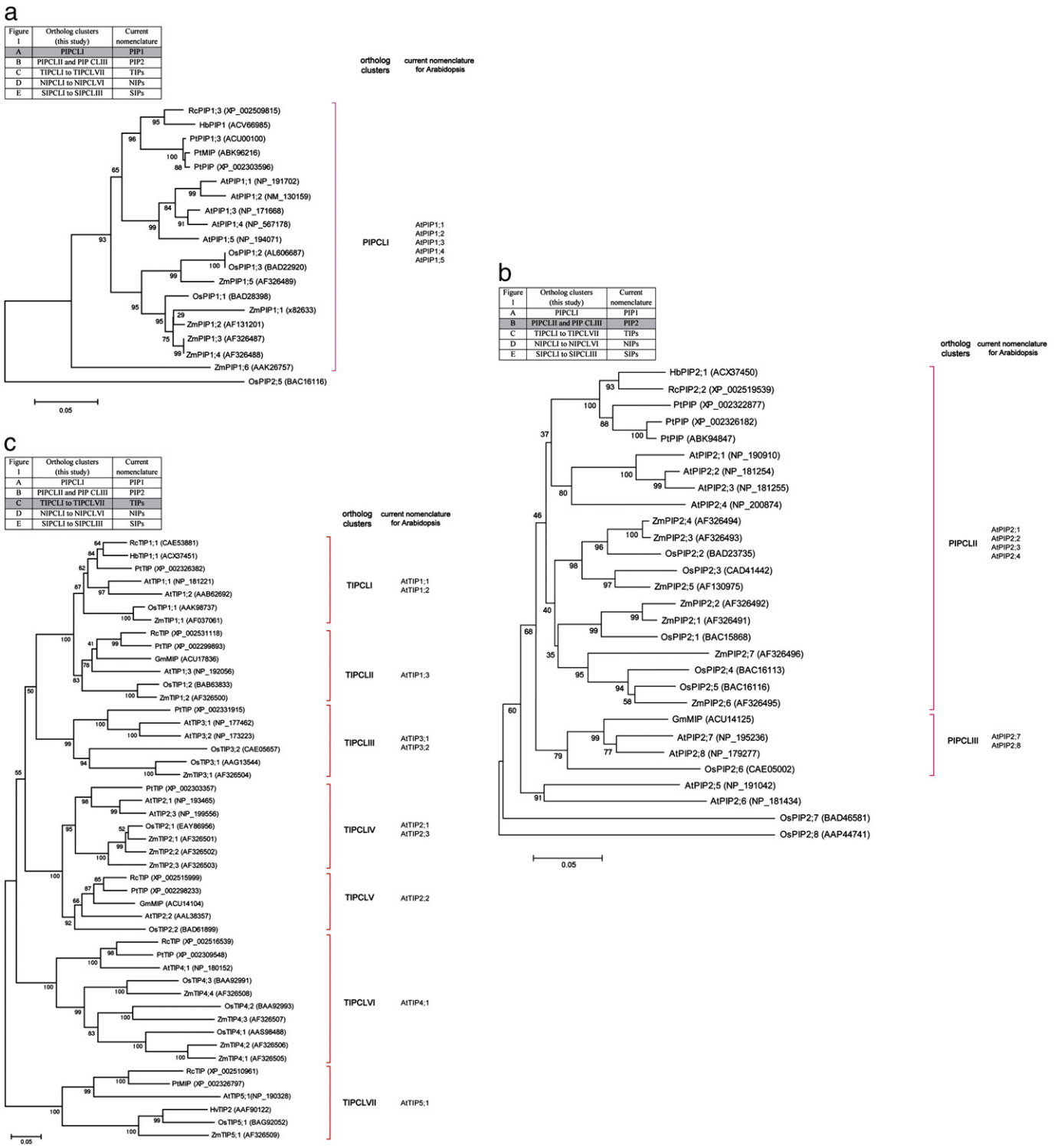


Fig. 1. Phylogeny of AQPs in plants. Phylogenetic trees of PIPs1 (a), PIPs2 (b), TIPs (c), NIPs (d), SIPs (e) and organism (f) from representative taxa based on NJ method. Bootstrap percentages are indicated at the branch points. Orthologous gene clusters (CL) and current nomenclature of Arabidopsis are found on right. Tree topology obtained using NJ method, Minimum evolution and Maximum parsimony methods were identical.

