**Title:** **Over 100 million years of enzyme evolution**

**underpinning the production of morphine**

**in the Papaveraceae family of flowering plants**

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**Running Title:** Evolution of morphine production in opium poppy

**Short Summary**

Annotated genomes of five benzylisoquinoline alkaloid (BIA) producing species allowed us to conduct gene tree analysis that uncovered the events leading to emergence of major BIA biosynthetic pathways in Ranunculales. Ks estimation of key paralogous pairs revealed that morphine biosynthesis evolved more recently than 18 MYA in the *Papaver* genus. Contrary to previous reports this study indicates that BIA biosynthesis evolved independently in the Ranunculales and Proteales orders.

**Abstract**

Phylogenetic gene tree analysis of whole genomes from five benzylisoquinoline alkaloid (BIA) producing species from the Ranunculales and Proteales orders of flowering plants revealed the sequence and timing of evolutionary events leading to the diversification of these compounds. (*S*)-Reticuline is a pivotal intermediate in the synthesis of many BIAs and our analyses revealed parallel evolution between the two orders, which diverged ~122 MYA. Berberine is present in species across the entire Ranunculales and we found co-evolution of genes essential for production of the protoberberine class. The benzophenanthridine class, which includes the antimicrobial compound sanguinarine, are specific to the Papaveraceae family of Ranunculales and biosynthetic genes emerged after the split with the Ranunculaceae family ~110 MYA but before the split of the three Papaveraceae species used in this study at ~77 MYA. The phthalideisoquinoline noscapine and morphinan class of BIAs are exclusive to the opium poppy lineage. Ks estimation of paralogous pairs indicate that morphine biosynthesis evolved more recently than 18 MYA in the *Papaver* genus. In the preceeding 100 MY gene duplication, neofunctionalisation and recruitment of additional enzyme classes, combined with gene clustering, gene fusion and gene amplification resulted in emergence of medicinally valuable BIAs including morphine and noscapine.

**Keywords:** benzylisoquinoline alkaloids, plant genomes, gene tree analysis, metabolic pathway evolution, gene clusters, gene fusion

**Introduction**

BIAs[[1]](#footnote-1) are a structurally diverse class of tyrosine-derived specialised metabolites occurring primarily in the order Ranunculales (Figure 1). Many of them possess pharmaceutical activities, among which the potent analgesics codeine and morphine belonging to the morphinan subclass of BIAs and the antitussive and anti-cancer noscapine belonging to the phthalideisoquinoline subclass are produced exclusively in opium poppy for the pharmaceutical industry.

The pathways of some common intermediates/end-products and the corresponding genes have been elucidated from a few representative species from the order Ranunculales, providing a blueprint to investigate their evolutionary origins. In addition to these functionally characterized individual genes, draft genome assemblies of five BIA producing species have been recently reported (Ming *et al.,* 2013; Liu *et al.,*2017; Filiault *et al.*,2018; Gui *et al.,* 2018; Hori *et al.,*2018; Guo *et al.*, 2018).

Whole genome sequence assemblies provide the full set of paralogous sequences of gene families for accurate comparison of orthologous relationships between species. They also allow estimation of species divergence times. We performed phylogenetic analyses of the gene families containing functionally characterized genes in combination with the complete paralogue sets from the draft genomes to better understand and date key events in BIA pathway evolution.

The first BIA in the biosynthetic pathway, (*S*)-norcoclaurine, is understood to be formed through the condensation of tyrosine derived dopamine and 4-hydroxyphenylacetaldehyde catalysed by NORCOCLAURINE SYNTHASE (NCS) (Stadler *et al.*, 1989; Samanani and Facchini, 2001). Intricate combinations of reactions including *N*- and *O*-methylations, hydroxylations, carbon-carbon couplings, reductions and acetylations generate the diversity of BIAs found across the Ranunculales. Many structurally distinct subclasses of BIA are derived from (*S*)-reticuline, a 1-benzylisoquinoline alkaloid that serves as a central branch point intermediate to other BIA subclasses such as protoberberines, benzophenanthridines, phthalideisoquinolines and morphinans (Figures 1, 2C; Hagel and Facchini 2013). In this work, we focus our analyses on the genes that are involved in the biosynthetic pathways leading to the important BIAs berberine, sanguinarine, noscapine and morphine that represent these different structural subclasses.

Berberine belongs to the protoberberine subclass of BIAs with a tetracyclic ring system. It is widely distributed among many different plant species, especially in Ranunculaceae, Papaveraceae, Berberidaceae and Menispermaceae families in the order Ranunculales (Neag *et al.*, 2018). (*S*)-reticuline is first converted to (*S*)-scoulerine through a methylene-bridge-forming reaction catalysed by BERBERINE BRIDGE ENZYME (BBE). After O-methylation a methylenedioxy bridge is added to the isoquinoline moiety by (*S*)-Canadine Synthase (CS), a cytochrome P450 CYP719 protein. The product, (*S*)-canadine, is then converted to berberine in a final oxidation step.

Sanguinarine belongs to the benzophenanthridine subclass of BIAs that has so far only been reported from members of the Papaveraceae family within the order Ranunculales (Krane *et al.*,1984; Sariyar 2002). Its biosynthetic pathway also involves the conversion of (*S*)-reticuline to the tetrahydroprotoberberine alkaloid (*S*)-scoulerine, which is followed by CYP719 catalysed methylenedioxy bridge formation, *N*-methylation, cytochrome P450 CYP82N protein catalysed oxidative ring opening and further hydroxylation to form its distinctive tetracyclic structural skeleton.

The phthalideisoquinoline alkaloid noscapine has a chemical structure that has a two ring phthalide moiety connected to its isoquinoline moiety. Noscapine is derived through further chemical modifications from tetrahydroprotoberberines. Unlike other phthalideisoquinoline alkaloids, noscapine and some of its intermediates bear an additional hydroxyl/*O*-methyl group at the C-8 position of the isoquinoline benzyl ring (Figure 2C). In opium poppy (*Papaver somniferum*), all but two of the genes required for noscapine biosynthesis from (*S*)-reticuline occur as a cluster in the genome (Winzer *et al*., 2012). Noscapine occurs in some but not all species within the genus Papaver of the Papaveraceae and the same is true for the morphinan subclass of BIAs (Sariyar 2002). The biosynthesis of morphinans requires a two step epimerisation of (*S*)-reticuline to (*R*)-reticuline. The reactions are catalysed by STORR, a unique bifunctional cytochrome P450-oxidoreductase fusion protein (Winzer *et al.*, 2015; Farrow *et al.*, 2015). The opium poppy genome has revealed that the noscapine biosynthetic genes are part of a larger BIA gene cluster that also includes *STORR* and all four other genes required for the production of the first morphinan alkaloid thebaine from (*S*)-reticuline (Guo *et al.*, 2018).

