Norwegian University
of Life Sciences

Master's Thesis 2018 60 ECTS

Faculty of Biosciences
Siri Fjellheim

Conservation and regulation of key photoperiod regulators in the Pooideae subfamily of grasses

Darshan Anthony Young

Master of Plant Biotechnology
Faculty of Biosciences

Abstract

Plants have adapted to seasonal constraints of the temperate environment by exploiting annual fluctuations in day length. Well timed flowering is essential to maximize reproductive output in temperate regions with short growing seasons. Many temperate plants time their flowering response with lengthening photoperiods to anticipate the favorable growing seasons in late spring and early summer. Photoperiodic regulators are the keys that unlock the flowering response mechanism. The key photoperiodic regulators being investigated in thesis are the genes *CONSTANS* (*CO*), *PHOTOPERIOD 1* (*PPD1*) and *PHYTOCHROME C* (*PHYC*). As a study system, I will use the Pooideae subfamily of grasses, which dominate northern temperate and arctic environments. This largely long-day flowering subfamily evolved from a short-day flowering ancestor. Phylogenetic analysis of the *CO1* and *CO2* paralogs revealed a duplication at the base of the Pooideae, however while *CO2* was well represented in the core Pooideae species only a few basal species possessed the *CO2* paralog. This may indicate that CO2 evolved a function in flowering relatively late in the evolutionary history of Pooideae. *PPD1* was highly expressed in long days in comparison with short days in the basal Pooideae *Piptatherum milaceum.* This is similar with expression patterns in long day flowering Pooideae model species. This suggests that this gene evolved to be a key photoperiodic regulator in long-day flowering, implying that the evolution of long day flowering in the Pooideae subfamily may be centered on this gene.

Acknowledgements

I would like to thank my supervisors Siri Fjellheim and Marian Schubert for their amazing advice and support through my master's project. I would also like to thank Jill Preston for her advice and providing material I needed. I am grateful to Yara Norway and NMBU's research Foundation for financial support. Also, Sylvia Johnsen for direction in the lab. I would also like to thank "The Cartel" Dajana, Nico and Sheona for their support academic and otherwise. My lab group, Ursula, Camila, Martin and Erica. Thanks to my family and friends back home and Maddie for bringing me food. I would also like to thank Half Nelson who will get me a cool beverage at the end. Also, Uncle Mike and Kirsti. Lastly I would like to thank Anja for her unwavering support through this process. One love!

Table of Contents

Introduction

Flowering

Plants in temperate environments are adapted to seasonal constraints such as varying photoperiod and temperature, which increases with distance from the equator (Willig et al., 2003). Because the annual fluctuation in photoperiod follows a very predictable pattern, many plants assimilate photoperiod information into developmental responses such as flowering (Imaizumi and Kay, 2006). Photoperiodism, which is a plants response to day-length (Gassner, 1918), allows temperate plants to identify the start of the growing season. The day-length dependent flowering response was first discovered in 1920 by Garner and Allard.

Plants that respond to short photoperiods are referred to as short-day plants (SDPs) and in fact measure increasing night length, until a threshold is reached that initiates flowering (Garner, 1933). SDPs are mostly found in the tropics where lower seasonality is observed. Photoperiodism in SDPs may be utilized to facilitate concerted flowering, which promotes cross pollination (Calle et al., 2010), to avoid high temperature and also to escape extreme rainfall (Kholová et al., 2013). Annual plants from the family Asteraceae, which include Ambrosia (ragweed), Helianthus (sunflower), Verbesina (wingstem) and Xanthium (cocklebur), rapidly grow during summer and initiate flowering late in the growing season using the shortening days as its photoperiodic cue. This strategy allows the large plants to produce many flowers and enhances cross pollination. Flowering precision is crucial to ensure seed development is completed before winter (Calle et al., 2009). The *rabi* variety of Sorghum flowers during the cooler and shorter days in late summer, avoiding the hot rainy season in India (Kholová et al., 2013). Sorghum is very sensitive to variations in photoperiod that facilitates synchronized flowering at the end of the rainy season irrespective of the planting dates. For example, pinnacle emergence is reduced from 54 to 22 days when day length only changes by 20 minutes (Folliard et al., 2004).

The contrasting reaction is found in long-day plants (LDPs), which measure increasing photoperiod before initiating flowering (Garner, 1933). Timing flowering with increasing day-length allows LDPs to avoid the major annual stress of the temperate zone, winter (Thomas and Vince-Prue, 1996). Cold and short winter days in the northern hemisphere limit the growing season to the summer months, unlike the tropics where the growing season is not limited to a specific time of year. Cold temperatures destroy fragile reproductive organs, therefore temperate plants will remain in a vegetative state during the winter (Fowler et al., 1996, Fjellheim et al., 2014). In temperate regions, many plants flower following a two-step

induction process where vernalization (a long period of cold i.e. winter) prevents flowering in the autumn and makes the plants competent to flower in the spring when day-length reach a certain threshold. *Arabidopsis thaliana* is a model LDP that germinates in autumn, survives the winter in vegetative form and delays flowering until spring in response to photoperiodic and temperature cues. This winter annual is classified as a facultative LDP, where short days delay flowering (Rédei, 1962). According to Heide (1994) the long day photoperiodic response is more stringent in latitudes that experience milder and less stable winters to prevent precocious flowering. In higher latitudes with stable seasonality, the combination of vernalization and photoperiodism to initiate flowering may be completely absent (Heide, 1994). *Phleum pratense* (Timothy grass) for example, is a forage grass that grows in extremely high latitudes and only depends on long-day photoperiodism to flower(Heide, 1994).

The external coincidence model

The external coincidence model (Pittendrigh, 1972), as described by Imaizumi and Kay (2006), is used to explain the molecular basis of photoperiodism in the model plant *Arabisopsis thaliana* and *Oryza sativa* (rice), LDP and SDP respectively. The daily expression pattern of the key regulator of photoperiodism is controlled by circadian clock genes and sets the peak of the regulator when daylight is observed in the late afternoon. In LDPs the regulator promotes flowering in long days while it suppresses flowering in SDPs under similar conditions. This mechanism provides a molecular explanation for the external coincidence model (Imaizumi and Kay, 2006). Research in *A. thaliana* shows that at the center of precise timing of flowering is the gene *CONSTANS* (*CO*). Under long days, expression of *CO* is regulated by circadian clock genes and the coinciding expression of *CO* and the main floral integrator gene in angiosperms *FLOWERING LOCUS T* (*FT*), induces flowering (Suárez-López et al., 2001, Imaizumi and Kay, 2006).

Long day gene expression

CO acts as a transcriptional activator that governs the day-length dependent induction of *FT* (Samach et al., 2000, Song et al., 2012, Valverde et al., 2004). Circadian clock genes coupled with light during dusk in summer controls the transcription of *CO*. The buildup of *CO* that starts in the late afternoon and continues into the night, leads to high levels of CO protein in the afternoon, which promotes the initiation of flowering (Song et al., 2012, Suárez-López et al., 2001, Valverde et al., 2004).

In *A. thaliana*, *CO*'s transcriptional activity is restricted to long-day afternoons. CYCLING DOF FACTORS (CDFs) restrict the transcription of *CO* in the morning and the PSEUDO-RESPONSE REGULATOR (PRR) family represses *CDF* transcription in the late afternoon (Nakamichi et al., 2010) allowing *A. thaliana* to differentiate between long and short days. Blue-light dependent formation of FLAVIN-BINDING, KELCH REPEAT, F-BOX1 (FKF1)–GIGANTEA (GI) complex, regulated by the circadian clock, releases *CO* transcriptional repression (Sawa et al., 2007). Releasing *CO* from CDF repression allows for transcription induction by FLOWERINGBHLH1 (FBH1; Ito et al., 2012). CO protein is under light dependent control where various signals through far-red and blue light stabilize CO in long days, while red-light signals cause destabilization. Day-length affects the abundance of CO, which exhibits drastic variation in concentration between day and night (Möglich et al., 2010, Valverde et al., 2004). Morning degradation of CO is regulated by CONSTITUTIVE PHOTOMORPHOGENIC 1 (COP1), HIGH EXPRESSION OF OSMOTICALLY RESPONSIVE GENES 1 (HOS1) and PHYTOCHROME B (PHYB) (Jang et al., 2008, Lazaro et al., 2012, Liu et al., 2008, Valverde et al., 2004). Due to red-light repression CO accumulation is restricted to the late afternoon in long-days leading to high *FT* expression at the end of the photoperiod. FKF1 along with PHYTOCHROME A (PHYA) and cryptochromes (CRY) play important roles in CO stabilization in long-day afternoons. FKF1 and CO even share a similar diurnal rhythm (Valverde et al., 2004). *FT* transcription is not limited to the effect of *CO*, but also a plethora of environmental and endogenous factors, which allows for precise seasonal responses (Andrés and Coupland, 2012, Castillejo and Pelaz, 2008).

Short day gene expression

The model plant for short-day photoperiodic flowering is rice (*Oryza sativa*; Izawa, 2007). Short-day flowering follows an external coincidence model with the opposite effect of *A. thaliana.* Flowering is still mediated by an external photoperiodic stimuli that coincides with the internal circadian clock (Song et al., 2015). The ortholog of *A. thaliana FT* in rice is *Heading date 3a* (*HD3a*) and is controlled by the key photoperiodic regulator *Heading date 1* (*Hd1*), an ortholog of *CO* (Izawa et al., 2002). Like *CO*, *Hd1* is highly expressed during the afternoon of long-days, however *Hd1* acts as a repressor of flowering under these conditions. When *Hd1* expression coincides with darkness in short days it acts as a flowering promotor, activating *Hd3a*. The ortholog of *A. thaliana GI*, *OsGI*, is the circadian clock component that regulates *Hd1* (Hayama et al., 2003). Phytochromes, specifically PHYB, are responsible for converting Hd1 from a promotor to a repressor in long-day afternoons (Ishikawa et al., 2011,

Izawa et al., 2002), however the mechanism underlying this conversion has not been determined (Song et al., 2015).

Pooideae

The global expanse of grasslands occurred from the Oligocene (34 million years ago; MYA) until the early Miocene (23 MYA) period (Strömberg, 2011). This periods also represents the transition from a more tropical world to an increase in the abundance of temperate ecosystems (Bouchenak-Khelladi et al., 2010). Even though the majority of extant grass subfamilies inhabit tropical and subtropical climates, the largest grass subfamily Pooideae predominantly inhabits temperate as well as arctic biomes (Hartley, 1973, Visser et al., 2013). In their natural range the Pooideae are exposed to high seasonality (GPWG, 2011, Edwards et al., 2010). The subfamily Pooideae belongs to the grass family Poaceae, which is divided into two monophyletic clades. The BOP clade comprises the subfamilies Bambusoideae, Oryzoideae, and Pooideae and the PACMAD clade comprises the subfamilies Panicoideae, Aristidoideae, Chloridoideae, Micrairoideae, Arundinoideae, and Danthonioideae. The predominantly tropical PACMAD grasses share a common ancestor with the BOP clade between 50 and 65 MYA. Within the Pooideae subfamily the core Pooideae and Brachypodieae diverged from their common ancestor 35 MYA (Bevan et al., 2010), subdividing the subfamily into the core and basal Pooideae (figure 1). The core Pooideae further evolved into tribes, such as Triticeae and Poeae, which include some of the most commercially significant crops like wheat, barley and oats (Soreng et al., 2017, Hartley, 1973, Visser et al., 2013). Increasing latitudes mark the transition from tropical to arctic conditions. The northern temperate zone extends from 23.5˚ north to the Arctic Circle and includes climate from sub-tropical to boreal. Characteristics of the temperate zone are lower temperatures, drier climates, greater seasonal variability with a limited annual growing seasons (Wiens et al., 2010). This is further attenuated in the arctic zone. Plants that live in temperate and arctic regions have therefore adapted to high seasonality. To adjust their growth and development with favorable environmental conditions throughout the seasons, the plants make among others use of photoperiodic cues.