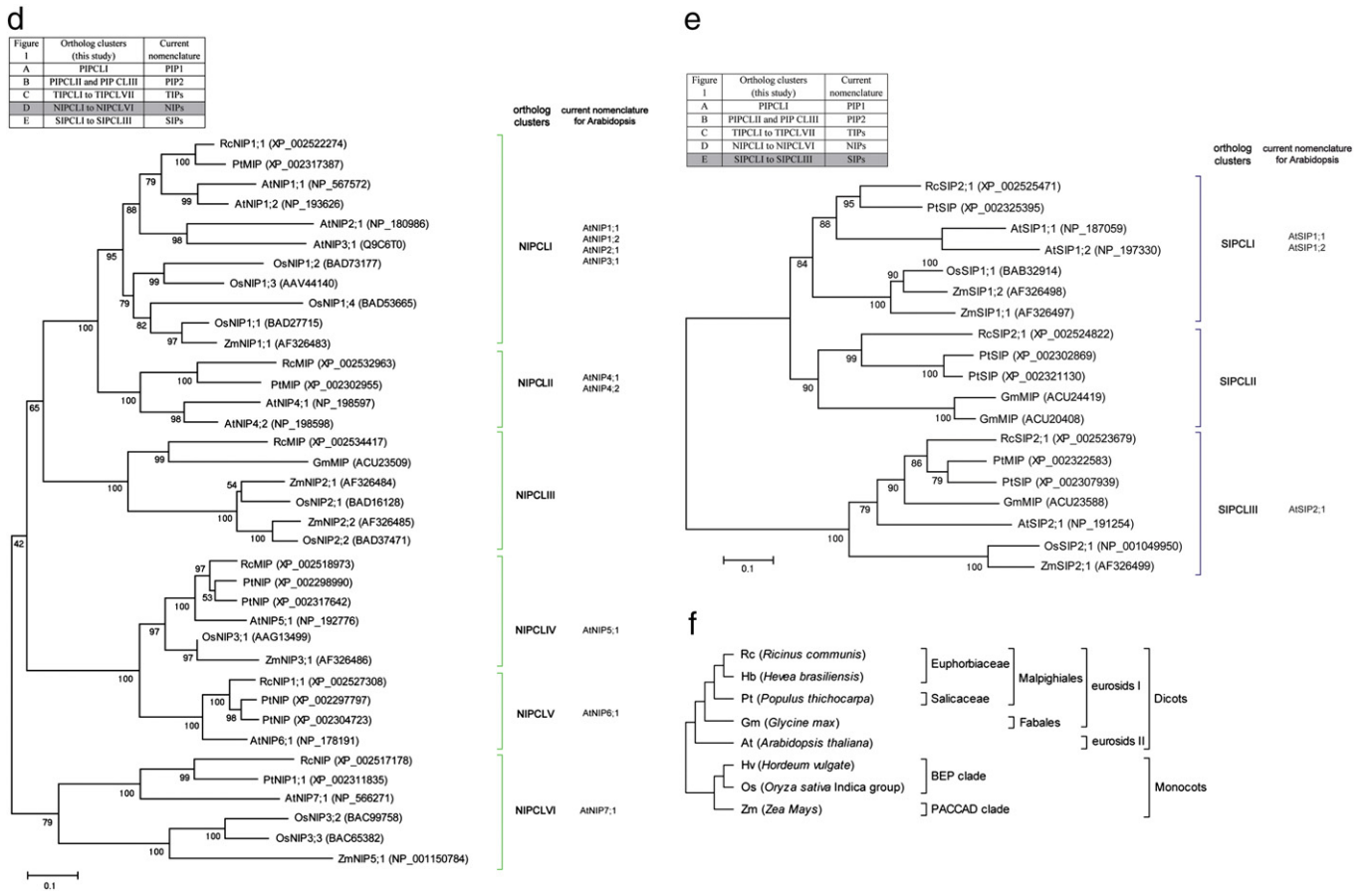


Fig. 1 (*continued*).

and OsPIP2;8 have shown unusual functions such as adaptation to abiotic stress (Alexandersson et al., 2010; Li et al., 2008a,b; Matsumoto et al., 2009).

TIPs analysis showed 7 clusters and not 5 as stated in the classical nomenclature (Johanson et al., 2001). Fig. 1c showed that the classical TIP1 to TIP5 groups are consistent according to their bootstrap values; however, according to the clustering criteria mentioned above, TIP1 and TIP2 members were split into two separate but closely related clusters, CLI and CLII for TIP1 and CLIV and CLV for TIP2.

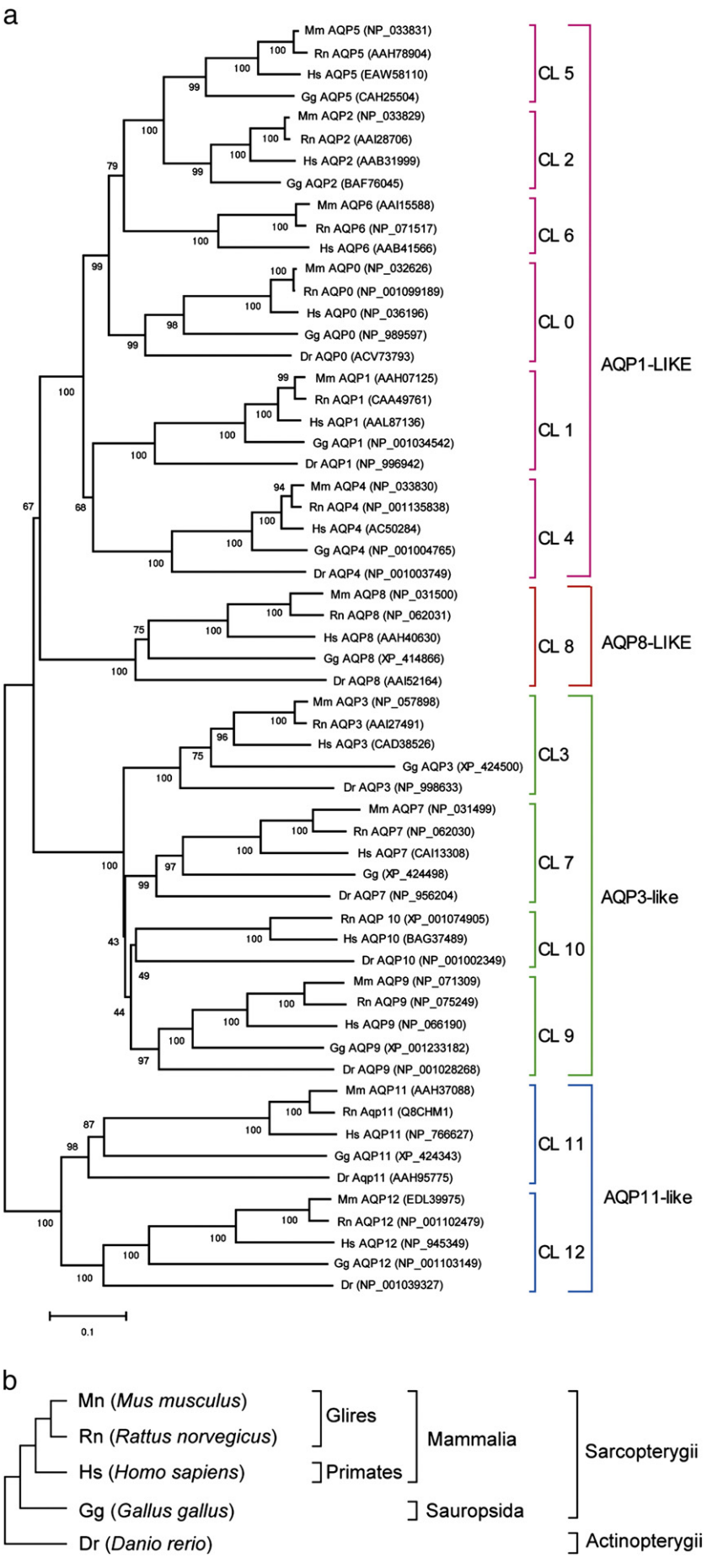
The NIPs subfamily was divided into 6 clusters (Fig. 1d), unlike the previous classification that grouped them into 7 different clusters (Johanson et al., 2001). The SIP subfamily showed 3 clusters instead of 2 (Fig. 1e). It is interesting to mention that the SIPCLII and NIPCLIII were the only clusters with proteins that are not present in *A. thaliana* (Figs. 1d and e).