Aside from opium poppy, the draft genomes of BIA producing species have become available for *Eschscholzia californica* and *Macleaya cordata* of the Papaveraceae family (Liu *et al.*, 2017; Hori *et al.*, 2018) and *Aquilegia coerulea* of the Ranunculaceae family (Filiault *et al.*,2018). Other than these plant species in the order Ranunculales, sacred lotus (*Nelumbo nucifera*) from the order Proteales is also rich in BIA alkaloids and genome assemblies of this species have also been reported (Ming *et al.*,2013; Gui *et al.*,2018). Even though BIA-biosynthetic genes have yet to be functionally characterized in sacred lotus, the complete coverage of the gene space provided by the whole genome assembly is a valuable and useful source for inferring potential orthologous history at the sequence level for BIA genes characterized in other plants. Although BIA structures are also present sporadically in some species of other plant orders such as Cornales, Laurales, Magnoliales, Piperales and Sapindales, there are no draft genome assemblies yet available from such species. Furthermore there are no gene sequences functionally characterized to be involved in BIA biosynthesis from these orders. Despite the lack of a comprehensive genome coverage of BIA producing plant species in all of the above orders, the four draft genome assemblies of Ranunculales as well as that of sacred lotus have allowed us to perform thorough phylogenetic analyses on the gene families related to BIA biosynthesis and investigate the origin and orthologous history of key gene functions in these pathways.

We also estimate the time frame for the emergence of a metabolic pathway by dating the STORR gene fusion in the morphinan pathway and gene duplications leading to paralogues such as *CYP82X1*/*CYP82X2*/*CYP82Y1* in the noscapine pathway and *THEBAINE 6-O-DEMETHYLASE* (*T6ODM*)*/CODEINE 3-O-DEMETHYLASE* (*CODM*) in the morphinan pathway in opium poppy (Winzer *et al*., 2012; Winzer *et al*., 2015; Guo *et al*., 2018). This analysis allows us to determine the order and timing of key events that give rise to the biosynthetic pathways to noscapine and morphine.

**Results and discussion**

**Phylogenomic analysis of BIA producing plants**

Phylogenomic analysis was used to determine the evolutionary relationship and date divergence times of all five BIA producing plants for which draft assembly genomes have been reported so far. Four genomes are from species in the order Ranunculales. Of these, *P. somniferum*, *E. californica* and *M. cordata* belong to the Papaveraceae family, whereas *A. coerulea* is from the Ranunculaceae family. *N. nucifera* is a member of the Nelumbonaceae family in the order Proteales. Because of its taxonomic relationship with *N. nucifera*, *Macadamia integrifolia* was also included in our analysis since it is the only other member of Proteales with a published genome assembly (Nock *et al*., 2016). However, no BIAs have been reported from this species. The genome of the monocot rice was used as an outgroup. We identified 117 single-copy orthologous groups in these seven genomes using OrthoFinder software (Supplemental Table S1) and then used these to estimate species divergence times with the BEAST2 software (Figure 2A; Emms and Kelly, 2015; Bouckaert *et al*., 2019). The resulting species tree shows that the Ranunculales clade diverged from the Proteales ~122 MYA and the three Papaveraceae species from *A. coerulea* of the Ranunculaceae family ~110 MYA. In addition, *P. somniferum* is estimated to have split from the other two Papaveraceae species, *E. californica* and *M. cordata* about 77 MYA. This provides the time frame for studying the BIA evolution in these plants.

Figure 2B summarises the distribution of the key BIA metabolites, including the central intermediate (*S*)-reticuline and other important end-products such as berberine, sanguinarine, noscapine and morphine, in these five BIA-producing species as reported in the literature: the presence of the 1-benzylisoquinolines (*S*)-reticuline is well documented in all three Papaveraceae species (Winzer *et al.*, 2015; Hagel *et al.,* 2015; Liu *et al.*, 2017). Berberine is one of the main BIAs found in *A. coerulea* (Winek *et al.*, 1964), but very little else is known about its biosynthesis or intermediates such as (*S*)-reticuline in this species. Although not the dominant BIA in the three papaveraceae species, berberine has been reported to occur in trace amounts in these plants (Schmidt *et al.,* 2007; Hagel *et al.,* 2015; Och *et al.*,2017). As such they should all have the biosynthetic capability of producing berberine. Both *E. californica* and *M. cordata* are rich in benzophenanthridine alkaloids, including sanguinarine (Liu *et al.*, 2017; Hori *et al.*, 2018). Opium poppy has also been reported to accumulate sanguinarine in cell cultures (Desgagné-Penix *et al*., 2010), whereas noscapine and morphinans such as morphine and codeine are the most dominant BIAs found in aerial tissues such as stem and capsules (Winzer *et al.*, 2012; Winzer *et al.*, 2015). However, none of the above BIAs have been detected in sacred lotus, even though other BIAs belonging to aporphine, bisbenzylisoquinoline and 1-benzylisoquinoline subclasses have been reported (Menéndez-Perdomo and Facchini 2018). This suggests alternative BIA biosynthetic pathways may be present in *N. nucifera*.

**Biosynthesis of (*S*)-Reticuline evolved in Ranunculales after divergence from Proteales 122 MYA**

The distribution of common BIA metabolites such as (S)-reticuline, berberine and sanguinarine across Ranunculales plants suggests shared evolutionary history of their biosynthetic pathways. We used gene tree analysis to shed light on key evolutionary events shaping the emergence of new BIA pathways in these plants. Figure 2C shows our current biochemical and molecular understanding of BIA biosynthesis. This has overwhelmingly come from Ranunculales species, *P. somniferum,* *E. californica* and *Coptis japonica* in particular. The biosynthetic pathways and all genes involved in the biosynthesis of berberine, sanguinarine, noscapine and morphine have been functionally characterized (Figure 2C). We used the genomes of the five BIA producing species in conjunction with other available data in the Genbank NR database to carry out gene tree analyses of these BIA pathway genes. As such, the gene search space has covered over one hundred annotated plant genomes in addition to other single entries of both functionally characterised or uncharacterised sequences in the NR database. This has provided insight into the timing of gene duplications, gene fusion and neofunctionalisation events as well as evolutionary relationship of these pathways in the context of BIA biosynthesis.

(*S*)-NORCOCLAURINE SYNTHASE (NCS) is the key enzyme catalysing the first step in BIA biosynthesis, the condensation of dopamine and 4-hydroxyphenylacetaldehyde to form (*S*)-norcoclaurine (Figure 3). The *NCS* gene has been functionally characterised from a number of species in the order of Ranunculales (Figure 2C; Samanani *et al.,*2004; Minami *et al.,*2007; Lee and Facchini 2010; Lichman et al., 2017).

The NCS gene tree show a clade of sequences exclusively from families in the order Ranunculales (Figure 3). A single Papaveraceae subclade contains the functionally characterised PSNCS1 sequence from opium poppy and orthologous sequences from the other two genomes as well as other Papaveraceae sequences. Similarly, a single sequence from the *A. coerulea* genome (AC3G218400) forms a single group exclusively with sequences of other Ranunculaceae species, which in turn group with a sequence from the Berberidaceae species *Sinopodophyllum hexandrum* (Figure 3), suggesting monophyletic origin of this NCS in the common ancestor of Papaveraceae, Ranunculaceae and Berberidaceae.