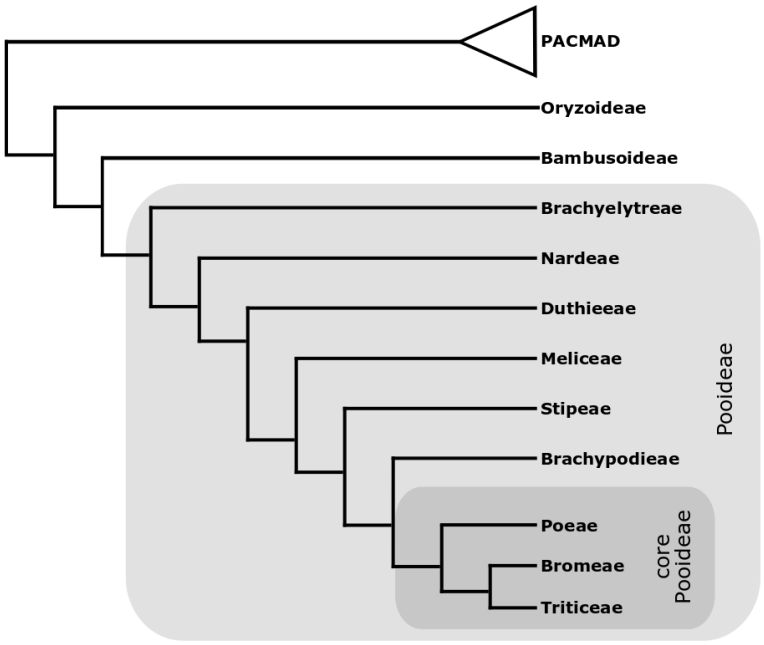


Figure 1. Phylogenetic tree highlighting the tribes of the Pooideae subfamily of grasses and defining the core Pooideae (dark grey box). Adjacent to the Pooideae are the sister clades within the BOP clade, Bambusoideae and Oryzoideae, and the neighboring PACMAD clade

The core and basal Pooideae are generally LDPs found in temperate climates (Fjellheim et al. 2014, Fjellheim unpublished). The Pooideae sister subfamily Oryzoideae are SDPs mainly confined to the tropics (GBIF, 2017, Imaizumi and Kay, 2006). Consequently, it was inferred that long-day photoperiodism evolved in a Pooideae ancestor (Fjellheim et al., 2014). How the Pooideae, whose common ancestor is likely a tropical SDP, evolved into temperate plants flowering in long days is unknown.

The ortholog of *CO* from *A. thaliana* has two paralogs in the core Pooideae denoted *CO1* and *CO2* (Shimada et al., 2009). Homologous to *CO* promotion of *FT* in *Arabidopsis* overexpression of the *CO* ortholog in barley, *HvCO1*, activates *HvFT1* under long-day treatment (Campoli et al., 2012). The diurnal expression of *CO1* in wheat is most highly expressed approximately 16 hours after dawn regardless of the photoperiod, suggesting circadian clock entrainment (Chen et al., 2014b, Shaw et al., 2012). Once transition to the reproductive stage is reached, a decline in *CO1* expression is observed suggesting *FT1* (*FT* ortholog in core Pooideae) has a negative feedback mechanism that represses *CO1* (Kitagawa et al., 2012, Shaw et al., 2013). *CO2* may be a candidate for *FT1* maintenance after the decline of *CO1* (Kitagawa et al., 2012).

The gene *PHOTOPERIOD 1* (*PPD1*) regulates photoperiodic sensitivity in long-day cereals (Nishida et al., 2013) and is upregulated during both long and short days, which is accompanied by the upregulation of *FT1* (Chen et al., 2014a, Kitagawa et al., 2012, Shaw et al., 2012).

During the night *PPD1* is only expressed at basal levels. The homolog in *A. thaliana* is *PRR7* whose gene family represses *CDF*. In rice the *PPD1* homolog *PRR37* represses flowering in long-days. *PRR37* expression affect the daily expression of floral regulators and may directly affect *FT*. In wheat upregulation of *FT1* is coupled with the upregulation of *PPD1* (Song et al., 2015). The action of red light via PHYTOCHROME C (PHYC) and *PPD1* regulates *FT1* (Song et al., 2015). Red light leads to the dimerization of PHYC with either itself or PHYB and operates as a transcriptional activator of both *PPD1* and *FT* (Chen et al., 2014b). The interaction of PHYC, PPD1 and CO orthologs is still unknown, however null *PHYC* mutants in core Pooideae affected the expression of *CO1* and *CO2* (Chen et al., 2014a, Woods et al., 2014)

Motivation

The Pooideae subfamily of grasses have adapted to the highly seasonal northern temperate climate using predictable annual photoperiodic patterns to precisely time the initiation of flowering at the start of the short growing season (Willig et al., 2003, Imaizumi and Kay, 2006). My aim is to elucidate the Pooideae's evolutionary history by investigating photoperiodic flowering in a basal Pooideae tribe, Stipeae. Because of the short growing season in the temperate and arctic regions, Pooideae evolved the long-day flowering trait which is preserved in the core Pooideae, (Garner, 1933, Fjellheim et al., 2014). Thus, it is plausible that long-day flowering evolved in the common ancestor of all Pooideae tribes. Some basal Pooideae species however, including *Nassella pubiflora* from the Stipeae tribe, exhibit short-day flowering patterns (Fjellheim, unpublished), whereas other basal lineages respond to long photoperiods. One of these species is *Piptatherum miliaceum*, also belonging to the Stipeae tribe. Whether the long photoperiod response has evolved from a short photoperiod response or vice versa is unknown. At the core of photoperiodc flowering in grasses are the photoperiod regulators, *CO1*/*CO2*, *PPD1* and *PHYC* (Song et al., 2015). I will examine the evolutionary history of these genes in a phylogenetically widespread set of Pooideae species, representing most tribes. I hypothesize the phylogeny of these genes will reflect the phylogeny of the investigate Pooideae species. The Pooideae share a common ancestor with the predominantly SDPs of the Oryzoideae (Soreng et al., 2017), thus the short-day flowering pattern of *N. pubiflora* might reflect an ancient short-day pathway homologous to the one in Oryzoideae species. In that case, I expect the gene expression of the key photoperiodic regulators in *N. pubiflora* to reflect the expression patterns observed in Oryzoideae. LDPs are found in the majority of Pooideae species. If indeed long-day photoperiodism evolved once in the common ancestor of Pooideae,

basal LDPs Pooideae, such as *P. miliaceum*, should express the key photoperiodic genes as observed in the core cereals. To analyze the expression patterns and diurnal rhythms between short-day and long-day Pooideae RT-qPCR experiments were carried out on *PPD1*, *PHYC* and *CO* in the basal Pooideae species from the Stipeae tribe, *P. miliaceum* and *N. pubiflora*, which express long- and short-day photoperiodism respectively (figure 2). The expression patterns will be compared to published expression patterns of *Oryza sativa* and *Hordeum vulgare* to establish if respective expression patterns are conserved or derived.

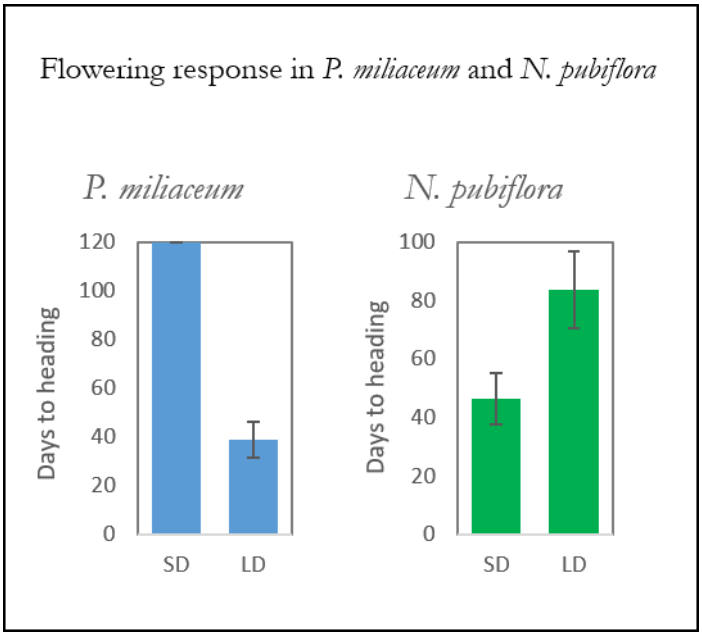


Figure 2. Flowering response of *N. pubiflora* and *P. miliaceum* showing days to heading after the initiation of photoperiodic treatment. SD = Short day treatment (8 hrs light, 16 hrs dark), LD = Long-day treatment (16 hrs light, 8 hrs dark)

Materials and Methods

Phylogenetic analysis

Transcriptome data from species in appendix 2 was used as a database for the phylogenetic study of the *CO* gene family, *PPD1* and *PHYC*. As reference, coding sequences of *H. vulgare* identified by Griffith et al., 2003 were downloaded from GenBank (Benson et al., 2012). These sequences acted as queries in a BLAST search (Altschul et al., 1990) for coding sequences from *Brachypodium distachyon* and outgroup reference species, which included Bambusoideae, Oryzoideae and Panicoideae representatives. The outgroup sequences were the best coding sequences (cds) BLAST hits for the *H. vulgare* reference sequence. To ensure proper rooting of the phylogenetic tree for *CO1* and *CO2*, I also included two coding sequences from *A. thaliana CO* orthologs. A custom BLAST database was built from our *de novo* transcriptome data (appendix 2). The *H. vulgare* reference sequences were used to query our database with BLAST (Altschul et al., 1990) to extract target sequences from the transcriptome data resulting is several transcripts from each species and target sequence. For each species and target gene all BLAST hits (Altschul et al., 1990) were filtered by retaining sequences with a bitscore of at least 300 and an e-value equal to or below 1e-81. An initial alignment including *de novo* transcripts, outgroup and Pooideae reference sequences was generated with the open reading frame aware alignment program MACSE (Ranwez et al., 2011) accounting for frameshifts and stop codons. The nuclear substitution model that fit best to each alignment was the GTR+Gamma substitution model. This was determined using the JModelTest 2 (Moumene and Meyer, 2016). The GTR+Gamma nuclear substitution model was used to build an initial maximum likelihood tree with PhyML (Guindon et al., 2010) to identify the smallest clade that included all the outgroup reference species plus *H. vulgare* and *B. distachyon* reference sequences. Branch supports were estimated by an approximate likelihood ratio test that returned a Chi2 based metric. A custom script was used to remove the transcripts that were not part of the previously identified clade using the 'mcra' function in the 'ape' package. Subsequently, highly identical *de novo* transcripts were clustered for each species and only the best representative per transcript cluster was chosen using CD-HIT-EST (available from http://weizhongli-lab.org/cd-hit/, accessed 13.08.2018) with the c function set to 0.9 (i.e. 90% sequence identity threshold). The program exonerate v.2.2.0 (Slater and Birney, 2005) was used to identify intron-exon boundaries and remove putative introns and untranslated region (UTR) from *de* novo sequences. After another round of CD-HIT-EST clustering using a sequence identity threshold of 98% *de novo* transcript sequences containing less than one third

of the number of nucleotides of the reference sequence were discarded. The remaining *de* novo transcript sequences were aligned with outgroup and Pooideae reference sequences using MACSE, treating *de novo* sequences as less reliable sequences, i.e. the cost for introducing framshifts and gaps was lower for this set of sequences (Ranwez et al., 2011). Final phylogenetic trees were constructed using PhyML as previously described and visually adjusted in figtree v1.4.3 (available from, http://tree.bio.ed.ac.uk/software/figtree/, accessed 13.08.2018) and inkscape v0.92.3(available from https://inkscape.org/en/, accessed 13.08.2018)

Plant growth experiments

Plant growth experiments and cDNA preparation was done prior to the start of my project at the University of Vermont (UMV, Fjellheim and Preston unpubl). Seventy two plants of each *N. pubiflora* and *P. miliaceum* were grown in 12 hour light treatment prior to the start of the experiment at 20˚C for 4 weeks. This temperature was maintained throughout the duration of the experiment. Subsequently, half of the plants from each species were transferred to long-day treatment (16 h light, 8 h dark) while the remaining samples were transferred to short-day treatment (8 h light 16 h dark). Starting on the second day of the respective photoperiodic regiments three plants (biological repeats) were removed from each treatment for RNA extraction at four different time-points, six hours apart, beginning at 10:00 hrs. RNA was extracted from the selected samples that was used to generate cDNA. The same sampling procedure and RNA extraction was repeated 14 and 28 days after plants were transferred to their respective treatment. The generated cDNA was used to determine the relative expression of the key photoperiod genes in question.

RT-qPCR

Relative expression data of key photoperiod regulator genes was investigated in both long- and short-day treatments of Pooideae species from the Stipeae tribe that are known long-day (*P. miliaceum*) and short-day (*N. pubiflora*) responsive plants (figure 2, Fjellheim and Preston unpubl.). Primers for *CO1* were used as described in Fjellheim and Preston (unpubl., appendix 1) Key photoperiod mediating genes *PPD1* and *PHYC* were extracted from *N. pubiflora* and *P. miliaceum* transcriptome data sets by identifying homologous targeted gene sequences from phylogenetic trees, as described above. Target gene sequences generated from transcriptome data were aligned in Unipro UGENE (Okonechnikov et al., 2012) to form consensus sequences, which were subsequently used to design gene-specific, RT-qPCR primers (appendix 1) using Primer3Plus v2.4.2 (Untergasser et al., 2012). The housekeeping genes *EF1* (Scoville Alison

et al., 2011, Woods et al., 2016) and *E1F4a* (Fjellheim and Preston unpubl.) were used as references to relatively quantify the expression of the target genes (appendix 1). PCRs were run to ensure that primers specifically amplified one amplicon resulting in one distinct band on electrophoresis gels. Primer efficiency (E) was tested for each target-species combination using a 2-fold dilution series. Ideally E should range from 0.90 – 1.10, where 1.00 represents doubling of the target sequence amplicons after each thermal cycle.