In addition, phylogenetic relationships between flowering plant AQPs can be established with different depths for each cluster. Some clusters showed several duplication events occurred in some species (e.g. PIPCLII,) while other clusters revealed only one protein from each species (e.g. TIPCLVII). In the first case, this result suggested that AtPIP2;1, AtPIP2;2, AtPIP2;3 and AtPIP2;4 from Arabidopsis are equally related to OsPIP2;1, OsPIP2;2, OsPIP2;3, OsPIP2;4, and OsPIP2;5 from rice (Fig. 1b). In the second case, the absence of paralogous genes within TIPCLII showed that there is only one gene in the rice genome (OsTIP1;2) evolutionary equivalent to AtTIP1;3 from Arabidopsis (Fig. 1c). The fact that Arabidopsis has 17 of the 19 clusters of orthologous genes of flowering plant AQPs suggests that it is possible to extrapolate the function of Arabidopsis AQPs to any plant AQP.

In vertebrates, AQP tree is completely consistent with the organismal tree (Fig. 2). Unlike plants, it was possible to include all animal AQPs even from evolutionarily very distant organisms such as mammals-birds (about 300 million years) and tetrapods-fishes (about 400 million years), with no distortion in the evolutionary reconstruction. This difference with plant AQPs could be attributed to the low evolutionary rate and gene duplication described for animals (Hasegawa et al., 2003; Hoshiyama et al., 2001).

Animal AQPs have usually been divided into two subfamilies: the orthodox group associated with transport of water (including AQP0, AQP1, AQP2, AQP4 and AQP5), (Ishibashi et al., 2009) and the aquaglyceroporins that mediate the transport of water and/or small, uncharged solutes; including AQP3 which transports water and glycerol (Echevarria et al., 1994; Ishibashi et al., 1994), AQP7 and AQP10 which transport water, glycerol and urea (Ishibashi et al., 1997, 2002) and finally AQP9 which transports water, glycerol, urea and other solutes (Tsukaguchi et al., 1998). AQP8, AQP11 and AQP12 have been described as AQPs, but their classification is under discussion. AQP8 is often included as an orthodox aquaporin (Zardoya, 2005) while AQP11 and AQP12 are known as super-aquaporins (Ishibashi, 2006). Fig. 2 showed that there are 4 vertebrate

Fig. 2. Phylogeny of AQPs in vertebrate animals. Phylogenetic tree of animal AQPs (a) and organism (b) from representative taxa based on NJ method. Bootstrap percentages are indicated at the branch points. Orthologous gene clusters (CL) and AQP subfamilies (AQPX-like) are found on right. Tree topology obtained using NJ method, Minimum evolution and Maximum parsimony methods were identical.


a
Mm AQP5 (NP_033831)
Rn AQP5 (AAH78904)
Hs AQP5 (EAW58110)
Gg AQP5 (CAH25504)
Mm AQP2 (NP_033829)
Rn AQP2 (AAI28706)
Hs AQP2 (AAB31999)
Gg AQP2 (BAF76045)
Mm AQP6 (AAI15588)
Rn AQP6 (NP_071517)
Hs AQP6 (AAB41566)
Mm AQP0 (NP_032626)
Rn AQP0 (NP_001099189)
Hs AQP0 (NP_036196)
Gg AQP0 (NP_989597)
Dr AQP0 (ACV73793)
Mm AQP1 (AAH07125)
Rn AQP1 (CAA49761)
Hs AQP1 (AAL87136)
Gg AQP1 (NP_001034542)
Dr AQP1 (NP_996942)
Mm AQP4 (NP_033830)
Rn AQP4 (NP_001135838)
Hs AQP4 (AC50284)
Gg AQP4 (NP_001004765)
Dr AQP4 (NP_001003749)
Mm AQP8 (NP_031500)
Rn AQP8 (NP_062031)
Hs AQP8 (AAH40630)
Gg AQP8 (XP_414866)
Dr AQP8 (AAI52164)
Mm AQP3 (NP_057898)
Rn AQP3 (AAI27491)
Hs AQP3 (CAD38526)
Gg AQP3 (XP_424500)
Dr AQP3 (NP_998633)
Mm AQP7 (NP_031499)
Rn AQP7 (NP_062030)
Hs AQP7 (CAI13308)
Gg (XP_424498)
Dr AQP7 (NP_956204)
Rn AQP 10 (XP_001074905)
Hs AQP10 (BAG37489)
Dr AQP10 (NP_001002349)
Mm AQP9 (NP_071309)
Rn AQP9 (NP_075249)
Hs AQP9 (NP_066190)
Gg AQP9 (XP_001233182)
Dr AQP9 (NP_001028268)
Mm AQP11 (AAH37088)
Rn Aqp11 (Q8CHM1)
Hs AQP11 (NP_766627)
Gg AQP11 (XP_424343)
Dr Aqp11 (AAH95775)
Mm AQP12 (EDL39975)
Rn AQP12 (NP_001102479)
Hs AQP12 (NP_945349)
Gg AQP12 (NP_001103149)
Dr (NP_001039327)
CL 5
CL 2
CL 6
CL 0
CL 1
CL 4
CL 8
CL3
CL 7
CL 10
CL 9
CL 11
CL 12
AQP1-LIKE
AQP8-LIKE
AQP3-like
AQP11-like
0.1
b
Mn (Mus musculus)
Rn (Rattus norvegicus)
Hs (Homo sapiens)
Gg (Gallus gallus)
Dr (Danio rerio)
Glires
Primates
Mammalia
Sauropsida
Sarcopterygii
Actinopterygii

AQP subfamilies: AQP1-like, AQP8-like, AQP3-like and AQP11-like. These subfamilies have been also suggested in a recent phylogenetic analysis (Danielson and Johanson, 2010). Our phylogenetic analysis is consistent with the null hypothesis (vertical transfer) suggesting that all animal AQPs are truly homologous. Contrary to plants, it is not necessary to describe new clusters of orthologous animal AQP genes. All vertebrate AQPs have their orthologous in humans (Fig. 2a) and the location of all vertebrate AQPs in the clusters can be predicted by comparing their amino acid identity with human AQPs (Table S1). Fig. 2a showed that AQP2 and AQP5 appeared in tetrapods while AQP6 in mammals. AQP10 was lost in bird's linage since it is present in mammals and fishes.