The four other enzymes required for the subsequent *O*-methylation, *N*-methylation and hydroxylation steps in the biosynthesis of (*S*)-reticuline are NORCOCLAURINE 6-*O*-METHYLTRANSFERASE (6OMT), COCLAURINE-*N*-METHYLTRANSFERASE (CNMT), cytochrome P450 HYDROXYLASE (CYP80B) and 3-HYDROXY-*N*-METHYLCOCLAURINE 4’-*O*-METHYLTRANSFERASE (4’OMT) ) (Loeffler and Zenk, 1990; Frenzel and Zenk, 1990a; Frenzel and Zenk, 1990b). The genes encoding these enzymes have been characterised from the Ranunculaceae species *C. japonica* and the Papaveraceae species *P. somniferum* and *E. californica* (Pauli and Kutchan 1998; Huang and Kutchan 2000; Morishige *et al.*, 2000; Choi *et al.*, 2002; Ikezawa *et al.,*2003). Similar to the NCS gene tree, the respective trees for the genes encoding these enzymes show clades containing sequences exclusively from Ranunculales. Orthologous sequences from the genomes of all four species from the Ranunculales order are represented in these clades (Supplemental Figures S1-S3). Furthermore, the clades of 6OMT/4’OMT and CNMT resemble that of the NCS gene in that in each gene clade Papaveraceae sequences form a subclade that is sister to the Ranunculaceae subclade with a single gene sequence from *S. hexandrum* representing the Berberidaceae. In the CYP80 tree (Supplemental Figure S1), the CYP80B clade contains a single Papaveraceae subclade that groups with a Ranunculaceae subclade with no data being available from other Ranunculales families. In summary, this analyses suggest all five genes encoding enzymes required for the biosynthesis of (*S*)-reticuline are likely to be present in the Ranunculales common ancestor before the divergence of the Papaveraceae and Ranunculaceae 110 MYA.

Five NCS like sequences have been reported in the sacred lotus genome based on sequence homology and considered as norcoclaurine synthases (NnNCS) but only by association of the gene expression profiles and alkaloid content (Vimolmangkang *et al.,* 2016). To understand the relationship between the Ranunculales NCSs and these NnNCSs, we extended our analyses to include sequences that fell into other clades in an expanded NCS gene tree (Supplemental Figure S4). The five paralogous sequences identified in sacred lotus appear in the extended tree but not in the Ranunculales NCS clade (Supplemental Figure S4) suggesting NCS activity may have evolved independently in these two orders. However, it should be noted that gene function of these candidates remains to be fully characterised at the biochemical level. Furthermore, no orthologues of the other genes involved in (*S*)-reticuline biosynthesis are present in the sacred lotus genome except for the *6OMT* gene (Supplemental Figures S1-S3), which is consistent with the observation that (*S*)-reticuline is absent from this species. This suggests the biosynthetic pathway for (*S*)-reticuline evolved in the order Ranunculales after the divergence of Proteales and Ranunculales about 122 MYA.

In sacred lotus, many 1-benzylisoquinoline alkaloids have both (*R*) and (*S*) stereochemical configurations (Menéndez-Perdomo, 2018). In contrast, in Ranunculales only (*S*) enantiomers have been detected in the 1-benzylisoquinolines intermediates in early biosynthetic steps up to (*S*)-reticuline. This is consistent with the stereospecificity of NCS activity in Ranunculales (Lee and Facchini 2010). Moreover, the major sacred lotus aporphines such as nuciferine are (*R*) enantiomers and they often lack the hydroxyl group corresponding to the 4’ position of the 1-benzylisoquinoline scaffold as shown in Figure 1. Therefore it is possible the benzylisoquinolines in *N. nucifera* are not derived from the same NCS catalysed condensation of dopamine and 4-hydroxyphenylacetaldehyde. Instead, the biosynthesis of BIAs in this species may start with an enzyme with similar catalytic activity to NCS but with less stringent stereochemical requirement and/or with different substrate specificity. This analysis therefore suggests that while BIA biosynthesis has a monophyletic origin within the order Ranunculales it may have paraphyletic origins in angiosperms outside the order such as sacred lotus. This is in contrast to the previous suggestion that BIA metabolism has a monophyletic origin across all angiosperms (Liscombe *et al*., 2005). Functional characterization of genes that are responsible for the NCS like activities in sacred lotus as well as BIA producing plants of other families will provide more definitive answers to the above.

**The common pathway for protoberberine biosynthesis evolved in the common ancestor of the Ranunculales about 110-122 MYA**

Four enzymes are required for berberine biosynthesis from (*S*)-reticuline (Figure 2C). Of these, CS catalyses the formation of a methylenedioxy bridge to produce (*S*)-canadine and is a member of the CYP719 subfamily of the cytochrome P450s (Rueffer and Zenk 1994; Ikezawa *et al.*, 2003).

Phylogenetic analysis shows the robustly supported CYP719A/B clade (Figure 4), containing sequences exclusively from Ranunculales. This clade contains all functionally characterized CYP719s, including CYP719A1 and CYP719A21, the CS enzymes that have been characterised from *C. japonica* and *P. somniferum* respectively.

In the CYP719A/B clade, CYP719A1forms a CS subclade exclusively with CYP719 sequences identified either from the genome of *A. coerulea* or from other Ranunculaceae species in the Genbank NR database, suggesting a monophyletic origin in Ranunculaceae. The subclade that includes functionally characterized (-)-PLUVIATOLIDE SYNTHASE (PS) contains sequences exclusively from the Berberidaceae family and is strongly supported. However, there is no bootstrap support for a sister relationship of the Berberidaceae PS clade to the Ranunculaceae CS subclade. Taking the species phylogeny of these taxonomic groups and the gene tree topology into account, we postulate the sister relationship of the Ranunculaceae CS and the Berberidaceae PS subclades is obscured in the gene tree by long branches in members of the PS subclade. PS, such as CYP719A23 and CYP719A24, is capable of converting (-)-matairesinol into (-)-pluviatolide by catalyzing methylenedioxy bridge formation in the biosynthesis of the phenolic aryltetralin lignan podophyllotoxin in Berberidaceae species *Podophyllum hexandrum* and *Podophyllum peltatum* (Marques *et al.,* 2013). The difference in substrate structures to BIAs may have required substantial changes at the sequence levels which may result in masking of the sister grouping with the Ranunculaceae CS subclade in our analysis.

All remaining sequences group strongly together and are exclusively from Papaveraceae species, forming a sister relationship to the Ranunculaceae CS and Berberidaceae PS subclades. This Papaveraceae subgroup is further divided into two strongly supported subclades both containing sequences from the three Papaveraceae genomes. This indicates a gene duplication in the Papaveraceae lineage following divergence of the Papaveraceae and Ranunculaceae/Berberidaceae.

It has been reported that CYP719A13 from *Argemone mexicana* converts (*S*)-tetrahydrocolumbamine to (*S*)-canadine, (*S*)-cheilanthifoline to (*S*)-stylopine and (*S*)-scoulerine to (*S*)-nandinine, thus CYP719A13 can be involved in both sanguinarine and berberine formation in *A*. *mexicana* (Díaz Chávez *et al*., 2011). The opium poppy CS (CYP719A21) and the trifunctional (*S*)-canadine synthase/(*S*)-stylopine synthase/(*S*)-nandinine synthase CYP719A13 from *A. mexicana* fall in only one of the Papaveraceae subclades, suggesting that the canadine synthase activity was present in the common ancestor of Ranunculaceae and Papaveraceae and this activity has been maintained in some Papaveraceae gene members after gene duplication events.