To determine threshold cycle (C_t) values that were accurate throughout the gene expression analysis a consistent threshold value was selected that gave a significant signal over the calculated baseline signal. The □□C_t method was employed, where calibration of the sample genes were carried out with a normalizer (housekeeping gene) to determine the relative expression (Livak and Schmittgen, 2001). A 20 ul reaction mixture with SYBR™ Select Master Mix (Thermofisher Scientific, Carlsbad, CA, USA) from Applied Biosystems was used following the manufacturer's instructions. Three technical replicates were quantified per biological replicate grown in either long- or short-day treatments. RT-qPCR for gene expression quantification and primer efficiency were carried out on an Applied Biosystems 7500 Fast instrument (ThermoFisher Scientific, Waltham, MA, USA). Once the RT-qPCR reaction was complete melt curves were examined to ensure a single peak and the appropriate melting temperature for the amplicon was attained.

Statistical testing

The statistical test selected for the relative gene expression data was a two-way ANOVA. *CO1*, *PPD1* and *PHYC* were analyzed to test if they were differentially expressed between short-day and long-day photoperiod, across the duration of the experiment and the interaction between photoperiod and duration. For each of the four time points per day the two-way ANOVA was carried out per species and gene. Raw data was analyzed where the residuals fit a normal distribution. The stats and car packages in R (RStudio v1.1.447, RStudio team, 2015) were the platforms for all ANOVAs, and the residual plotting was done using the rcompanion package in R (RStudio v1.1.447, RStudio team, 2015). The expression data was scaled and centered using the R's scale function (RStudio v1.1.447, RStudio team, 2015) for all genes and days for each species. Centering and standardizing effectively disregarded sampling days and variations in biological replicates to visualize whether relative expression patterns followed diurnal patterns.

Results

Phylogenetic analysis

To resolve the phylogenetic history of the *CO1*/*CO2* paralogs a combined gene tree was constructed (figure 3) using maximum likelihood analysis. *A. thaliana* cds sequences for *CO* were introduced to root the gene tree, due to *CO2* not being represented in outgroups or sister clades. Two distinct monophyletic clades were reconstructed that represented each of the *CO* paralogs (probability = 1.00). *CO1* genes from the outgroup subfamilies Panicoideae, Bambusoideae and Oryzoideae formed a monophyletic clade with the Pooideae *CO1* othrologs. Core Pooideae species were identified in both *CO* clades (p = 1.00; grey box, figure 3). The topology of the *CO1* clade followed the Pooideae species tree topology and copies of *CO1* were identified in all the Pooideae transcriptomes used for this study. However, *CO2* sequence data was not identified in all the Pooideae transcriptomes. Species not present in the *CO2 clade* were *Nardus stricta*, *Duthiea brachypodium* and from the Stipeae tribe, *Stipa lagascae*, *Nassella pulchra* and *Piptatherum miliaceum*. The only Stipeae species present in the CO2 clade was *Piptatherum aequiglume*. The other remaining *CO2* sequences identified represented members of the core Pooideae, the Meliceae tribe, *Brachypodium distachyon* and *Brachyelytrum aristosum*. The blue arrow in figure 3 represents the suspected point of duplication of the Pooideae *CO* paralogs that will be further discussed.

The phylogenetic analysis of *PPD1* (figure 4), alternatively denoted as *PRR37*, resembled the topology to the Pooideae species phylogeny. The tree was rooted with the *PRR37* paralog *PRR73* from outgroup members *O. sativa* and *Z. maize*, and also Pooideae species *H. vulgare*, *Aegilops tauschii and B. distachyon*. No gene losses or major duplication events were observed, which suggests high sequence conservation of *PPD1* in Pooideae.

The *PHYC* phylogenetic analysis suggests a partially conserved Pooideae topology. Outgroups from the Panicoideae subfamily and *Oryza* rooted the tree. The core Pooideae, *Brachypodium*, *Nardus* and *Brachyelytrum* were found in the typical Pooideae topological positions. However, the placement of members of the Stipeae tribe was not clearly resolved. Although highly similar *de novo* transcripts were identified in Meliceae species, none of them satisfied the criteria of the filtering pipeline (i.e. they were too short) and were discarded from the final alignment used to generate the phylogenetic tree (figure 5). Thus the *PHYC* gene tree lacks sequences representing the Meliceae tribe.

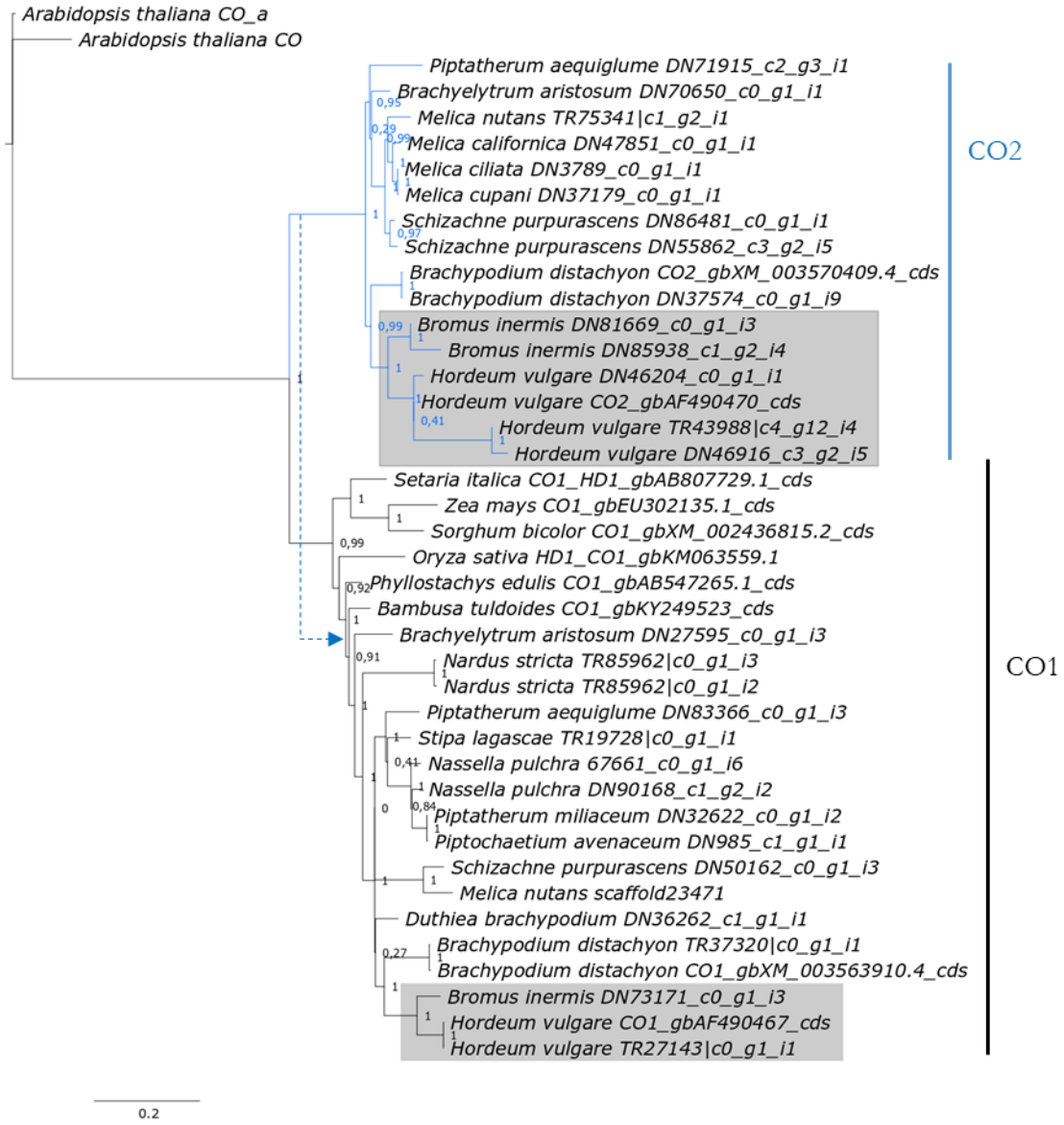


Figure 3 Phylogenetic tree of *CO1*/*CO2* gene family. Maximum likelihood tree was estimated using PhyML 3.0 including outgroup sequences from non-Pooideae species and *A. thaliana*, reference sequences from various core Pooideae, *B. distachyon* and *de novo* transcript sequences. Branch support is shown as node labels displaying the results of the approximate likelihood ratio test. Dashed arrow indicates the proposed position of the *CO2* clade. Shaded boxes mark the core Pooideae.

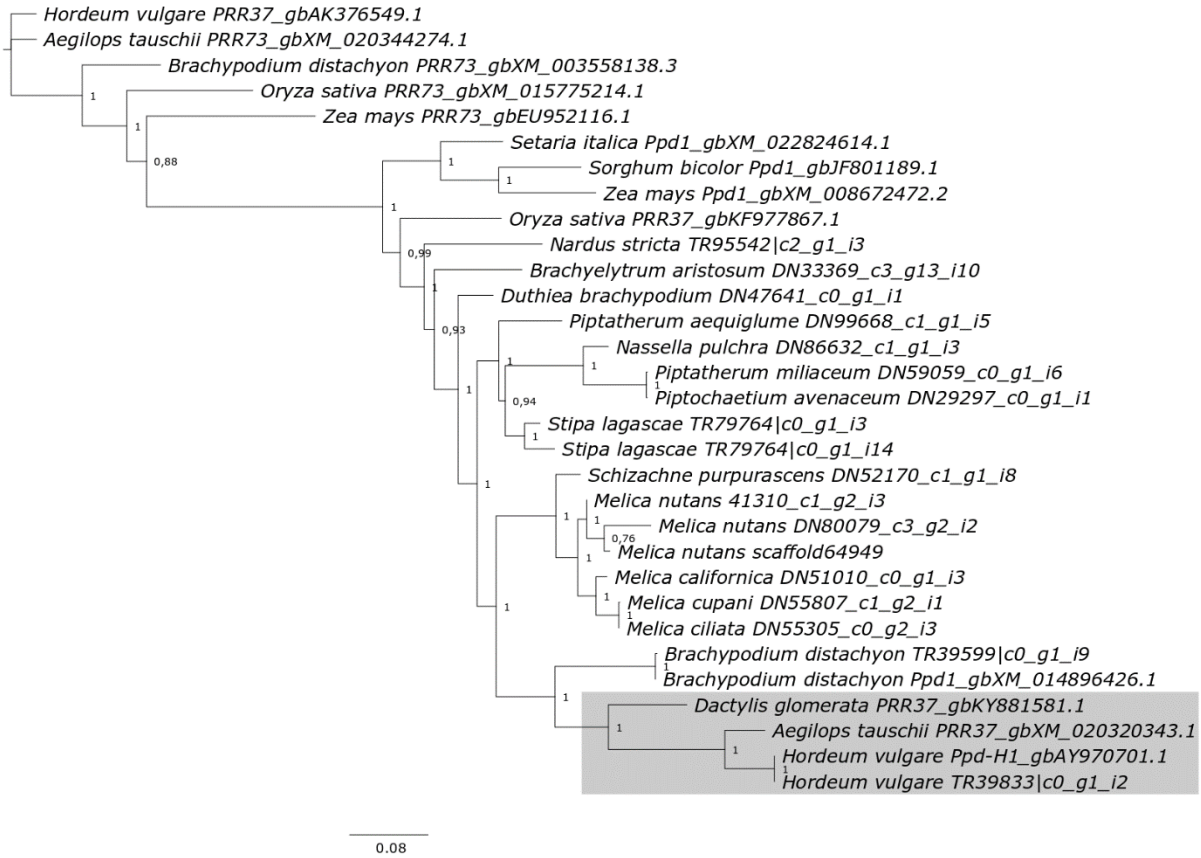


Figure 4 Phylogenetic tree of the *PPD1* (*PRR37*) gene. Maximum likelihood tree was estimated using PhyML 3.0 including outgroup sequences from non-Pooideae species, reference sequences from various core Pooideae, *B. distachyon* and *de novo* transcript sequences. Branch support is shown as node labels displaying the results of the approximate likelihood ratio test. Shaded boxes mark the core Pooideae.