3.2. Identification of motifs in plant AQPs

In order to evaluate the possibility of quickly identifying flowering plant AQP subfamilies and their orthologous clusters, we analyzed the amino acid identity of all AQPs used in phylogenetic trees with respect to Arabidopsis AQPs (Table S1). The location in clusters of 20% of flowering plant AQPs is not predictable by the amino acid identity with respect to Arabidopsis AQPs. For example, the amino acid identity of ZmTIP2;2 (cluster TIPCLIV) compared to AtTIP2;1, AtTIP2;3 of the same cluster and to AtTIP2;2 of the cluster TIPCLV was 69.4, 71.0 and 74.1%, respectively.

Assessment of the correct orthology requires rigorous and time-consuming phylogenetic analyses of individual genes. We proposed here an alternative automatic approach. We found differential conserved motifs for each subfamily and for each cluster of flowering plant AQPs (Fig. 3a). These motifs were found in the "N-t AEF" and "NPA domains" regions both in TIPs, NIPs and SIPs (Zardoya and Villalba, 2001). N-terminal motifs can be distinguished in PIPCLI, PIPCLII and PIPCLIII, potentially involved in PIPs subcellular localization (Postaire et al., 2010; Zelazny et al., 2009). Despite the functional relevance of these motifs, they could be used to automatically classify flowering plant AQPs. For this, we subjected a comparison of the AEF and NPA motifs in a random sample of 48 AQPs analyzing the phylogenetic reconstruction. Table S1 showed that these two motifs were able to predict 100% of the subfamilies and 96% of the clusters. Fig. 3b showed four examples for the automatic assignment of orthologs using motifs. It is important to note that in all cases, motif predictions coincided with the phylogenetic tree location (Table S1). Our analyses showed that 36% of the proteins chosen at random (Table S1), have not been assigned yet to a particular subfamily. Thus, these motifs could be used to work in a more accurate AQP classification.

3.3. Evolutionary constraint on plant AQPs

Recent work has determined that NIP-like AQPs had high divergence in function and expression during evolution (Liu et al., 2009). Nevertheless, evolutionary constraint on flowering plants AQP family have not been studied due to incongruence between organismal and AQPs phylogenetic trees. The time divergence between orthologous AQPs from monocots and dicots for each cluster is coincident with the time these lineages have diverged. Thus, we compared the percentage of amino acid identity among monocot and dicot AQPs for each cluster as an estimate of the evolution rate. Fig. 4 showed three different constraint sets: high ($77.7 \pm 3.2\%$) in PIPCLI, PIPCLII, PIPCLIII, TIPCLIV, TIPCLV, TIPCLII and NIPCLIV clusters; medium ($50.7 \pm 3.1\%$) in TIPCLIII, NIPCLI, NIPCLIII, TIPCLVI, TIPCLVII, SIPCLI and SIPCLIII clusters; and low (29%) in the cluster NIPCLVI. While the three PIP clusters are homogeneous in terms of sequence identity, the other subfamilies showed different identity ranges for their clusters, suggesting that PIPs have a greater evolutionary constraint compared with other subfamilies. Previously, two hypotheses have been proposed to explain the high identity of PIPs: i — PIPs have a high evolutionary constraint or ii — PIPs have been recently emerged (Zardoya and Villalba, 2001). The identification and analysis of orthologous gene clusters presented here and the presence of PIP group in *Physcomitrella patens* (Danielson and Johanson, 2008) support the first hypothesis.

The high evolutionary constraint of PIPs may be due to functional constraint. PIP subfamily has some members that transport different molecules suggesting that high transport selectivity is not a particular characteristic of this subfamily. However, the high evolutionary constraint can be related to the reported physical interaction that occurs between different members of the subfamily and that module their activity. It has been described that proteins that are part of complexes tend to evolve at a relatively slow rate, in order to improve the co-evolution with their interacting partners (Mintseris and Weng, 2005). Interestingly, physical interaction between different aquaporins has been only described for PIPs. Co-expression of two different PIPs in the same cell showed higher water fluxes than the expression of any of them alone. Co-expression analysis was reported for *Z. mays* PIPs, for VvPIP1;1 (PIPCLI) with VvPIP2;2 (PIPCLIII), for tobacco NtPIP1;1 (PIPCLI) with NtPIP 2;1 (PIPCLIII), and for red beet BvPIP1;1 (PIPCLI) with BvPIP2;2 (PIPCLII) confirming that the physical interaction occurs in all the three PIP clusters (Bellati et al., 2010; Bots et al., 2005; Cavez et al., 2009; Fetter et al., 2004; Mahdieh et al., 2008; Vandeleur et al., 2009; Zelazny et al., 2009). Further studies will be necessary to analyze physical interactions in members of the clusters TIPCLII, TIPCLIV, TIPCLV, and NIPCLIV, which also showed low evolution rate.

3.4. Evolutionary relationship between plant and animal AQPs

The high evolutionary rate of plant AQPs excludes the possibility of building a single tree containing all animal and plant AQPs. Nevertheless, taking advantage of the low evolutionary rate of animal AQPs it is possible to analyze the evolutionary relationship between animal and flowering plant AQPs. Fig. 5 showed individual phylogenetic trees of each subfamily of flowering plant AQPs within the animal AQPs tree. Interestingly, we found that each AQP plant subfamily can be grouped with each subfamily of animal AQPs. PIP-like were associated with AQP1-like subfamily, TIP-like with AQP8-like subfamily, NIP-