BBE and (*S*)-SCOULERINE 9-O-METHYLTRANSFERASE (9OMT) catalyse the first two steps in the biosynthesis of berberine from (*S*)-reticuline. The former catalyses a methylene-bridge-forming step to convert (*S*)-reticuline to (*S*)-scoulerine, resulting in a first tetrahydroprotoberberine structure while the latter adds a methyl group to the 9-hydroxyl group of (*S*)-scoulerine (Dittrich and Kutchan, 1991; Takeshita *et al.,* 1995). Gene tree analyses of these two genes show a similar tree topology to that of the (*S*)-reticuline biosynthetic genes, supporting a single copy origin of *BBE* and *9OMT* genes in the Ranunculales lineage (Supplemental Figure S5). Orthologues of both genes were found in all four Ranunculales genome assemblies, apart from *E. californica* lacking an orthologue of 9OMT. In this case, a similar activity to that of 9OMT was reported being carried out by a methyltransferase, G3OMT, belonging to a different subfamily (Purwanto *et al*., 2017). Taken together, we speculate that a functional replacement and gene loss of the original *9OMT* may have occurred in the *E. californica* lineage.

In summary, since orthologs of *BBE*, *9OMT* and *CS* exist in the assembled genomes of all four species from the order Ranunculales, we propose that the protoberberine biosynthetic pathway in the Ranunculales evolved after the split of Ranunculales/Proteales around 122 MYA and prior to the divergence of Ranunculaceae and Papaveraceae at 110 MYA. The occurrence of berberine in species among most families in the order of Ranunculaes is consistent with this proposal.

**The sanguinarine pathway emerged in the Papaveraceae lineage 77 MYA**

The first two steps involved in the conversion of tetrahydroprotoberberine (*S*)-scoulerine to the benzophenanthridine alkaloid sanguinarine are catalysed by two members of the cytochrome P450 CYP719 subfamily, (*S*)-CHEILANTHIFOLINE SYNTHASE (CFS) and (*S*)-STYLOPINE SYNTHASE (SPS) (Figure 2C; Bauer and Zenk 1989; Ikezawa *et al.,*2003; Ikezawa *et al.,*2009). All functionally characterized CFS enzymes fall into the afore-mentioned CFS subclade in the CYP719 tree that included sequences exclusively from Papaveraceae (Figure 4; Ikezawa *et al.*, 2007; Ikezawa *et al.*, 2009; Díaz Chávez *et al.*, 2011; Yahyazadeh *et al.*, 2017). On the other hand, all reported SPS are members of the sister SPS/CS subclade that is also comprised exclusively of Papaveraceae sequences with all three genomes represented. This suggests that the neofunctionalisation leading to CFS and SPS activities occurred soon after the gene duplication event discussed above before the divergence of *P. somniferum, E. californica* and *M. cordata* approximately 77 MYA. Interestingly, we found that the CFS and SPS genes are closely associated in the genomes of both *P. somniferum* and *M. cordata* suggesting they arose by tandem duplication.

The major structural conversions from *N*-methylated tetrahydroprotoberberine to protopine and subsequently benzophenanthridine subclass of BIAs including sanguinarine are catalysed by (*S*)-N-METHYLSTYLOPINE HYDROXYLASE (MSH) and PROTOPINE-6-HYDROXYLASE (P6H) (Figure 2C). Both belong to the CYP82N/R/X/Y subfamily of the cytochrome P450 enzymes (Rueffer and Zenk 1987; Tanahashi and Zenk 1990; Takemura *et al.*, 2012; Beaudoin and Facchini 2013). Gene tree analyses show that the members of the CYP82N/R/X/Y subfamily are exclusively from species of Ranunculaceae and Papaveraceae to date (Figure 5). Two sequences from the genome of *A. coerulea* form an outgroup to two sister clades. The MSH clade contains the functionally characterised MSH, CYP82N4, from opium poppy and sequences from the three Papaveraceae genomes as well as two other sequences from *P. bracteatum* and *P. rhoeas*. The functionally uncharacterized CYP82R1 is the only Ranunculaceae sequence in this clade and it is sister to all Papaveraceae sequences. On the other hand, the P6H clade is comprised of sequences exclusively from the three Papaveraceae genomes including the functionally characterized P6Hs, CYP82N2v2 and CYP82N3, from *E. californica* and *P. somniferum,* respectively*.* Thus both MSH and P6H activities are also likely to have emerged from single ancestral orthologues before the divergence of *P. somniferum*, *E. californica* and *M. cordata* 77 MYA. The presence of the CYP82R1 sequence from the Ranunculaceae species C. japonica in the MSH clade indicates that both MSH and P6H orthologues must have existed in the common ancestor of the Ranunculales. Both orthologues seem to have been lost in the lineage of *A. coerulea*.

In the sanguinarine pathway, (*S*)-stylopine is *N*-methylated by (*S*)-TETRAHYDROPROTOBERBERINE-*N*-METHYLTRANSFERASE (TNMT) after the conversion from (*S*)-scoulerine by CFS and then SPS, and before being hydroxylated by MSH to form protopine (Figure 2C Rueffer *et al.*, 1990; O'Keefe and Beecher 1994; Liscombe and Facchini 2007). In the TNMT clade of the *N*-methyltransferase tree, the four *A. coerulea* sequences form a robust orthologous group, sister to other sequences exclusively from Papaveraceae species including all TNMTs characterised to date (Supplemental Figure S3). Since sequences from the genomes of all three Papaveraceae species are represented, this TNMT activity must have evolved before the divergence of these three lineages.

In summary, our analyses suggest the pathways leading to the biosynthesis of the benzophenanthridine alkaloids such as sanguinarine evolved after gene duplication/neofuntionalisation in the CYP719, TNMT and CYP82N/R/X/Y families before the divergence of *P. somniferum*, *E. californica* and *M. cordata* 77 MYA and after the split of Papaveraceae from Ranunculaceae around 110 MYA. Consistent with this, the benzophenanthridine subclass of BIAs and enzyme activities of CFS, SPS, TNMT, MSH and P6H have so far only been detected in the Papaveraceae family (Krane *et al.*, 1984; Rueffer and Zenk 1987; Bauer and Zenk 1989; Tanahashi and Zenk 1990; Bauer and Zenk 1991; Sariyar 2002; Liscombe and Facchini 2007).