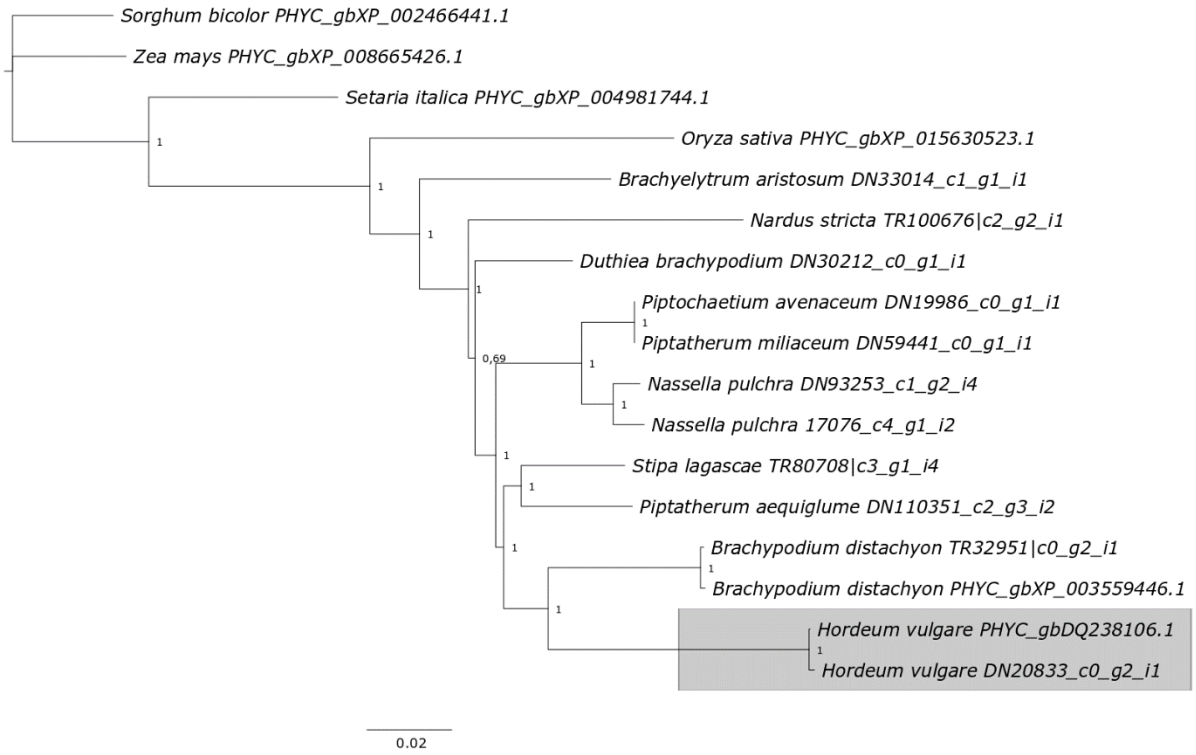


Figure 5 Phylogenetic tree of *PHYC* gene. Maximum likelihood tree was estimated using PhyML 3.0 including outgroup sequences from non-Pooideae species, reference sequences from various core Pooideae, *B. distachyon* and *de novo* transcript sequences. Branch support is shown as node labels displaying the results of the approximate likelihood ratio test. Shaded boxes mark the core Pooideae.

Phylogenetic analyses were also performed for *CO* paralogs *CO3*, *CO4*, *CO5*, *CO6* and *CO8* (appendix 3). All except *CO6* resembled the typical Pooideae topology. However, at least one of the basal Pooideae tribes investigated in this study was not represented in each of those gene trees.

Gene expression of *CO1*

CO1 in *N. pubiflora* was most highly expressed after 22:00 hrs in each day of short-day treatment while being expressed relatively low at 10:00 and 16:00 hrs. A downward trend in relative expression of *CO1* was observed between day two and day 28, however, this decrease was not statistically significant. In long-day treatment daily *CO1* expression in *N. pubiflora* also peaked after 22:00 hrs on day 2 and day 14. On day 28 *CO1* expression was relatively high at 22:00 but peaked at 04:00 hrs. A statistically significant difference was observed between the two daylength treatments at 16:00 hrs and 22:00 hrs ($P < 0.05$), where *CO1* expression was lower in short-day treatment. The diurnal rhythms observed between the two treatments were mostly in sync (figure 6). *CO1* in *P. miliaceum* was also highly expressed after 22:00 hrs in each days of long-day treatment. The peak on day 2 and day 28 was at 04:00 hrs, while on day

14 the peak was observed at 22:00 hrs. In long-day treatment between day 2 and day 28 a downward trend was observed in the relative expression of *CO1*. A statistically significant difference was observed over the three days at the 10:00 hrs time point ($P < 0.05$). No data was generated for the 10:00 hrs time point on day 2 during long-day treatment. The diurnal rhythm observed between the two treatments were mostly in sync (figure 6).

Gene expression of *PPD1*

The relative expression of *PPD1* in *N. pubiflora* was relatively low in both treatments. Over the course of each day the highest expression was observed at 10:00 hrs and steadily declined until it was most lowly expressed at 04:00 hrs. Statistical analysis showed a marked difference in expression at 22:00 hrs between long and short-days ($P < 0.05$), but no distinction could be made between the diurnal rhythms of the two treatments. In *P. miliaceum* a significant statistical difference between treatments was observed at time points 10:00 hrs, 16:00 hrs and 22:00 hrs ($P < 0.01$). At 04:00 hrs expression was at its lowest in both treatments. Expression was consistently higher in long-day treatment with time points between 10:00 and 16:00 hrs displaying the highest expression. A significant statistical difference was also observed in the expression of *PPD1* at 10:00 hrs between the three days ($P < 0.01$), where a decline in relative expression was observed. Also, a statistical differnece in the interaction of treatment and duration at 10:00 hrs was observed ($P < 0.01$). Diurnal rhythms for the most part were in sync between treatments, except on day 28 when the peak expression of *PPD1* in long day treatment was observed to be 16:00 hrs (figure 7).

Gene expression in *PHYC*

No statistical differences in *PHYC* were observed in treatment, duration or their interaction in both species. In *N. pubiflora* the diurnal pattern between the two treatments were mostly in sync where relative expression peaked at 16:00 hrs and 04:00 hrs each day. A discrepancy between treatments was observed at 16:00 hrs on day two, but lacked statistical support. Diurnal expression in *P. miliaceum* appreared out of sync, the only commonality was observed at 10:00 hrs when PHYC was lowly expressed (figure 8).

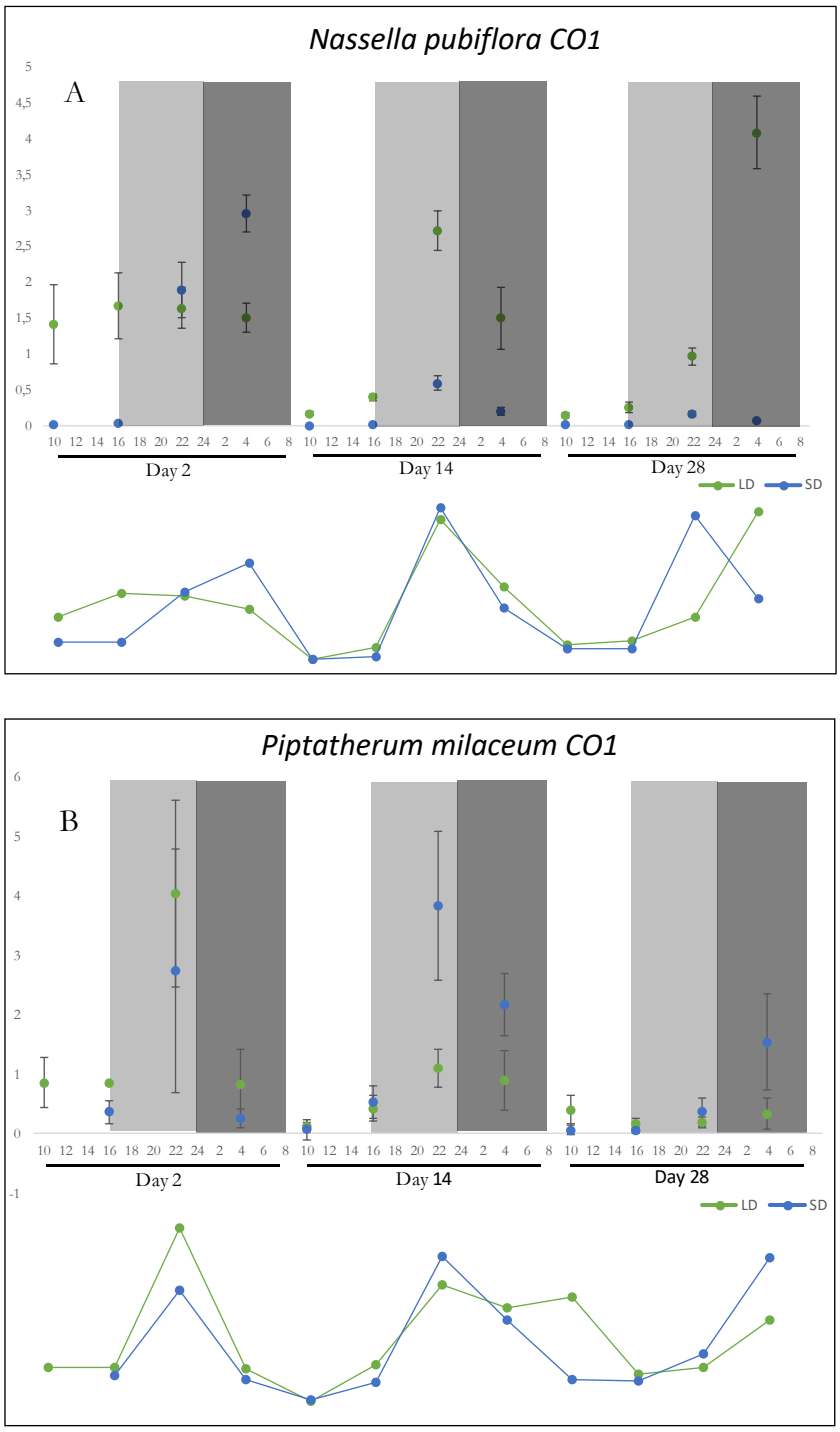
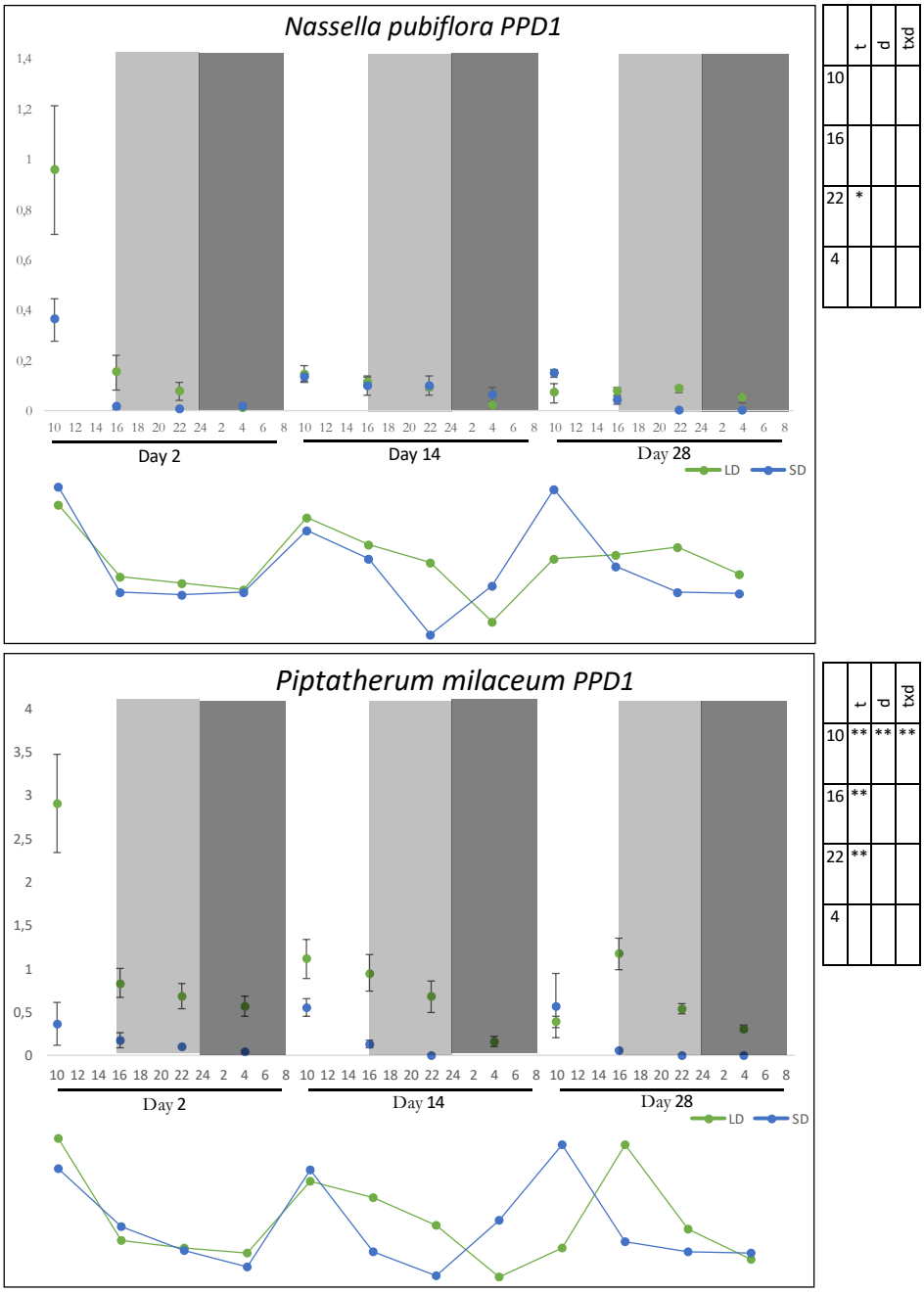