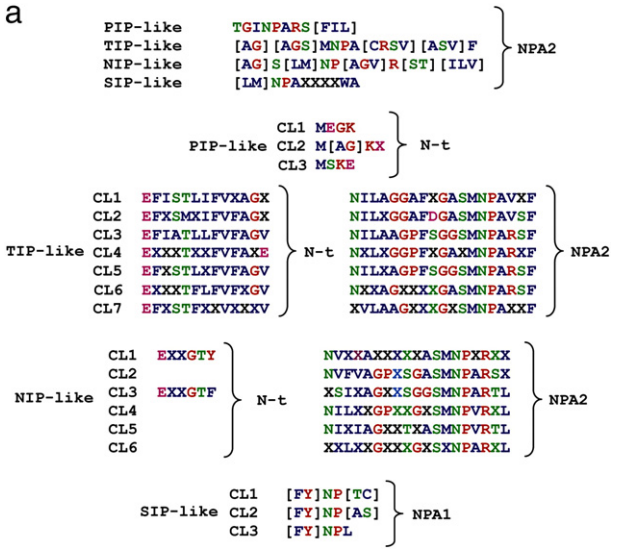


Fig. 3. Motif identification of plant AQPs. (a) In this figure we showed amino acid motifs for each subfamily (PIPs, TIPs, NIPs and SIPs) and each cluster (CL), as described previously in phylogenetic trees. On the right, we showed the region in which each motif is located. The letter X represents similar amino acids. Blue, fuchsia, green, pink and red letters represents hydrophobic (ACFILVWM), large hydrophobic (FIWLM), polar (NQST), negative (DE) and positive (KRHGPY) aminoacids, respectively. (b) In this figure we showed the use of motifs for orthologous genes assignment. Motifs of four plant AQPs were evaluated in sequences selected at random and contrasted with phylogenetic tree location. Vv: *Vitis vinifera*, Mt: *Medicago truncatula* and Bo: *Brassica oleracea*.

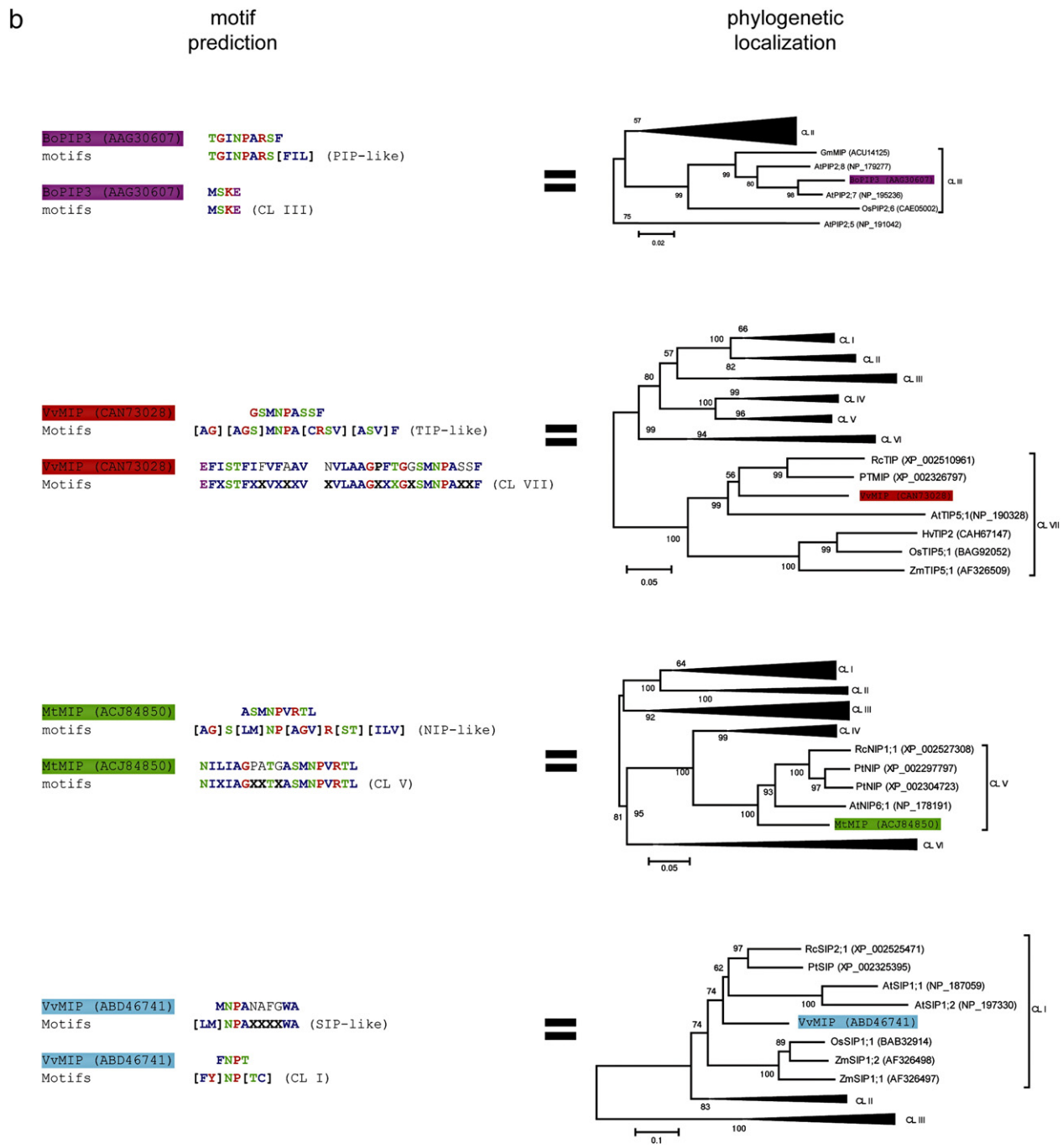


Fig. 3 (*continued*).

like with AQP3-like subfamily and SIP-like were more related with AQP11-like subfamily. These results suggest that the eukaryotic common ancestor of plant and animal AQPs had at least four subfamilies: A (PIP-like and AQP1-like), B (TIP-like and AQP8-like), C (NIP-like and AQP3-like) and D (SIP-like and AQP11-like) (Fig. 6). Recently, it was proposed that GIPs are the most similar plant MIPs to the AQP3-like cluster (Danielson and Johanson, 2010). Contrarily, we observed that AQP3-like is closer to NIP-like (Fig. S2). These contrasting results show that the phylogenetic reconstruction of aquaporins is heavily dependent on protein selection criteria used to make phylogenetic trees.