**Biosynthesis of the phthalideisoquinoline alkaloid noscapine evolved in the opium poppy lineage following specific gene family expansion/neofunctionalisation**

With the exception of *BBE* and *TNMT*, all genes required for the synthesis of noscapine and the first morphinan alkaloid thebaine from the branch point intermediate (*S*)-reticuline occur as a major BIA gene cluster in the opium poppy genome (Winzer *et al.*, 2012; Guo *et al.*, 2018). Of these, CYP82X1, CYP82X2 and CYP82Y1 are involved in converting the *N*-methyl tetrahydroprotoberberine alkaloid (*S*)-*N*-methylcanadine to the phthalideisoquinoline alkaloid noscapine (Figure 2C, Winzer et al., 2012; Dang and Facchini, 2014). All three are members of the cytochrome P450 CYP82N/R/X/Y subfamily. Gene tree analyses show they are all in a well supported subgroup which is sister to that containing orthologous MSH sequences in the MSH clade (Figure 5). No sequences from the *E. californica* or *M. cordata* genomes are present in this subgroup. This suggests the gene duplications and neofunctionalisation resulting in *CYP82X1*, *CYP82X2* and *CYP82Y1* occurred in the opium poppy lineage after its split from the other two Papaveroideae species.

Likewise, gene tree analyses of PSAT1 and PSCXE1, two other genes encoding proteins involved in noscapine biosynthesis (Winzer *et al.*, 2012), support the emergence of these activities after gene duplication and neofunctionalisation specifically in the opium poppy lineage (Supplemental Figures S6-7) with no orthologous sequence being found in the genomes of *E. californica* and *M. cordata*.

Noscapine biosynthesis shares the first three enzymatic steps with the protoberberine biosynthetic pathway converting (*S*)-reticuline to (*S*)-canadine (Figure 2C). In the opium poppy genome the genes encoding two of these activities from (*S*)-scoulerine to (*S*)-canadine, the opium poppy *9OMT* orthologue *PSMT1* and *CYP719A21* are adjacent to each other head-to-head in opposite directions and separated by 1.6kb in the BIA gene cluster. We observed that the orthologues of both genes are present in the same orientation 80kb apart in the *M. cordata* genome assembly with only one annotated hypothetical gene in between. We hypothesize that these two protoberberine pathway genes represent an ancient ‘seeding’ gene pair for the BIA gene cluster to which other genes have been recruited during the evolution of noscapine and morphinan biosynthesis.

In addition to PSMT1, PSMT2 and PSMT3 are also involved in noscapine biosynthesis. They are all members of the same *O*-methyltansferase family with PSMT2 and PSMT3 being more closely related to each other than to PSMT1 (Cabry *et al.*, 2019). PSMT2 and PSMT3 share the same exon/intron structure (Winzer *et al.*, 2012). However, our gene tree analyses show that they fall into separate clades with orthologues from Ranunculaceae/Berberidaceae species (Supplemental Figures S2, S5 and S8 ), suggesting they derived from different copies in the common ancestor of the Ranunculales. As discussed above, PSMT1 activity, also required for berberine biosynthesis, evolved in the Ranunculales common ancestor. PSMT2 is the only functionally characterized member in its clade (Supplemental Figure S6). PSMT3 is a member of the 6OMT clade and is sister to the 6OMTs from other *Papaver* species that are required for the biosynthesis of the central intermediate (*S*)-reticuline. We therefore propose *PSMT3* arose in the opium poppy lineage through neofunctionalsation following gene duplication from an ancestral *6OMT* copy. It has been found that the formation of a heterodimer of PSMT2 and PSMT3 is required for the substrate specific *O*-methylation reaction of the hydoxyl group introduced at the C-8 position by CYP82Y1 (Li and Smolke, 2016; Li *et al.*, 2018; Park *et al.*, 2018). *In vitro* assays showed that 6OMT can replace PSMT3 but not PSMT2 in the heterodimer with it maintaining the same substrate specificity, consistent with our analyses that PSMT3 and 6OMT are closely related.

All three *O*-methyltransferases are present within a 150 kilobases segment in the genome as part of the BIA gene cluster with PSMT2 and PSMT3 being 77 kilobases apart, only separated by CYP82Y1 (Winzer *et al.*, 2012; Guo *et al.*, 2018). Despite the physical proximity and sharing the same exon/intron structure, our gene tree analyses show PSMT2 and PSMT3 must have been recruited to the BIA gene cluster independently.

Taken together, our analysis suggests that the components of the noscapine biosynthetic pathway following conversion of (*S*)-canadine to (*S*)-N-methylcanadine by TNMT have evolved in the opium poppy lineage after divergence from *E. californica* and *M. cordata* at 77 MYA. Analysis of the genomes from more species within the opium poppy lineage would be necessary to improve the resolution of the evolutionary time frame leading to the emergence of the noscapine biosynthetic pathway and the assembly of the BIA gene cluster.

**Biosynthesis of the morphinan alkaloids emerged in the *Papaver* genus**

All five genes, including *STORR*, *SALUTARIDINE SYNTHASE* (*SALSYN*) and *SALUTARIDINOL-7-O-ACETYLTRANSFERASE* (*SALAT*), *SALUTARIDINE REDUCTASE* (*SALR*) and *THEBAINE SYNTHASE* (*THS*), required for the first morphinan alkaloid thebaine from (*S*)-reticuline are part of the BIA gene cluster in opium poppy. The emergence of the *STORR* fusion is considered a key event for the evolution of morphinan biosynthesis in opium poppy, enabling the conversion of (*S*)-Reticuline to (*R*)-Reticuline (Figure 2C; Winzer *et al.*, 2015; Farrow *et al.*, 2015). The CYP82N/R/X/Y gene tree shows that CYP82Y2, the cytochrome P450 module of the opium poppy STORR fusion protein, groups strongly with those of STORR orthologues from *P. setigerum* and *P. bracteatum*. Both CYP82Y2 and its closest paralogue, PS0216860, belong to the same subgroup as CYP82X1, CYP82X2 and CYP82Y1 which is sister to the MSH orthologues in the MSH clade (Figure 5). No sequences from the genomes of *E. californica* and *M. cordata* are present in this subgroup. Thus, we conclude that the gene duplication giving rise to the CYP82Y2 module of STORR occurred in the lineage leading to opium poppy after its split from *E. californica* and *M. cordata*.

The oxidoreductase module of the STORR fusion protein and its closest paralogue PS0216870 are members of an oxidoreductase family closely related to the CODEINONE REDUCTASE (COR) proteins, the enzymes involved in the last steps of morphine biosynthesis (Figures 2C and 6). The COR clade contains sequences exclusively from the genus *Papaver*, mostly from the opium poppy genome. The analyses of both modules show specific gene family expansion in the opium poppy lineage for both families, and a fragmental genome duplication occurred prior to the STORR fusion event.

SALSYN and SALAT are two enzymes involved in the structural conversions of the 1-benzylisoquinoline alkaloid (*R*)-Reticuline to promorphinans (Figure 2C). Both are in groups containing exclusively *Papaver* sequences in their respective gene trees, further supporting our hypothesis that the morphinan pathway evolved within the opium poppy lineage (Figure 4; Supplemental Figure S6). SALSYN (CYP719B1) exclusively converts the R-epimer of reticuline to salutaridine (Gesell *et al.*,2009), a reaction requiring intramolecular C-C phenol-coupling. This reaction would have required extensive neofunctionalization as other functionally characterised CYP719 proteins all catalyse the formation of methylenedioxy bridges (Figure 4). SALAT carries out the *O*-acetylation of salutaridinol and is one of the closest paralogues to PSAT1, an enzyme involved in the noscapine pathway (Supplemental Figure S6; Lenz and Zenk 1995; Grothe *et al.,* 2001). Dating the divergence time between SALAT and PSAT1 would require additional genomes in the opium poppy lineage and this would provide useful insight into the evolutionary timing and relationship of the morphinan and noscapine biosynthetic pathways.