Figure 6 Relative expression of *CO1* including diurnal rhythms below in (A) *Nassella pubiflora* and (B) *Piptatherum miliaceum*. The diurnal rhythm is the scaled and centred data from the first panel. Light grey bar represents night in only short-day treatment and the dark grey bar represents night in both treatments. Days after the start of the photoperiodic treatement indicated below the x-axis. Green and blue data points represent long- and short-day treatment respectively. Error bars indicate the standard deviation of the three technical replicates averaged across the three biological samples. Statistical significance on treatment (t), duration (d) and the interaction of t and d. *Significance levels*: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ (Two-way ANOVA)



	t	d	txd
10			
16			
22	*		
4			

	t	d	txd
10	**	**	**
16	**		
22	**		
4			

Figure 7 Relative expression of *PPD1* including diurnal rhythms below in (A) *Nassella pubiflora* and (B) *Piptatherum miliaceum*. The diurnal rhythm is the scaled and centred data from the first panel. Light grey bar represents night in only short-day treatment and the dark grey bar represents night in both treatments. Days after the start of the photoperiodic treatement indicated below the x-axis. Green and blue data points represent long- and short-day treatment respectively. Error bars indicate the standard deviation of the three technical replicates averaged across the three biological samples. Statistical significance on treatment (t), duration (d) and the interaction of t and d. *Significance levels*: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ (Two-way ANOVA)

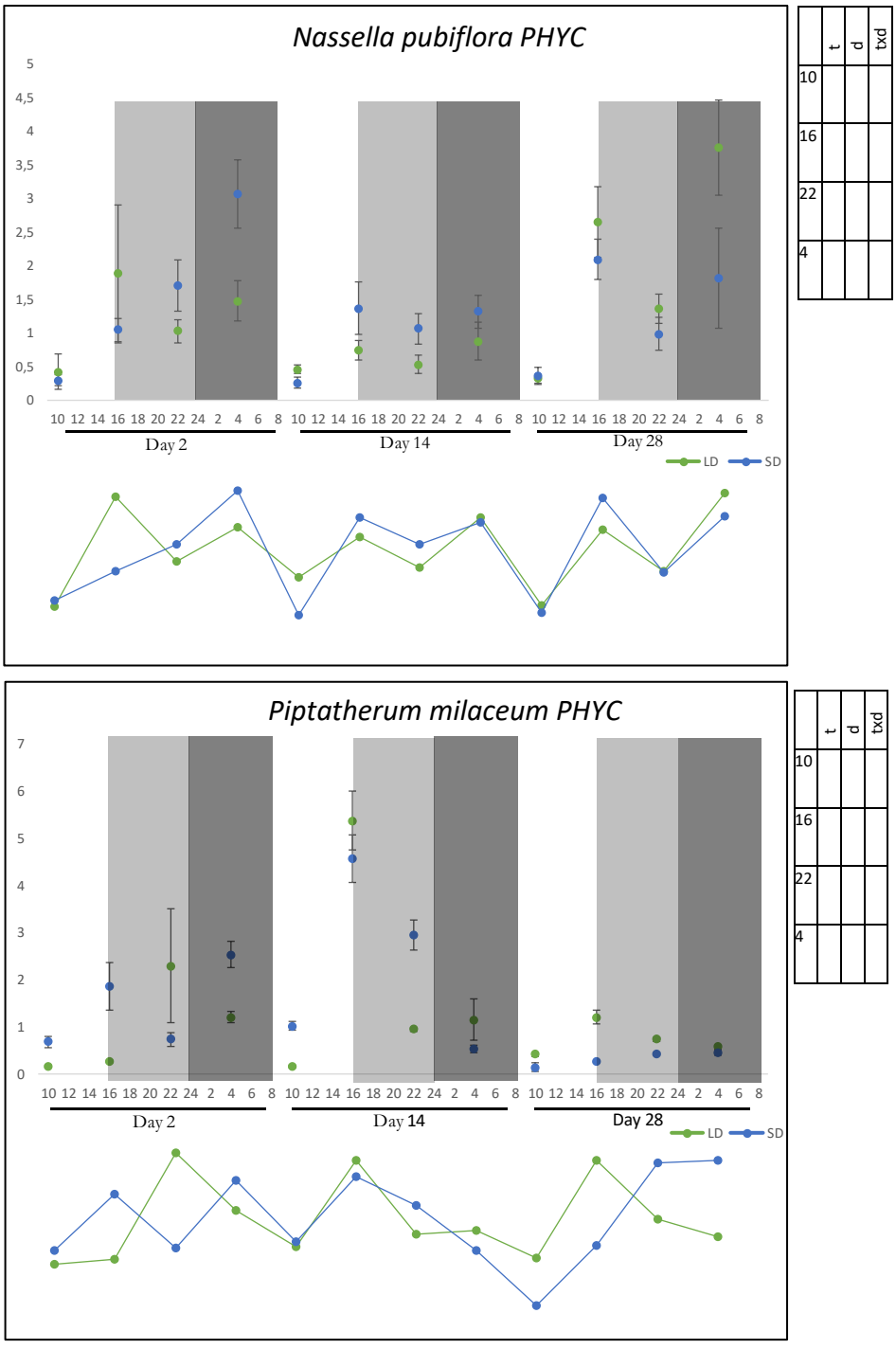


Figure 8 Relative expression of *PHYC* including diurnal rhythms below in (A) *Nassella pubiflora* and (B) *Piptatherum miliaceum*. The diurnal rhythm is the scaled and centred data from the first panel. Light grey bar represents night in only short-day treatment and the dark grey bar represents night in both treatments. Days after the start of the photoperiodic treatement indicated below the x-axis. Green and blue data points represent long- and short-day treatment respectively. Error bars indicate the standard deviation of the three technical replicates averaged across the three biological samples. Statistical significance on treatment (t), duration (d) and the interaction of t and d. *Significance levels*: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ (Two-way ANOVA)

Discussion

CONSTANS

The phylogenetic analysis of the *CO1/CO2* paralogs suggests a duplication at the base of the Pooideae, as only one copy of the gene exist in the outgroup species of the Panicoideae, Oryzoideae and Bambusoideae subfamilies and both genes exist in *Brachyelytrum aristosum*, which belongs to the earliest diverging Pooideae tribe (Fig. 2).

The phylogeny of the *CO1* clade shows the presence of *CO1* copies in all Pooideae species investigated and reflects the species tree topology. This indicates the central and conserved role of *CO1* in flowering throughout the Pooideae subfamily. *CO2* is well represented in the core Pooideae, *Brachypodium* and Meliceae, where all investigated species possess a copy of the gene. However, even if the duplication happened at the base of the Pooideae subfamily, I was unable to identify the *CO2* gene among most transcriptomes and genomes of Stipeae tribe members as well as in *Duthiea* and *Nardus*, leaving only *Piptatherum aequiglume* and *B. aristosum* from the early diverging lineages of this study. The absence of *CO2* transcripts in many of the basal Pooideae lineages suggests a loss of the gene or the gene transcription. Among the two low-coverage genomes that were screened for presence of *CO2* (*Nardus stricta* and *Stipa lagasceae*; appendix 2) none of them included *CO2*, supporting the notion that the gene was lost. Multiple independent losses of gene or gene expression within the Pooideae would suggest that the function of *CO2* is less critical than that of other key photoperiodic regulators. In barley and other long-day cereals *CO2* promotes *FT* once *CO1* expression begins to decline (Song et al., 2015). The presence of *CO2* in all species of Meliceae, *Brachypodium* and core Pooideae may indicate that a function in long day flowering for *CO2* evolved at the base of this clade. The phylogenetic analysis of the *CO1*/*CO2* paralogs was not able to reconstruct the putative true history of the genes because only coding sequences were used. The high sequence conservation in the *CO1* copies, which is likely due to purifying selection pressure caused by its central role in regulation of flowering, masked reduced the phylogenetic signal supporting its sister relationship to the *CO2* clade. However, previous studies (Griffiths et al., 2003) have shown that *CO1* and *CO2* are indeed Pooideae-specific paralogs. Thus, I manually indicated the most likely position of *CO2* (Fig. 2). To resolve this conflict in my gene tree topologies non-coding regions of the *CO1* and *CO2* genes would also have to be analyzed due to their lower degree of sequences conservation and stronger phylogenetic signal.

The Pooideae have independent pathways that maintain photoperiodic regulated flowering centered on *PPD1* and *CO1* (Song et al., 2015). In wheat, *PPD1*, *CO1* and *CO2* all act to upregulate the central flowering gene *FT* in long days (Li et al., 2011). However, *CO2* is expressed at basal levels while *CO1* is highly expressed prior to the initiation of flowering. Once *FT* expression is initiated, *CO1* expression begins to decline and *CO2* expression starts to increase. This trend continues through the initiation of flowering until *CO2* is more highly expressed at terminal spikelet formation and heading. The timing of *CO2* upregulation during the decline of *CO1* expression may be a recently evolved function of *CO2*

in long-day cereals, conserving *CO2* expression (Song et al., 2015). *HvCO2* when overexpressed in barley causes overexpression of *HvFT1* (Mulki and von Korff, 2015) Analysis of *CO2* expression between basal and core Pooideae would show if the function observed in long-day cereals is conserved throughout the Pooideae.

The diurnal rhythm of *CO1* was maintained in both photoperiodic treatments of *N. pubiflora* and *P. miliaceum* where low relative expression values were observed in the day compared to high values at night. In long-day cereals *CO1* expression is very similar to *A. thaliana* where it peaks approximately 16 hours after dawn (24:00 hrs) regardless of the photoperiod (Chen et al., 2014, Shaw et al., 2012). In *Oryza* a conserved diurnal rhythm is also observed where *Hd1*, the *CO1* ortholog, peaks at a similar time. (Osugi et al., 2011, Song et al., 2015).This closely corresponds to the peaks observed in both *N. pubiflora* and *P. miliaceum* and does not differentiate between long-day and short-day treatments. Similar expression pattern may influence contrasting functions as seen in *Oryza* and *Hordeum* where *CO1* can either repress or promote the expression of *FT* (Song et al., 2015). From this, I conclude that a shift in expression pattern of *CO1* is not responsible for the difference in photoperiodic flowering in *N. pubiflora* and *P. miliaceum.*

Flowering data shows that *N. pubiflora* flowers earlier under short-day than long-day treatment (figure 2; Fjellheim unpubl). A statistically significant difference in *CO1* expression was observed at 16:00 hrs and 22:00 hrs between short-day and long-day treatments in *N. pubiflora*, where *CO1* expression was lower in short day treatment at these time points. Also, a downward trend in the relative expression of *CO1* in short-day treatment between day 2 and day 28 was observed. In *O. sativa* the diurnal rhythm of *Hd1* between short-day and long-day treatment is similar. To regulate flowering, *PHYB* at the end of long-days acts on *Hd1*, converting it to a repressor of *Hd3a*. *Hd1* promotes flowering when the effect of *PHYB* is absent during short days, allowing the highly expressed *Hd1* to act as a promotor of *Hd3a* (Osugi et al., 2011, Song et al., 2015). In *N. pubiflora* the higher expression of *CO1* during long-day treatment coincides with a delayed flowering response. This suggests that *CO1* may act as a repressor of flowering in *N. pubiflora*. Although not statistically significant, the difference in expression between treatments highlighted a downward trend in short-day treatment of *CO1*. The lowering of *CO1* expression in short days suggests that the expression of this key regulator may be similar to the expression pattern found in the LDPs of the core Pooideae. (Song et al., 2015). Reduced *CO1* expression after the initiation of flowering has not been reported in *Oryza*. In *P. miliaceum* a significant difference was observed between day two and day 28 at the 10:00 hrs time point, possibly caused by a missing data point on day two in long-day treatment. A downward trend in *CO1* expression was observed that corresponded with long-day treatment and early flowering, similar to the response in *N. pubiflora* during short-day treatment. The downward trend in expression of *CO1* in *P. miliaceum* mirrors the trend observed in the core Pooideae when flowering is initiated (Song et al., 2015). Long-day treatment also initiates flowering in *P. miliaceum* suggesting that *CO1* may have a role in the promotion of flowering.