In previous phylogenetic analysis of AQPs, trees constructed using proteins from distant taxa (Bacteria and Eukaya domains) were incongruent with rRNA data (Danielson and Johanson, 2010; Heymann and Engel, 1999; Johanson et al., 2001; Quigley et al., 2002; Zardoya et al., 2002). For example, the glycerol facilitator from *Escherichia coli* (EcGlpF) and bacterial NIP-like proteins were associated with AQP3-like and plant NIPs, respectively (Danielson and Johanson, 2010; Park and Saier, 1996; Zardoya et al., 2002). These relationships are supported strongly by statistics (Danielson and Johanson, 2010; Park and Saier, 1996). Nevertheless, an unexpected position of a protein within a phylogenetic tree may also be explained by gene duplication, lineage-specific gene loss events (Koonin, 2003) and high amino acidic distances (Abby et al., 2010; Andersson, 2005). Moreover, it is important to mention that obtaining a strongly supported tree does not necessarily mean that the tree is correct; one should be aware that an incorrect tree can receive strong statistical support if the method used does not correctly handle properties of the data (Delsuc et al., 2005). Thus, the

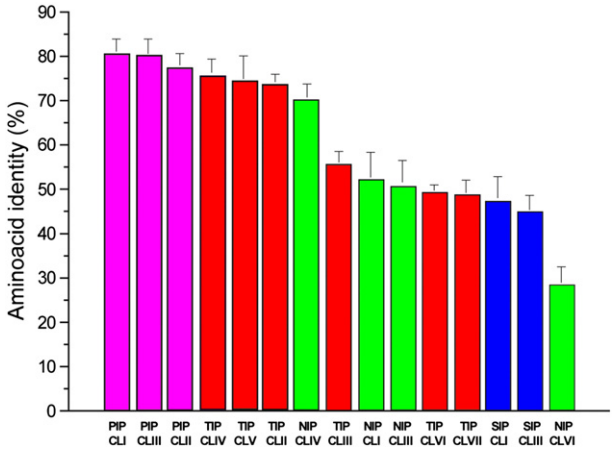


Fig. 4. Analysis of evolutionary constraint in plant AQPs. In order to estimate the evolutionary constrain in plant AQPs, we compared the percentage of amino acid identity among monocots and dicots within each orthologous gene clusters. Values represent media ± SD of triplicate or quadruplicate measures.

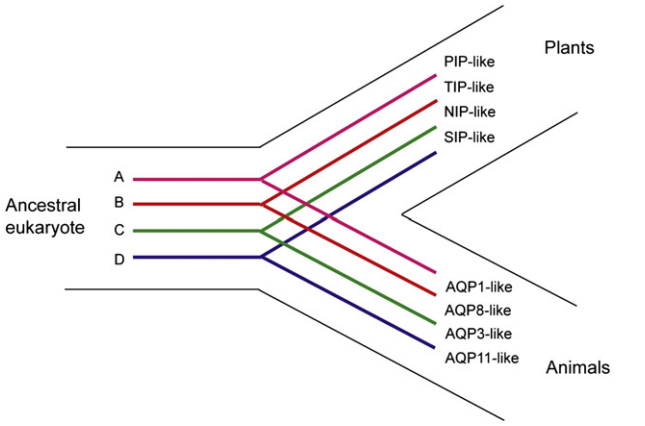


Fig. 6. The evolution of the MIP superfamily in flowering plants and vertebrates animals. A hypothetical ancestral eukaryote which has at last four AQP subfamilies is described in this scheme.

horizontal transfer of AQPs between Bacteria and Eukaya domains could be an artifact. To avoid overestimation of horizontal gene transfer, a general congruence with the organismal tree must be observed, except for the transfer event. It is also necessary to find independent evidence such as localization within genomic islands (Ayub et al., 2007) or take advantage of powerful algorithms specifically developed for statistical support of gene transfer events (Abby et al., 2010).

The analysis performed in this work was restricted to well-characterized species belonging to flowering plants and vertebrates, then, we report a general pattern of vertical transfer (Figs. 1, 2 and 5). It is known that the number of possible trees grows exponentially with the number of proteins (Li, 1997); 210 proteins were analyzed in this work, then, the probability that the congruent pattern is by chance is practically null.

3.5. Correlation between evolution analysis and functional data

In order to integrate evolutionary information reported in this work with functional data, we analyzed the putative ancestral features of each AQP subfamily. We summarized the most relevant functional data found for each cluster in Table 1 and showed the hypothetical ancestral functions in Fig. 7.

Subfamily A (PIP-like and AQP1-like): The putative ancestral characteristic shared by AQPs belonging to the subfamily A is the ability to transport water (Fig. 7). Both in animals and plants different water transport activities have been reported, showing a varied rate of water transport. Regarding solute transport, transport of CO_2 was detected for both animal AQP1 and plant NtAQP1 (PIPCLI) (Endeward et al., 2006; Nakhoul et al., 1998; Uehlein et al., 2008), possibly being an ancestral feature for the subfamily A (Fig. 7). Anions, glycerol and hydrogen peroxide transport was observed in some members of this subfamily (Table 1).

Subfamily B (TIP-like and AQP8-like): This subfamily B showed at least two putative ancestral characteristics, transport of water and urea. This kind of transport was found in AQP8 (mammals and fish) and almost in all plant TIP-like clusters (Fig. 7). Recently, we demonstrated that AtTIP5;1 is a urea transporter that is located in pollen mitochondria and related to pollen nitrogen recycling (Soto et al., 2008, 2010). Based on these results we proposed that AtTIP5;1 is involved to the efflux of urea from mitochondria during the urea

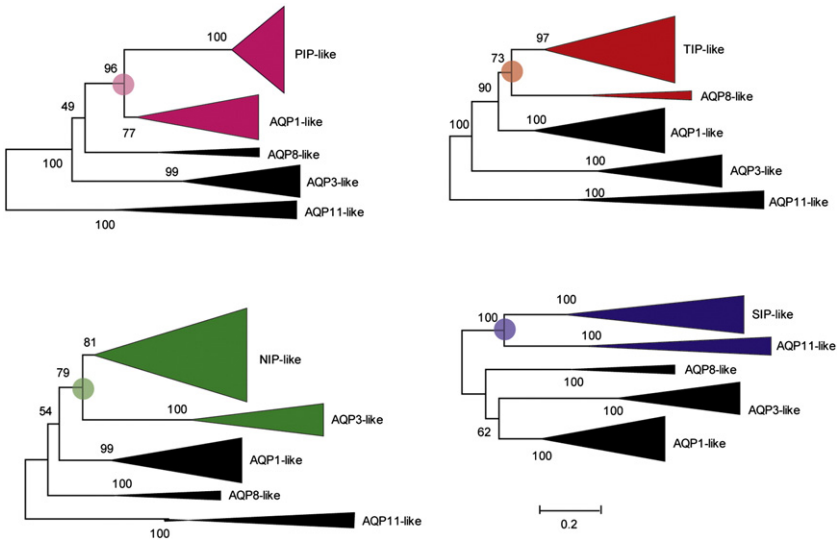


Fig. 5. Phylogenetic relationship between flowering plant and vertebrate animal AQP subfamilies. Vertebrate AQP tree was used as framework to study the relationship between plant and animal AQP subfamilies. In each vertebrate AQP tree, we include a subfamily of plant AQPs. Animal and plant subfamilies are found on the right. Tree topology obtained using NJ method, Minimum evolution and Maximum parsimony methods were identical.