While the emergence of STORR opened the gateway for promorphinan biosynthesis and ultimately the first morphinan alkaloid thebaine, the evolution of T6ODM and CODM was crucial for the production of the morphinan alkaloid morphine (Farrow and Facchini 2013). T6ODM, CODM and COR are not part of the BIA gene cluster in opium poppy but each occurs in multiple locally linked copies (Guo et al., 2018). Both CODM and T6ODM are members of the 2-OXOGLUTARATE/Fe(II)-DEPENDENT DIOXYGENASE family that also went through recent rapid family expansion in the opium poppy lineage, similar to the COR clade (Supplemental Figure S9).

In the absence of other genome assemblies from species within the opium poppy lineage we calculated the number of synonymous substitutions per synonymous site (Ks) values between paralogous gene pairs to gain insight about the timing of these gene duplication events. Previously, we found that the closest paralogues (PS0216860 and PS0216870) of the STORR P450 and oxidoreductase modules are located just 865 base pairs apart on a different chromosome of the opium poppy genome (Guo *et al*., 2018). From this we hypothesized that the *STORR* gene fusion event must have followed a segmental duplication. We have now calculated the Ks values of CYP82Y2/PS0216860 and STORR-oxired/PS0216870 paralogue pairs with all 17 different substitution models with the KaKs\_Calculator software (Wang *et al*., 2010). These are all in the range of 0.279456 - 0.387986 and 0.290438 - 0.356423 respectively (Supplemental Table S2). Based on a synonymous substitution rate (r) of 6.98 per billion years (109) (Guo *et al*., 2018), the time (T) for this duplication would be in the ranges of 20.0 - 27.8 and 20.8 - 25.5 MYA respectively (T=Ks/2r). Thus, based on our reasoning that the segmental duplication event must have occurred prior to the *STORR* gene fusion we propose that the emergence of the pathway to morphinan biosynthesis occurred more recently than 20 MYA.

The gene duplication of the common ancestor that eventually gave rise to both *CODM* and *T6ODM* through neofunctionalisation can also provide an important time point for the evolution of morphine biosynthesis. We calculated the pairwise Ks between the three *CODM* and five *T6ODM* gene copies. All are between 0.245096 - 0.327976, pointing to a possible time of most recent ancestral gene duplication giving rise to these two enzyme activities at 17.6 - 23.5 MYA.

Similar to noscapine, our gene tree analysis of CYP82N/R/X/Y, CYP719, ACETYLTRANSFERASE, COR and CODM/T6ODM indicate that the morphine biosynthetic pathway arose through gene duplications, gene fusion and neofunctionalisation events that built on activities derived from the existing protoberberine and sanguinarine biosynthetic pathways in the opium poppy lineage after the divergence from the *E. californica* and *M. cordata* lineages at 77 MYA. Ks estimation of the paralogous pairs of the STORR modules and CODM/T6ODM has enabled us to speculate the morphine pathway may have arisen more recently than 18 MYA, which is consistent with the proposed emergence of the *Papaver* genus around 19-29 MYA (Xie *et al*., 2012; Valtueña *et al*., 2012).

Concluding from our tree analyses, we propose a monophyletic origin for all five genes required for the biosynthesis of (*S*)-reticuline, the central intermediate of BIA metabolism in the order Ranunculales. The biosynthetic pathway leading to production of the protoberberine alkaloid, berberine, emerged before the divergence of the Papaveraceae and Ranunculaceae families between 110-122 MYA. BIA synthesis in *N. nucifera*, which belongs to the order Proteales, is very likely to have evolved through a parallel process (Figure 7). Emergence of the sanguinarine pathway occurred after the Papaveraceae Ranunculaceae divergence 110 MYA but before the split of the three Papaveraceae species at 77 MYA. We found that the gene duplications necessary for subsequent gene fusion and neofunctionalisation of essential genes involved in the production of both the phthalideisoquinoline alkaloid noscapine and morphinans including codeine and morphine production occurred exclusively in the opium poppy lineage. We therefore propose that the biosynthetic pathways involved in both noscapine and morphinans emerged more recently in the opium poppy lineage after its divergence from the *E. californica* and *M. cordata* lineages at 77 MYA. Based on Ks estimation of the paralogous pairs of the two *STORR* modules and *CODM*/*T6ODM* we further propose that morphine biosynthesis evolved more recently than 18 MYA in the *Papaver* genus.

Further validation of the conclusions drawn from our analysis of the available draft genome assemblies of five BIA-producing species will be possible once genome assemblies of other BIA-accumulating species from within the Papaveraceae and Ranunculaceae as well as those from other families in the Ranunculales such as Berberidaceae, Menispermaceae, Circaeasteraceae and Lardizabaloideae become available. In this respect we note that in our analysis, we infer the monophyletic origin of BIA biosynthetic pathways in Ranunculales based on the understanding that the Papaveraceae diverged from the Berberidaceae, Menispermaceae and Ranunculaceae families earlier than the latter three diverged from each other according to the Angiosperm Phylogeny Group (APG). Ultimately, functional characterisation of genes involved in BIA biosynthesis across the range of species that produce BIAs including *N. nucifera*, *A. coerulea* and *M. cordata* will be necessary in order to be absolutely definitive about our understanding of evolutionary events that resulted in the emergence of BIA metabolism in the Ranunculales. Despite these limitations we consider our analysis, representing as it does a complete coverage of the gene space provided by the whole genome assembly of five relevant species, has allowed us to propose an orthologous history at the sequence level for the BIA genes and reveal key events in the evolution of BIA biosynthetic pathways.

This study demonstrates the effectiveness of using gene tree analysis of whole genome assemblies to identify key evolutionary events leading to the emergence of the structural genes necessary for new biosynthetic pathways to evolve. The recruitment model of metabolic evolution proposes that the emergence of new biochemical pathways requires the co-evolution of structural genes and associated transcription factors resulting in a regulon of coordinately expressed genes (Shoji 2019). The metabolic evolution of noscapine and morphinan biosynthesis is particularly interesting in this regard as the regulon includes 15 genes in a localised genomic region of 600kb in the opium poppy genome plus additional genes such as *BBE* and *TNMT* that are not specific to noscapine or morphinan biosynthesis and others that are specific only to morphine production including *T6ODM*, *CODM* and *COR*. Now that we have established key evolutionary events leading to the emergence of the structural genes, the challenge is to integrate these events with those involved in gene regulation at both the transcription factor and genome organisation levels giving rise to the BIA 15 gene cluster.