The phylogenetic analysis of the *CO* paralogs (CO3, CO4, CO5, CO6 and CO8) mostly follow the Pooideae species tree. However, they are less conserved when compared to *CO1*. The gene trees lacked certain species or tribes and exhibited a more dynamic history, which may indicate that the functions of these paralogs are not as conserved in the Pooideae compared to other key photoperiod genes. The *CO6* tree, for example, was unable to be resolved (appendix 3).

PPD1

The phylogenetic analysis of *PPD1* in the Pooideae showed a highly conserved topology. The tree was rooted using the *PRR73* paralog from outgroup members *O. sativa* and *Z. maize*, and also *H. vulgare*, *Aegilops tauschii and B. distachyon*. No gene losses nor major duplications were observed that would be apparent if genes from the same species occupied distinct position in the tree, which suggests that *PPD1* is highly conserved in the Pooideae In both *N. pubiflora* and *P. miliaceum PPD1* is more highly expressed during the day than at night regardless of the treatments. This is similar to long-day cereals where *PPD1* is upregulated throughout the light period and its expression declines during the night (Chen et al., 2014).In wheat *PPD1* requires light to be expressed and will remain upregulated when exposed to continuous light and will decline to basal levels where it will remain if exposed to constant darkness (Chen et al., 2014). Additionally *PPD1* requires activation by *PHYC*, which leads to the promotion of *FT1* (Song et al., 2015).

The relative expression of *PPD1* in *P. miliaceum* was significantly higher in long-day treatment at each time point except 04:00 hrs where relative expression was lowest in both treatments. At 10:00 hrs a significant reduction in expression was observed from day two to day 28. *PPD1* directly effects *FT* in long-day cereals, the increased expression of *PPD1* in long day treatment corresponds to the early flowering in *P. miliaceum* (Figure 2) suggesting that *PPD1* acts to induce flowering. Relative expression analysis of the short-day species *N. pubiflora* (Figure 2) shows no difference between treatments and an overall low expression of *PPD1*. The ortholog of *PPD1* in Oryza, *PRR37* represses *HD3a* and *Hd1*. This repression is relieved during short days (Kim et al., 2013, Campoli et al., 2012). *PRR37* peaks 8 hours after dawn in long-days (Koo et al., 2013), which is equivalent to the 16:00 hrs time point in this experiment. In short days the *PRR37* peak is abolished (Kim et al., 2013). The overall low expression of *PPD1* suggests that *N. pubiflora* flowers independently of this gene. *N. pubiflora* short-day flowering or the delayed flowering in long-day treatment seems not to be influenced by *PPD1*. This suggests that the role of *PRR37/PPD1* in the putatively ancestral SD-flowering mechanism found in *Oryza* is not conserved in *N. pubiflora.* Rather, it acts as a promotor of flowering in *P. miliaceum* similar to long-day cereals. Distinguishing *PPD1* as a long-day *FT* promotor early in the evolution of the Pooideae supports the claim that a single event, centered on this gene, contributes the success of the Pooideae in the temperate and arctic regions. Based on my data, I claim that this function of *PPD1* was lost in the transition to SD flowering in *N. pubiflora* and even might have been causative for this transition.

PHYC

Conservation of Pooideae species topology in the *PHYC* gene tree was partially maintained. The core Pooideae, *Brachypodium, Nardus* and *Brachyelytrum* held typical Pooideae topological positions. However, the limited phylogenetic information in coding sequence may not be sufficient for the maximum likelihood to resolve the proper topology. Species belonging to the Stipeae tribe occupied uncharacteristic positions conflicting the topology of the species trees and no members of the Meliceae tribe were identified among the *de novo* transcriptomes and low coverage genome of *M. nutans*. Similar sequences to *PHYC* were identified in *Melica ciliata* however the custom pipeline removed them because they had less than a third of the nucleotides (nts) of the *H. vulgare* reference sequence (558 nts, 366 nts and 327 nts) where the respective threshold was set at 1148 nts. The short length increased the chance that these sequences belonged to a different phytochrome gene. Diurnal rhythms of *PHYC* in *N. pubiflora* showed peaks at 16:00 hrs and 04:00 hrs regardless of the photoperiodic treatment. A specific diurnal rhythm was not observed in *P. miliaceum*. Nishida et al. (2013) found that *PHYC* in barley showed diurnal fluctuations throughout the day where upregulation was observed around dusk and down regulation during the day (Nishida et al., 2013). *PHYC* encodes a photoreceptor which stands at the top of the photoperiod mediated cascade (2014). The effect expression of *PHYC* may have had on photoperiodism in both *N. pubiflora* and *P. miliaceum* was most likely concealed because the regulative function of phytochromes are influence by light, which changes the biochemical structure (Lin, 2000). Further investigation, possibly at the protein level, need to be done to determine its role in photoperiodic mediated flowering.

Summary

Based on the identification of *CO2* in the most basal Brachyelytreae tribe, I conclude that the CO1/CO2 duplication occurred early in the evolutionary history of the Pooideae. However, while *CO2* was identified in both in the early diverging line *Brachyelytrum* and also the core Pooideae there was a notable absence among the basal Pooideae species from which we have transcriptomes or low coverage genomes Only Meliceae and Brachypodieae and a single member from the Stipeae tribe, *P. aequiglume* possess a copy of the gene. The putative loss of *CO2* in some basal lineages and its conservation in the core Pooideae suggest that a more recently evolved function, possibly adaptation to the more northern temperate and arctic climate stabilized the presence of *CO2*. This is supported by the fact that in long-day cereals *CO2* maintains the promotion of *FT* as the expression of *CO1* declines, accelerating spikelet formation.

Differences in *CO1* expression patterns and flowering response between *N. pubiflora* (SDP), and *P. miliaceum* (LDP), suggests a role in photoperiod mediated flowering. Downward trends in *CO1* expression during inductive treatments possibly mark the initiation of flowering in *P. miliaceum and N. pubiflora* as seen in long-day cereals. However, *N. pubiflora* exhibited expression patterns that resembled, the more ancient short-day flowering mechanism where the ortholog of *CO1* acts as a repressor during long days. *CO1* expression was higher in long-day treatment where a slower flowering response was observed. Further analysis to pinpoint the role of *CO1* in SD flowering in *N. pubiflora* is necessary.

The conserved phylogenetic topology of *PPD1*, which resembles the Pooideae species tree, suggests a conserved function within the subfamily. In core Pooideae, *PPD1* is a key promotor of flowering while its ortholog outside this clade, *PRR37*, acts as a flowering repressor. Low *PPD1* expression indicates that this gene lost its role and is not involved in the photoperiodic regulation of flowering in *N. pubiflora*. Low PPD1 expression in both day lengths opposes the more ancient photoperiodic strategy in *Oryza* and *Sorghum*, where PRR37 expression represses flowering during long days. PPD1 is highly expressed in the flowering inducing long-day treatment of *P. miliaceum* confirming its conserved role in photoperiod mediated flowering. This observation in a more basal Pooideae species suggests that the role of *PPD1* as a promotor of *FT* is central to the evolution of long-day flowering in this subfamily.

The pooideae species tree topology was not maintained throughout the PHYC gene tree. Stipeae tribe species exhibited unresolved topology. A diurnal rhythm was observed in PHYC expression of *N. pubiflora* unlike *P. miliaceum*. How relative expression of PHYC affect photoperiodism remained uncertain. Due to its sensitivity to red and far red light it is necessary to investigate the biochemical structure of the PHYC protein.

References

ANDRÉS, F. & COUPLAND, G. 2012. The genetic basis of flowering responses to seasonal cues. *Nature Reviews Genetics,* 13**,** 627.

BEVAN, M. W., GARVIN, D. F. & VOGEL, J. P. 2010. *Brachypodium distachyon* genomics for sustainable food and fuel production. *Current Opinion in Biotechnology,* 21**,** 211-217.

BOUCHENAK-KHELLADI, Y., VERBOOM, G. A., SAVOLAINEN, V. & HODKINSON, T. R. 2010. Biogeography of the grasses (Poaceae): a phylogenetic approach to reveal evolutionary history in geographical space and geological time. *Botanical Journal of the Linnean Society,* 162**,** 543-557.

CALLE, Z., SCHLUMPBERGER, B. O., PIEDRAHITA, L., LEFTIN, A., HAMMER, S. A., TYE, A. & BORCHERT, R. 2010. Seasonal variation in daily insolation induces synchronous bud break and flowering in the tropics. *Trees,* 24**,** 865-877.

CAMPOLI, C., SHTAYA, M., DAVIS, S. J. & VON KORFF, M. 2012. Expression conservation within the circadian clock of a monocot: natural variation at barley *Ppd-H1* affects circadian expression of flowering time genes, but not clock orthologs. *BMC plant biology,* 12**,** 97.

CASTILLEJO, C. & PELAZ, S. 2008. The balance between *CONSTANS* and *TEMPRANILLO* activities determines *FT* expression to trigger flowering. *Current Biology,* 18**,** 1338-1343.

CHEN, A., LI, C., HU, W., LAU, M. Y., LIN, H., ROCKWELL, N. C., MARTIN, S. S., JERNSTEDT, J. A., LAGARIAS, J. C. & DUBCOVSKY, J. 2014. *PHYTOCHROME C* plays a major role in the acceleration of wheat flowering under long-day photoperiod. *Proceedings of the National Academy of Sciences*.

EDWARDS, E. J., OSBORNE, C. P., STRÖMBERG, C. A. E. & SMITH, S. A. 2010. The Origins of C_4 Grasslands: Integrating Evolutionary and Ecosystem Science. *Science,* 328**,** 587.

FJELLHEIM, S., BODEN, S. & TREVASKIS, B. 2014. The role of seasonal flowering responses in adaptation of grasses to temperate climates. *Frontiers in Plant Science,* 5.

FOLLIARD, A., TRAORÉ, P. C. S., VAKSMANN, M. & KOURESSY, M. 2004. Modeling of sorghum response to photoperiod: a threshold–hyperbolic approach. *Field Crops Research,* 89**,** 59-70.

FOWLER, D., CHAUVIN, L., LIMIN, A. & SARHAN, F. 1996. The regulatory role of vernalization in the expression of low-temperature-induced genes in wheat and rye. *Theoretical and Applied Genetics,* 93**,** 554-559.

GARNER, W. W. & ALLARD, H. A. 1920. Effect of the relative length of day and night and other factors of the environment on growth and reproduction in plants. *Monthly Weather Review,* 48**,** 415-415.

GARNER, W. W. 1933. Comparative responses of long-day and short-day plants to relative length of day and night. *Plant Physiology,* 8**,** 347-356.

GASSNER, G. 1918. *Beiträge zur physiologischen Charakteristik sommer-und winterannueller Gewächse, insbesondere der Getreidepflanzen*.

GBIF 2017. *Oryza* L. in GBIF Secretariat (2017). GBIF Backbone Taxonomy. Checklist dataset https://doi.org/10.15468/39omei accessed via GBIF.org on 2018-07-31.

GPWG, I. 2011. New grass phylogeny resolves deep evolutionary relationships and discovers C4 origins. *New Phytologist,* 193**,** 304-312.

GRIFFITHS, S., DUNFORD, R. P., COUPLAND, G. & LAURIE, D. A. 2003. The evolution of *CONSTANS-like* gene families in barley, *Oryza*, and *Arabidopsis*. *Plant Physiol,* 131**,** 1855-67.

HARTLEY, W. 1973. Studies on the origin, evolution, and distribution of the Gramineae. V. The subfamily Festucoideae. *Australian Journal of Botany,* 21**,** 201-234.

HAYAMA, R., YOKOI, S., TAMAKI, S., YANO, M. & SHIMAMOTO, K. 2003. Adaptation of photoperiodic control pathways produces short-day flowering in rice. *Nature,* 422**,** 719.

HEIDE, O. M. 1994. Control of Flowering and Reproduction in Temperate Grasses. *The New Phytologist,* 128**,** 347-362.

IMAIZUMI, T. & KAY, S. A. 2006. Photoperiodic control of flowering: not only by coincidence. *Trends in Plant Science,* 11**,** 550-558.