Table 1
Functional features of ortholog gene clusters in each AQP subfamily. H_2O: water. H_2O_2: hydrogen peroxide. CO_2: carbon dioxide. GLY: glycerol. NH_3: ammonia. As (III): arsenic. Bo: boric acid. (−): anion.

Subfamily (localization)	CL	Name	Transport								Reference
			H_2O	H_2O_2	CO_2	Urea	GLY	NH_3	$As_{(III)}$	Bo	-	
A (Mainly in PM, retention in ER, Chloroplast)	1	NtAQP1										Biela et al. 1999; Uehlein et al. 2008
	1	PIPs										Chaumont et al. 2001
	2	AtPIP2;1										Dynowski et al. 2008
	2	ZmPIP2;1										Fetter et al. 2004; Zelazny et al. 2009
	3	VvPIP2;2										Vandeleur et al. 2009
	3	NtPIP2;1										Bots et al. 2005
	0	AQP0										Verkman and Mitra 2000
	1	AQP1										Endeward et al. 2006; Preston et al. 1992
	2	AQP2										Fushimi et al. 1993
	4	AQP4										Verkman and Mitra 2000
	5	AQP5										Ma et al. 1999
	6	AQP6										Yasui et al. 1999
B (Tonoplast, Mitochondria)	1	AtTIP1;1										Bienert et al., 2007; Klebl et al., 2003; Liu et al., 2003; Loqué et al., 2005
	2	OsTIP1;2										Li et al., 2008a,b
	2	AtTIP1;3										Soto et al. 2008
	3	OsTIP3;2										Li et al., 2008a,b
	4	AtTIP2;1										Holm et al. 2005; Klebl et al. 2003; Liu et al. 2003
	4	AtTIP2;3										Bienert et al. 2007; Holm et al. 2005
	5	TaTIP2;1										Bertl and Kaldenhoff 2007; Jahn et al. 2004
	6	OsTIP4;1										Li et al., 2008a,b
	6	AtTIP4;1										Liu et al. 2003
	7	AtTIP5;1										Soto et al., 2008, 2010
	8	AQP8										Holm et al. 2005; Ma et al., 1997; Tingaud-Sequeira et al., 2010
C (Mainly in PM)	1	AtNIP1;1										Kamiya et al. 2009; Weig and Jakob 2000
	1	GmNOD26										Dean et al., 1999; Kaldenhoff and Fischer, 2006; Niemietz and Tyerman, 2000
	2	AtNIP4;1										Soto et al. 2008
	3	OsNIP2;1										Li et al. 2009
	4	AtNIP5;1										Kamiya et al. 2009; Takano et al. 2006
	4	LjNIP5;1										Bienert et al. 2008
	5	AtNIP6;1										Bienert et al. 2008; Tanaka et al. 2008
	6	AtNIP7;1										Isayenkov and Maathuis 2008
	3	DrAQP3										Hamdi et al. 2009
	3	HsAQP3										Liu et al. 2004
	3	MmAQP3										Ma et al. 2002
	7	HsAQP7										Liu et al. 2004
	7	MmAQP7										Ishibashi et al. 2002; Tsukaguchi et al. 1998
	9	RnAQP9										Tsukaguchi et al. 1998
	9	DrAQP9										Hamdi et al. 2009
	10	DrAQP10										Hamdi et al. 2009
	10	HsAQP10										Liu et al. 2004
	10	MmAQP10										Ishibashi et al. 2002; Tsukaguchi et al. 1998
D (ER)	1	AtSIP1;1										Ishikawa et al. 2005
	2	Not studied										
	3	AtSIP2;1										Ishikawa et al. 2005
	11	AQP11										Ishibashi 2009; Morishita et al. 2005
	12	Not studied										Ishibashi 2009

cycle. AtTIP5;1 belongs to most divergent clusters of TIPs (TIPCLVII) suggesting that this function could be ancestral to this group. The urea cycle is also present in animals but the urea transporter in mitochondria is still unknown. Due to urea transport and mitochondrial localization of AQP8 (Gena et al., 2009) it will be interesting to analyze the role of AQP8 in urea cycle. H_2O_2 and NH_3 could also be ancestral features of B subfamily, as these solutes have also been reported as substrates shared by several members of TIP-like and AQP8 (Fig. 7). TIP-like aquaporins that transport glycerol were found within clusters II, III and IV suggesting that transport of glycerol appeared after divergence of clusters I, II, III and IV with clusters V, VI and VII (Fig. 7).

Subfamily C (NIP-like and AQP3-like): Members of the subfamily C, usually called aquaglyceroporins, have been classically associated with bacterial glycerol transporters (EcGlpF). Moreover, it appeared that not only water and glycerol transport could be ancestral features of the subfamily C, but also the transport of metalloid compounds, particularly arsenic (As (III)) (Fig. 7) (Bienert and Jahn, 2010). The ancestral transport of arsenic for NIP subfamily has been previously suggested (Bienert et al., 2008; Ludewig and Dynowski, 2009). Human and rat AQP9 and AQP7 were found to be effective As (III) transporters (Liu et al., 2002, 2004). Recently, AQP3, AQP9 and AQP10 zebrafish orthologs showed that they all have both water and As (III) transport capacity (Hamdi et al., 2009). The arsenic transport is shared for almost all of the NIP-like clusters (Fig. 7). Urea transport was observed only in the animal members of C subfamily, suggesting that urea transport is not an ancestral function of this subfamily. Boric acid transport was observed in members of clusters IV and V which are intimately related respectively (Fig. 7), suggesting that boric acid transport arose before the gene duplication that gave rise to the ancestral gene of both clusters.