**Methods**

**Estimation of species divergence times of five BIA-producing plants**

Divergence of seven plant species was dated with BEAST2 v2.5.1 (Bouckaert *et al*., 2019) based on a concatenated dataset of orthologous groups from their draft genomes. The five BIA producing plants include four species in the order of Ranunculales and *N. nucifera*, a member of the Nelumbonaceae family in the order of Proteales. The four Ranunculales species are *P. somniferum*, *E. californica* and *M. cordata* from the Papaveraceae family and *A. coerulea* from the Ranunculaceae family. *Macadamia integrifolia*, the only other member of Proteales with its genome assembly available was included (Nock *et al*., 2016), whereas monocot rice was used as an outgroup. Annotation datasets of all seven genomes can be downloaded from publicly available sources (Supplemental Table S3).

OrthoFinderv2.0 (Emms and Kelly, 2015) was used to identify orthologous groups across the seven genomes with their annotated datasets. Protein sequence alignments were then constructed for each orthologous group with ClustalX(Thompson *et al.*,2002), and the conserved blocks in each alignment were evaluated and selected with Gblocks v0.91b by allowing gap positions within final blocks (Castresana, 2000). A final alignment was constructed by concatenating all conserved blocks and used in the subsequent analyses. This was then used to estimate species divergence times based on a Bayesian approach with BEAST2.

For the divergence time estimation, we used the birth-death model for tree priors and a Jones–Taylor–Thornton substitution model with a strict clock rate. To calibrate divergence times, an exponential model was chosen for monocot-dicot split time (mean: 150 MYA. Std dev: 4MYA) and Papaveraceae-Ranunculaceae split time (mean: 110 MYA. Std dev: 4MYA) (Guo *et al*., 2018). The Markov chain Monte Carlo was repeated 500,000,000 times with 1000 steps to allow chain convergence. Tracer v1.7.1 (Rambaut *et al*., 2018) was employed to check chain convergence and ensure satisfactory effective sampling sizes for all parameters. The maximum clade credibility tree was calculated with TreeAnnotator v2.5.1 within the BEAST2 software package, summarizing the estimated mean age and 95% confidence intervals from post-burn-in (20%) trees.

**Gene tree analyses**

We used a phylogenetic approach to examine the orthologous relationships of members of all gene families that contain functionally characterized genes involved in the biosynthesis of berberine, sanguinarine, noscapine and morphinan alkaloids in BIA-producing plants. All query sequences are listed in Supplemental Table S4. For each query sequence, local BLASTP searches were performed against the annotated protein datasets of all seven genomes described above (Altschul *et al.,* 1997). The sequences with an expected value lower than e-20 or within top 100 hits of the lowest expected values were then retrieved from each annotated protein datasets of all seven species. Parallel BLASTP searches for each query were also carried out against the non-redundant protein sequences (NR) database at the National Center for Biotechnology Information (NCBI). A maximum number of 400 sequences were retrieved with an expected value lower than e-20. These two sets of resulting sequences were then combined for each of the starting query sequences. Duplicated and partial sequences were removed together with other synthetic sequence entries in the NR database. Species identification associated with the NR database entries were verified and corrected where possible by checking the associated publications.

Protein sequence alignments were made firstly with ClustalX. Conserved blocks in each alignment were evaluated and selected with Gblocks analysis. The best-scoring maximum likelihood tree was inferred in conjunction with bootstrap analyses of 100 replicates using RAxMLv8.2.12 (Stamatakis, 2006). The substitution model was the same as used in the above BEAST2 analyses. The trees from this first round of analyses were then evaluated. A subset of sequences were selected for a further round of analyses if (1) it contains the query sequence; and (2) it forms a group with above 50% bootstrap value support. Not all gene tree analyses yielded informative results in terms of strong bootstrap support. This may be due to either too little starting data (short sequences) or too few informative sites caused by extreme mutation rates along the lineages. As such, the gene families containing *STOX*, *DBOX*, *SDR1*, *SALR*, *THS* and *NISO* did not give informative phylogenetic relationships, and were therefore not included in further analyses. For the gene families with a clear subset identified ,a further round of Gblocks analyses were performed to allow more residues to be included in the conserved blocks in order to achieve higher bootstrap support value in the subsequent phylogenetic analyses with RAxML. This process were reiterated whenever further improvements could be made. The final sets of sequences selected for the gene analyses were summarised in Supplemental Table S5. In the final trees, groups with above 70% bootstrap value were considered as strongly supported.

**Estimation of divergence times between paralogues using Ks analyses**

KaKs\_Calculator v2.0 (Wang *et al*., 2010) was used to estimate the timing of the gene duplication events between paralogue gene pairs in opium poppy genome. These include the pairings of the STORR P450 and oxidoreductase modules and their corresponding closest paralogues (PS0216860 and PS0216870) as well as the pairings between the T6ODM and CODM copies. The coding sequences of the paralogue pairs were aligned using MUSCLE alignment tool (Edgar, 2004). The alignments were then converted into the input format required for the KaKs\_Calculator software to estimate Ks values between paralogue pairs.

The divergence times of the gene duplication leading to the paralogue pair were then estimated with the formula T=Ks/2r, where T denotes divergence time, Ks the number of synonymous substitutions and r the substitution rate. With the absence of the absolute substitution rate for the specific gene families, we used a synonymous substitution rate (r) of 6.98 per billion (109) years that was estimated in the opium poppy lineage after its divergence from the Ranunculaceae species *A. coerulea* (Guo *et al*., 2018).

**Supplemental Information (SI)**

Document S1: Supplemental Tables S1-S5. Supplemental tables supporting the manuscript. (XLSX)

Document S2: Supplemental Figure S1-S9. Supplemental figures supporting the manuscript. (PDF)

**Authors’ contributions**

YL performed all species and gene tree analyses and ZH carried out the OrthoFinder analyse. YL, TW and IAG analyzed and interpreted results; and were major contributors in writing the manuscript. All authors read and approved the final manuscript.

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**Table 1. List of abbreviations**

|  |  |
| --- | --- |
| A**bbreviations** | **Full name** |
| 4’OMT | 3-HYDROXY-*N*-METHYLCOCLAURINE 4’-*O*-METHYLTRANSFERASE |
| 6OMT | NORCOCLAURINE 6-*O*-METHYLTRANSFERASE |
| 9OMT | (*S*)-SCOULERINE 9-*O*-METHYLTRANSFERASE |
| BBE | BERBERINE BRIDGE ENZYME |
| BIA | Benzylisoquinoline alkaloid |
| CFS | (*S*)-CHEILANTHIFOLINE SYNTHASE |
| CNMT | COCLAURINE-N-METHYLTRANSFERASE |
| CODM | CODEINE 3-*O*-DEMETHYLASE |
| COR | CODEINONE REDUCTASE |
| CS | (*S*)-CANADINE SYNTHASE |
| CYP80B | (*S*)-*N*-METHYLCOCLAURINE 3'-HYDROXYLASE |
| CYP82X1 | CYTOCHROME P450 CYP82X1 |
| CYP82X2 | CYTOCHROME P450 CYP82X2 |
| CYP82Y1 | CYTOCHROME P450 CYP82Y1 |
| DBOX | DIHYDROBENZOPHENANTHRIDINE OXIDASE |
| Ks | the number of synonymous substitutions per synonymous site |
| MSH | (*S*)-N-METHYLSTYLOPINE HYDROXYLASE |
| MYA | million years ago |
| NCBI | National Center for Biotechnology Information |
| NCS | (*S*)-NORCOCLAURINE SYNTHASE |
| NISO | NEOPINONE ISOMERASE |
| P6H | PROTOPINE-6-HYDROXYLASE |
| PS | (-)-PLUVIATOLIDE SYNTHASE |
| PSAT1 | ACETYLTRANSFERASE 1 |
| PSMT2 | *O*-METHYLTRANSFERASE 2 |
| PSMT3 | *O*-METHYLTRANSFERASE 3 |
| PSCXE1 | CARBOXYLESTERASE 1 |
| PSSDR1 | SHORT-CHAIN DEHYDROGENASE/REDUCTASE |
| SALAT | SALUTARIDINOL-7-*O*-ACETYLTRANSFERASE |
| SALR | SALUTARIDINE REDUCTASE |
| SALSYN | SALUTARIDINE SYNTHASE |
| SanR | SANGUINARINE REDUCTASE |
| SPS | (*S*)-STYLOPINE SYNTHASE |
| STORR | ((*S*)-TO-(*R*)-RETICULINE P450-OXIDOREDUCTASE |
| STOX | (*S*)-TETRAHYDROPROTOBERBERINE OXIDASE |
| T6ODM | THEBAINE 6-*O*-DEMETHYLASE |
| THS2 | THEBAINE SYNTHASE |
| TNMT | (*S*)-TETRAHYDROPROTOBERBERINE-*N*-METHYLTRANSFERASE |