ISHIKAWA, R., AOKI, M., KUROTANI, K.-I., YOKOI, S., SHINOMURA, T., TAKANO, M. & SHIMAMOTO, K. 2011. Phytochrome B regulates *Heading date 1* (*Hd1*)-mediated expression of rice florigen *Hd3a* and critical day length in rice. *Molecular Genetics and Genomics,* 285**,** 461-470.

ITO, S., SONG, Y. H., JOSEPHSON-DAY, A. R., MILLER, R. J., BRETON, G., OLMSTEAD, R. G. & IMAIZUMI, T. 2012. FLOWERING BHLH transcriptional activators control expression of the photoperiodic flowering regulator *CONSTANS* in *Arabidopsis*. *Proceedings of the National Academy of Sciences,* 109**,** 3582-3587.

IZAWA, T. 2007. Adaptation of flowering-time by natural and artificial selection in *Arabidopsis* and rice. *Journal of Experimental Botany,* 58**,** 3091-3097.

IZAWA, T., OIKAWA, T., SUGIYAMA, N., TANISAKA, T., YANO, M. & SHIMAMOTO, K. 2002. Phytochrome mediates the external light signal to repress *FT* orthologs in photoperiodic flowering of rice. *Genes & development,* 16**,** 2006-2020.

JANG, S., MARCHAL, V., PANIGRAHI, K. C., WENKEL, S., SOPPE, W., DENG, X. W., VALVERDE, F. & COUPLAND, G. 2008. *Arabidopsis* COP1 shapes the temporal pattern of CO accumulation conferring a photoperiodic flowering response. *The EMBO journal,* 27**,** 1277-1288.

KHOLOVÁ, J., MCLEAN, G., VADEZ, V., CRAUFURD, P. & HAMMER, G. L. 2013. Drought stress characterization of post-rainy season (rabi) sorghum in India. *Field Crops Research,* 141**,** 38-46.

KIM, S. L., CHOI, M., JUNG, K.-H. & AN, G. 2013. Analysis of the early-flowering mechanisms and generation of T-DNA tagging lines in Kitaake, a model Oryza cultivar. *Journal of experimental botany,* 64**,** 4169-4182.

KITAGAWA, S., SHIMADA, S. & MURAI, K. 2012. Effect of *Ppd-1* on the expression of flowering-time genes in vegetative and reproductive growth stages of wheat. *Genes & genetic systems,* 87**,** 161-168.

KOO, B.-H., YOO, S.-C., PARK, J.-W., KWON, C.-T., LEE, B.-D., AN, G., ZHANG, Z., LI, J., LI, Z. & PAEK, N.-C. 2013. Natural variation in *OsPRR37* regulates heading date and contributes to rice cultivation at a wide range of latitudes. *Molecular Plant,* 6**,** 1877-1888.

LAZARO, A., VALVERDE, F., PIÑEIRO, M. & JARILLO, J. A. 2012. The Arabidopsis E3 ubiquitin ligase HOS1 negatively regulates CONSTANS abundance in the photoperiodic control of flowering. *The Plant Cell***,** tpc. 110.081885.

LI, C., DISTELFELD, A., COMIS, A. & DUBCOVSKY, J. 2011. Wheat flowering repressor VRN2 and promoter CO2 compete for interactions with NUCLEAR FACTOR-Y complexes. *The Plant Journal,* 67**,** 763-773.

LIU, L.-J., ZHANG, Y.-C., LI, Q.-H., SANG, Y., MAO, J., LIAN, H.-L., WANG, L. & YANG, H.-Q. 2008. COP1-mediated ubiquitination of CONSTANS is implicated in cryptochrome regulation of flowering in *Arabidopsis*. *The Plant Cell,* 20**,** 292-306.

MÖGLICH, A., YANG, X., AYERS, R. A. & MOFFAT, K. 2010. Structure and function of plant photoreceptors. *Annual review of plant biology,* 61.

MULKI, M. A. & VON KORFF, M. 2015. CONSTANS controls floral repression by upregulating *VERNALIZATION 2* (*Vrn-H2*) in barley. *Plant physiology***,** pp. 01350.2015

NAKAMICHI, N., KIBA, T., HENRIQUES, R., MIZUNO, T., CHUA, N.-H. & SAKAKIBARA, H. 2010. PSEUDO-RESPONSE REGULATORS 9, 7, and 5 are transcriptional repressors in the *Arabidopsis* circadian clock. *The Plant Cell,* 22**,** 594-605.

NISHIDA, H., ISHIHARA, D., ISHII, M., KANEKO, T., KAWAHIGASHI, H., AKASHI, Y., SAISHO, D., TANAKA, K., HANDA, H. & TAKEDA, K. 2013. Phytochrome C is a key factor controlling long-day flowering in barley (*Hordeum vulgare* L.). *Plant physiology***,** pp. 113.222570.

OSUGI, A., ITOH, H., IKEDA-KAWAKATSU, K., TAKANO, M. & IZAWA, T. 2011. Molecular Dissection of the Roles of Phytochrome in Photoperiodic Flowering in *Oryza*. *Plant Physiology,* 157**,** 1128.

PITTENDRIGH, C. S. 1972. Circadian Surfaces and the Diversity of Possible Roles of Circadian Organization in Photoperiodic Induction. *Proceedings of the National Academy of Sciences,* 69**,** 2734.

RÉDEI, G. P. 1962. Supervital mutants of *Arabidopsis*. *Genetics,* 47**,** 443.

SAMACH, A., ONOUCHI, H., GOLD, S. E., DITTA, G. S., SCHWARZ-SOMMER, Z., YANOFSKY, M. F. & COUPLAND, G. 2000. Distinct Roles of CONSTANS Target Genes in Reproductive Development of *Arabidopsis*. *Science,* 288**,** 1613.

SAWA, M., NUSINOW, D. A., KAY, S. A. & IMAIZUMI, T. 2007. FKF1 and GIGANTEA complex formation is required for day-length measurement in Arabidopsis. *Science,* 318**,** 261-265.

SHAW, L. M., TURNER, A. S. & LAURIE, D. A. 2012. The impact of photoperiod insensitive Ppd-1a mutations on the photoperiod pathway across the three genomes of hexaploid wheat (*Triticum aestivum*). *The Plant Journal,* 71**,** 71-84.

SHAW, L. M., TURNER, A. S., HERRY, L., GRIFFITHS, S. & LAURIE, D. A. 2013. Mutant alleles of Photoperiod-1 in wheat (*Triticum aestivum L.*) that confer a late flowering phenotype in long days. *PLoS One,* 8**,** e79459.

SHIMADA, S., OGAWA, T., KITAGAWA, S., SUZUKI, T., IKARI, C., SHITSUKAWA, N., ABE, T., KAWAHIGASHI, H., KIKUCHI, R. & HANDA, H. 2009. A genetic network of flowering-time genes in wheat leaves, in which an *APETALA1/FRUITFULL-like* gene, *VRN1*, is upstream of *FLOWERING LOCUS T*. *The Plant Journal,* 58**,** 668-681.

SONG, Y. H., SHIM, J. S., KINMONTH-SCHULTZ, H. A. & IMAIZUMI, T. 2015. Photoperiodic flowering: time measurement mechanisms in leaves. *Annu Rev Plant Biol,* 66**,** 441-64.

SONG, Y. H., SMITH, R. W., TO, B. J., MILLAR, A. J. & IMAIZUMI, T. 2012. FKF1 conveys timing information for CONSTANS stabilization in photoperiodic flowering. *Science,* 336**,** 1045-1049.

SORENG, R. J., PETERSON, P. M., ROMASCHENKO, K., DAVIDSE, G., TEISHER, J. K., CLARK, L. G., BARBERÁ, P., GILLESPIE, L. J. & ZULOAGA, F. O. 2017. A worldwide phylogenetic classification of the Poaceae (Gramineae) II: An update and a comparison of two 2015 classifications. *Journal of Systematics and Evolution,* 55**,** 259-290.

STRÖMBERG, C. A. 2011. Evolution of grasses and grassland ecosystems. *Annual Review of Earth and Planetary Sciences,* 39**,** 517-544.

SUÁREZ-LÓPEZ, P., WHEATLEY, K., ROBSON, F., ONOUCHI, H., VALVERDE, F. & COUPLAND, G. 2001. CONSTANS mediates between the circadian clock and the control of flowering in Arabidopsis. *Nature,* 410**,** 1116.

THOMAS, B. & VINCE-PRUE, D. 1996. *Photoperiodism in plants*, Elsevier.

VALVERDE, F., MOURADOV, A., SOPPE, W., RAVENSCROFT, D., SAMACH, A. & COUPLAND, G. 2004. Photoreceptor regulation of CONSTANS protein in photoperiodic flowering. *Science,* 303**,** 1003-1006.

VISSER, V., CLAYTON, W. D., SIMPSON DAVID, A., FRECKLETON ROBERT, P. & OSBORNE COLIN, P. 2013. Mechanisms driving an unusual latitudinal diversity gradient for grasses. *Global Ecology and Biogeography,* 23**,** 61-75.

WIENS JOHN, J., ACKERLY DAVID, D., ALLEN ANDREW, P., ANACKER BRIAN, L., BUCKLEY LAUREN, B., CORNELL HOWARD, V., DAMSCHEN ELLEN, I., JONATHAN DAVIES, T., GRYTNES, J.-A., HARRISON SUSAN, P., HAWKINS BRADFORD, A., HOLT ROBERT, D., MCCAIN CHRISTY, M. & STEPHENS PATRICK, R. 2010. Niche conservatism as an emerging principle in ecology and conservation biology. *Ecology Letters,* 13**,** 1310-1324.

WILLIG, M. R., KAUFMAN, D. M. & STEVENS, R. D. 2003. Latitudinal Gradients of Biodiversity: Pattern, Process, Scale, and Synthesis. *Annual Review of Ecology, Evolution, and Systematics,* 34**,** 273-309.

WOODS, D. P., REAM, T. S., MINEVICH, G., HOBERT, O. & AMASINO, R. M. 2014. PHYTOCHROME C Is an Essential Light Receptor for Photoperiodic Flowering in the Temperate Grass, *Brachypodium distachyon*. *Genetics,* 198**,** 397.

Appendices

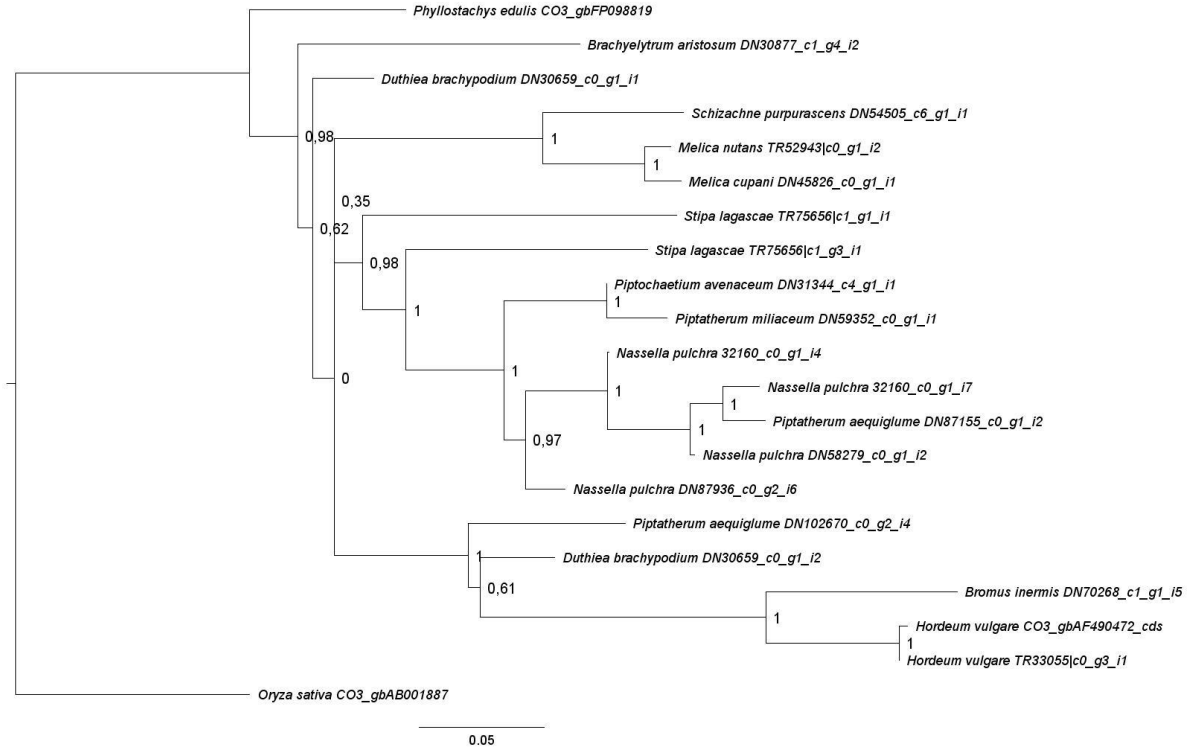
Appendix 1: Primers used for target gene quantification by RT-qPCR