Subfamily D (SIP-like and AQP11-like): The members of the subfamily D have been grouped together as a super family of AQPs named S-aquaporins by structural features and by the low identity with respect to the other aquaporins (Ishibashi, 2006). In all D subfamily members the first NPA motif is changed. As discussed previously, the

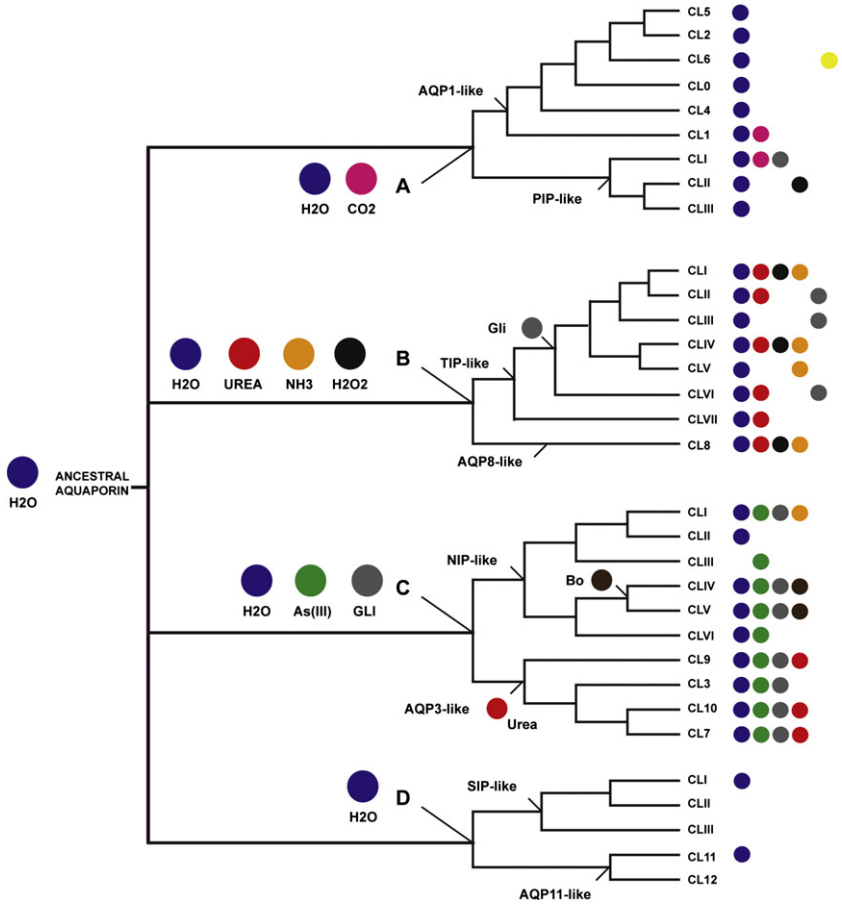


Fig. 7. Putative ancestral functions of AQP subfamilies. A schematic representation of putative ancestral features of aquaporins based on evolutionary and functional data is summarized in Table 1. Large and medium color circles indicate the position where each feature appeared in AQP evolution. Small color (right) circles indicate clusters where each transport feature is presented. Blue circle: water transport. Red circle: urea transport. Orange circle: NH_3 transport. Fuchsia circle: CO_2 transport. Black circle: H_2O_2 transport. Green circle: As(III) transport. Gray circle: glycerol transport. Yellow circle: anion transport. Only data about positive AQP transport was considered, results suggesting that a AQP do no transport some solute were avoided.

variation of the NPA motif might directly reflect the substrate specificity and/or velocity of the water transport (Ishibashi, 2006). Moreover, the N-terminal tail of all members of this subfamily is short, characteristic assigned to their intracellular destination (Maeshima and Ishikawa, 2008).

There is very little functional information on their transport capacity because of the difficulty of testing intracellular aquaporins, but it is well established that all the studied members localized to the endoplasmic reticulum (ER) (Ishikawa et al., 2005; Itoh et al., 2005; Morishita et al., 1995). Thus, ER localization together with water transport could be ancestral properties.

Taken together, the only ancestral feature shared by the four subfamilies is the water transport, suggesting that the ancestral AQP that originated the four AQP subfamilies has the capacity of transporting water.

3.6. Nomenclature update based on evolutionary relationships among AQPs

Protein nomenclature should provide information about the named protein and as far as from now, the current nomenclature for aquaporins is somehow confusing regarding this objective. In order to suggest a new aquaporin nomenclature that would reflect the studies here presented, some considerations must be taken: i — in flowering plants, AQP subfamilies are organized considering primarily their cellular localization, even though it has already been demonstrated multiple location within each subfamily (Maurel et al., 2009), ii — functional classification is hard to achieved, since individual proteins that belong to each subfamily are able to transport different molecules, and iii — our results showed that numbers in the classical nomenclature do not represent the evolutionary relationship in plants (Figs. 1a to e) while in animals the classical nomenclature is consistent with human orthologous genes. We believe that the phylogenetic framework shown in our work can help to reevaluate and consider a new classification of flowering plant and animal AQPs mainly based on evolution.

4. Conclusions

In this work, we have analyzed 210 sequences of aquaporin genes demonstrating congruence between AQPs and organismal trees. A total of 32 orthologous clusters, 19 in plants and 13 in animals, were identified. The existence of a congruent phylogenetic tree allowed a study of the evolutionary constraint of AQPs. In this regard, we found that PIP-like aquaporins showed a high functional constrain compared with other plant subfamilies. In addition, we described an automatic method for flowering plant AQP orthologous assignment based on motif identification. As orthologous proteins in different organisms are likely to share same function, the data presented in this work could be used as a starting point in the prediction of function and biological role of AQPs lacking of a functional analysis. In the future, the orthologous alignment can be critical in the identification of motifs related with structural and regulatory functions of AQPs. We propose

that the four AQPs subfamilies described in plants (PIP-like, TIP-like, NIP-like and SIP-like) and animals (AQP1-like, AQP8-like, AQP3-like and AQP11-like) are derived from four ancestral AQPs subfamilies (A–D). Finally, we open the discussion for an update of AQP nomenclature based on evolution.

Supplementary materials related to this article can be found online at http://dx.doi.org/10.1016/j.gene.2012.04.021.

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