**Figure Legends**

**Figure 1.** **Structural scaffolds of major subclasses of benzylisoquinoline alkaloids derived from 1-benzylisoquinolines.** Numbering of different carbon positions is shown for the scaffold of 1-benzylisoquinolines.

**Figure 2. The occurrence of benzylisoquinoline alkaloids in Ranunculales and Proteales species with published genome assemblies.** **(A).** Inferred phylogenetic tree with single-copy orthologous sequences across seven genomes (Supplemental Table S1). The scale bar shows 30.0 million years. Divergence timings are estimated using BEAST2 (Bouckaert *et al*., 2019) and shown at the nodes. Branch colours indicate species. Light blue bars at the nodes indicate the range with 95% highest posterior density. **(B).** Occurrence of selected alkaloids representing different benzylisoquinoline classes with respect to the species shown in Figure 2A. **(C).** BIA biosynthetic pathways and enzymes characterised from species belonging to the order Ranunculales. Enzyme names are provided in the list of abbreviations in Table 1 .

**Figure 3.** **Phylogenetic analyses show an NCS clade across species in the Ranunculales.** All functionally characterised NCSs are members of this NCS clade. Sequences from species of the same family form strongly supported subclades. The best-scoring maximum likelihood tree was inferred using RAxML (Stamatakis, 2006) All branches are drawn to scale as indicated by the scale bar (substitutions/site) and the bootstrap values from an analysis of 100 replicates are shown at the nodes. The solid diamonds indicates the root of the tree. The NCS clade is indicated with the dark grey bar. Branch colours indicate species. Each subclade that contains sequences from species of the same family is highlighted in colour. ACxG, EC, MC and PS prefixes in sequence identifiers denote sequences from the annotation datasets of *A. coerulea*, *E. californica*, *M. cordata* and *P. somniferum* respectively. For the remaining sequences a three letter code was used at the end of the sequence identifiers to indicate the respective species (Supplemental Document S2: General information for gene trees). Additional sequence information is in Supplemental Table S5. The inset shows the reaction catalysed by NCS.

**Figure 4. CYP719 tree analysis suggests key duplication events for the evolution of multiple BIA biosynthetic pathways.** All functionally characterised CYP719 proteins fall into the CYP719A/B clade which contains sequences exclusively from Ranunculales species. The CYP719A/B clade contains several strongly supported subclades. The CS subclade contains sequences exclusively from Ranunculaceae species and all of its functionally characterised members exhibit CS activity. Both the SPS/CS and CFS subclades consist of sequences exclusively from Papaveraceae species. All functionally characterized SPS proteins fall into the SPS/CS subclade, whereas all functionally characterized CFS proteins are members of the CFS subclade. The SPS/CS subclade also contains the opium poppy CS sequence, CYP719A21 and the trifunctional (*S*)-canadine synthase/(*S*)-stylopine synthase/(*S*)-nandinine synthase (CYP719A13) from *Argemone mexicana*. SALSYN (CYP719B1) falls into an opium poppy group inside the CFS subclade highlighted by the shaded box in the tree. The figure descriptors and legends are the same format as described in Figure 3. Extended information of the gene tree is included in Supplemental Document S2: General information for gene trees. The black arrow indicates the occurrence of the gene duplication event giving rise to SPP/CFS. The inset shows the reactions catalysed by functionally characterized members of the CYP719 subfamily with the reactive groups highlighted in red boxes.

**Figure 5. The CYP82N/R/X/Y tree shows recruitment of CYP82 enzymes into the noscapine and morphinan pathways.** Members of CYP82N/R/X/Y subfamily from the Papaveraceae family fall into either the MSH or P6H clade. Both clades are strongly supported. The P6H clade contains all functionally characterized P6H proteins and sequences from each of the three Papaveraceae species, *E. californica*, *M. cordata* and *P. somniferum*. The MSH clade contains two sister subclades. One subclade contains all functionally characterized MSH enzymes and all three Papaveraceae lineages are represented. The other is comprised of sequences from only Papaver species and is highlighted by the shaded box in the tree. Three CYP82 members, CYP82X1, CYP82X2 and CYP82Y1, involved in noscapine biosynthesis, fall into this subclade. CYP82Y2, the cytochrome P450 module of the STORR fusion protein is also a member of this subclade. The figure descriptors and legends are the same format as described in Figure 3. Extended information of the gene tree is included in Supplemental Document S2: General information for gene trees. The inset shows the reactions catalysed by functionally characterized members of the CYP82N/R/X/Y subfamily with the reactive groups highlighted in red boxes.

**Figure 6.** **Opium poppy lineage specific expansion of the oxidoreductase subfamily gave rise to the STORR oxidoreductase module.** The Papaveraceae oxidoreductase subfamily contains sequences mostly from opium poppy. A COR clade is nested within a much larger group containing only *Papaver* sequences and highlighted by the shaded box in the tree. The COR clade contains 11 sequences from the opium poppy genome including both the STORR oxidoreductase module and four COR isoforms. The figure descriptors and legends are the same format as described in Figure 3. Extended information of the gene tree is included in Supplemental Document S2: General information for gene trees. The inset shows the reaction catalysed by STORR.

**Figure 7. Key evolutionary events in benzylisoquinoline alkaloid metabolism as inferred from gene tree and Ks analyses.** Timing of evolutionary events are indicated by letters on the same species tree as shown in Figure 2A. Blue arrows highlight the estimated divergence time span from the analyses. The Ranunculales lineage is highlighted by an orange box, the Papaveraceae lineage in light green and the opium poppy lineage in dark green. The inset box shows a description of the evolutionary events with the font colour corresponding to specific lineages.

1. All abbreviations are listed in Table 1 [↑](#footnote-ref-1)