Primer	Sequence	Species	Reference
qPCR			
LolEf1αF	CCTTGCTTGAGGCTCTTGAC	*P. miliaceum* & *N. pubiflora*	(Woods et al., 2014)
LolEf1αR	GTTCCAATGCCACCAATCTT		
EIF4a_poace_F2	CGCAAGGTGGACTGGCTCAC	*P. miliaceum* & *N. pubiflora*	(Fjellheim unpubl)
EIF4a_poace_R2	GAACTCCCTCATGATGATGT		
CO1_NPUB_468_F	CAGTGAGAGCAACAACAGCA	*N. pubiflora*	
CO1_NPUB_650_R	ACACACTCGTTCCCTTCCTT		
CO1_PMIL_414_F	AAAGGAGGTGGAGTCTTGGC	*P. miliaceum*	
CO1_PMIL_645_R	CTCGCTCCCTTCCTTCTCTC		
PM ppd1 68 F	ACTCGCCATCTCTTCTCCCT	*P. miliaceum*	(Young unpubl)
PM ppd1 230 R	TTCTTGTGGAGGAAGCGGTC		
PM PHYC 3867 F	TGGGAGAGCCTAGCTGATGT		
PM PHYC 3950 R	TCCTGCTCCCCAAACATCAC		
NP ppd1 1601 F	CTGCTCCGATGAAACAGGGT	*N. pubiflora*	
NP ppd1 1790 R	TCACCCATCTTCTTGCCCAC		
NP PHYC 592 F	CAGCCTATCAGCCTCTGTGG		
NP PHYC 720 R	CCCGTCCTCCTCATCCTCAT		

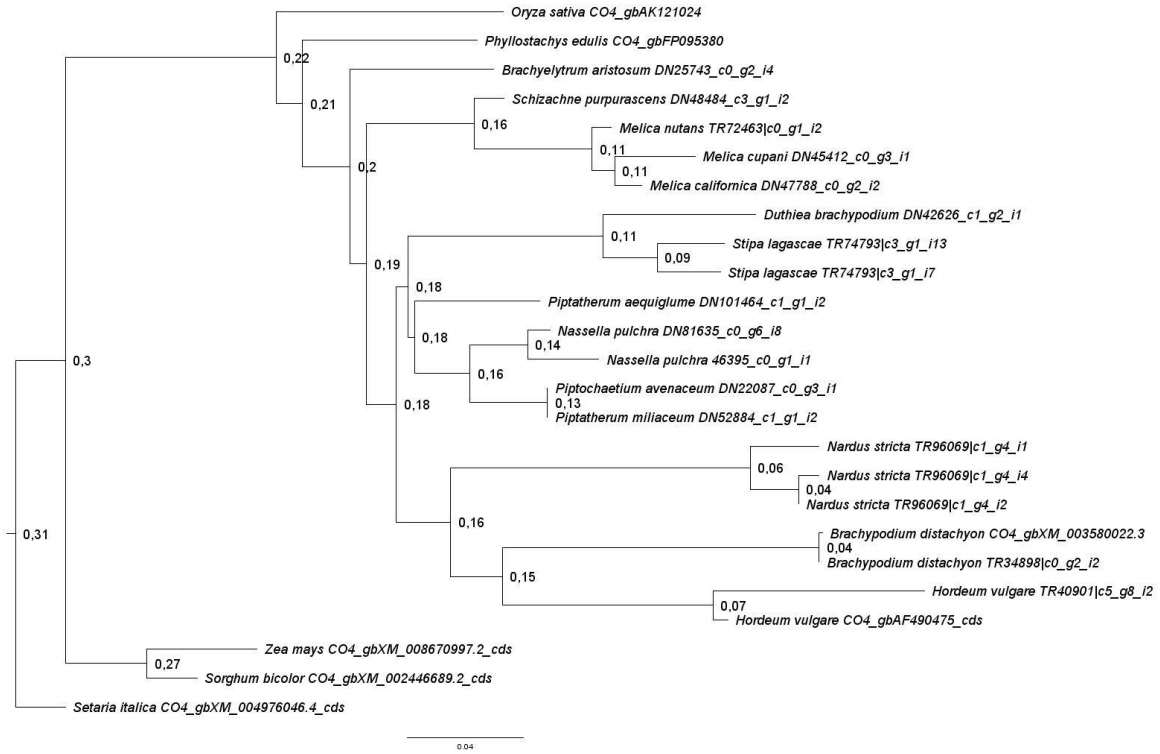
Appendix 2: Pooideae species where transcriptome and low coverage genome date was obtained

Genus	Species	Tribe	Type	Source
Melica	nutans	Meliceae	transcriptome	Grønvold et al., unpubl
Stipa	lagascae	Stipeae	transcriptome	Grønvold et al., unpubl
Nardus	stricta	Nardeae	transcriptome	Grønvold et al., unpubl
Brachypodium	distachyon	Brachypodieae	transcriptome	Grønvold et al., unpubl
Hordeum	vulgare	Triticeae	transcriptome	Grønvold et al., unpubl
Nassella	pulchra	Stipeae	transcriptome	Zhong et al., 2017
Brachyelytrum	arirosum	Brachyelytreae	transcriptome	Preston et al., unpubl
Melica	nutans	Meliceae	transcriptome	Zhong et al., 2017
Duthiea	brachypodium	Phaenospermatae	transcriptome	Leder et al., upubl
Melica	nutans	Meliceae	transcriptome	Leder et al., upubl
Nassella	pulchra	Stipeae	transcriptome	Leder et al., upubl
Piptatherum	aequiglume	Stipeae	transcriptome	Leder et al., upubl
Piptochaetium	avenaceum	Stipeae	transcriptome	Leder et al., upubl
Piptatherum	miliaceum	Stipeae	transcriptome	Leder et al., upubl
Schizachne	purpurascens	Meliceae	transcriptome	Leder et al., upubl
Brachypodum	distachyon	Brachypodieae	transcriptome	Leder et al., upubl
Hordeum	vulgare	Triticeae	transcriptome	Leder et al., upubl
Melica	ciliata	Meliceae	transcriptome	Schubert et al., unpubl
Melica	californica	Meliceae	transcriptome	Schubert et al., unpubl
Melica	cupani	Meliceae	transcriptome	Schubert et al., unpubl
Bromus	inermis	Bromeae	transcriptome	Schubert et al., unpubl
Nassella	pulchra	Stipeae	transcriptome	Schubert et al., unpubl
Hordeum	vulgare	Triticeae	transcriptome	Schubert et al., unpubl
Melica	nutans	Meliceae	low coverage genome	Leder et al., upubl
Nardus	stricta	Nardeae	low coverage genome	Leder et al., upubl
Stipa	lagascae	Stipeae	low coverage genome	Leder et al., upubl

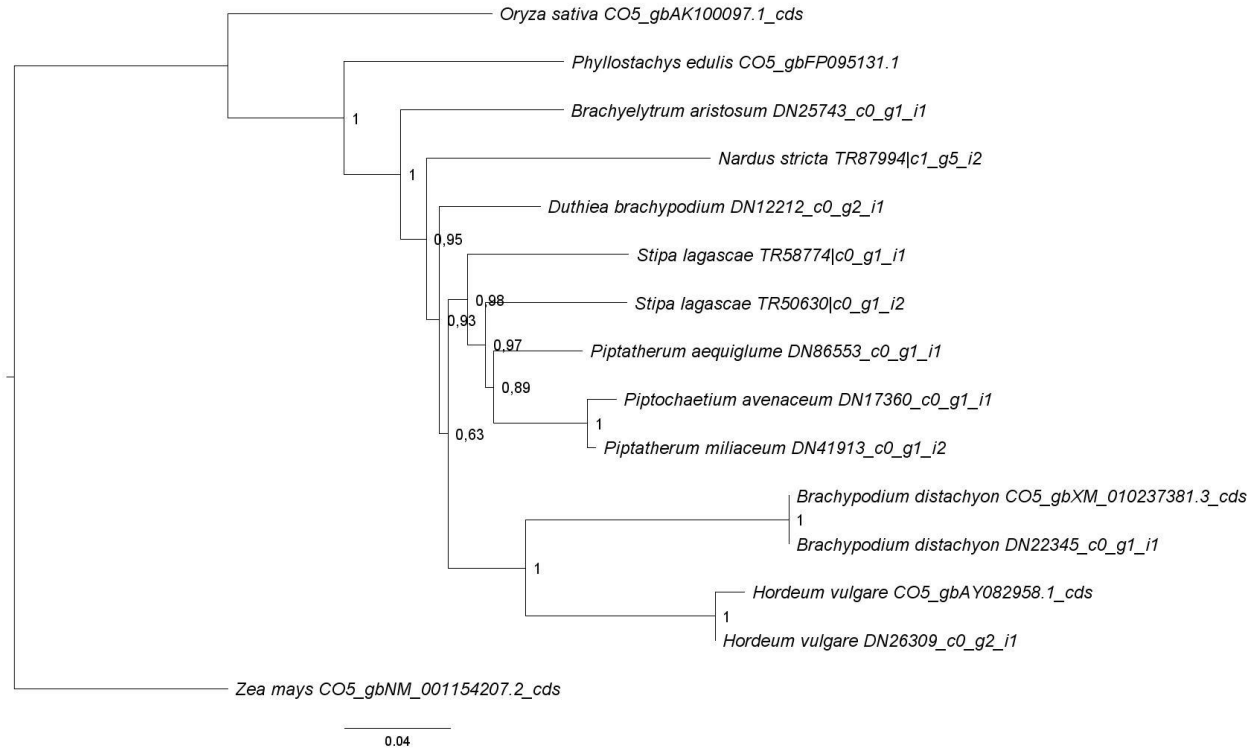
Appendix 3: Phylogenetic tree estimated using PhyML 3.0



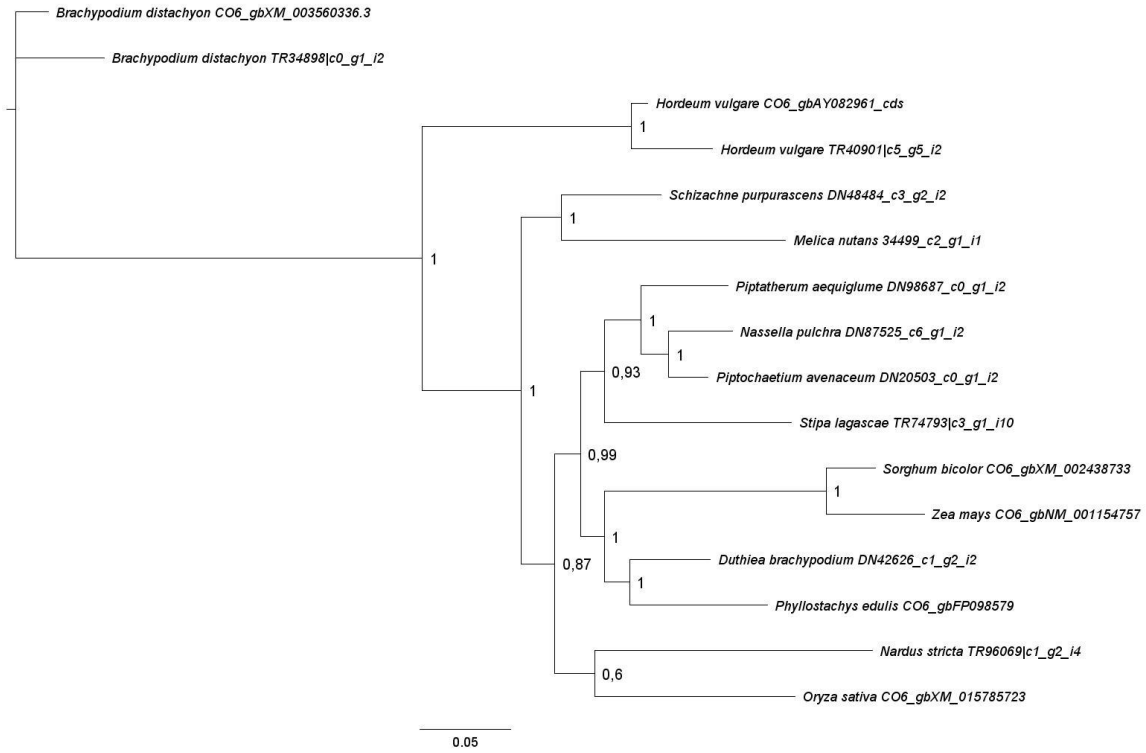
A. *CO3* Maximum likelihood tree including outgroup sequences from non-Pooideae species. Branch support is shown as node labels displaying the results of the approximate likelihood ratio test.



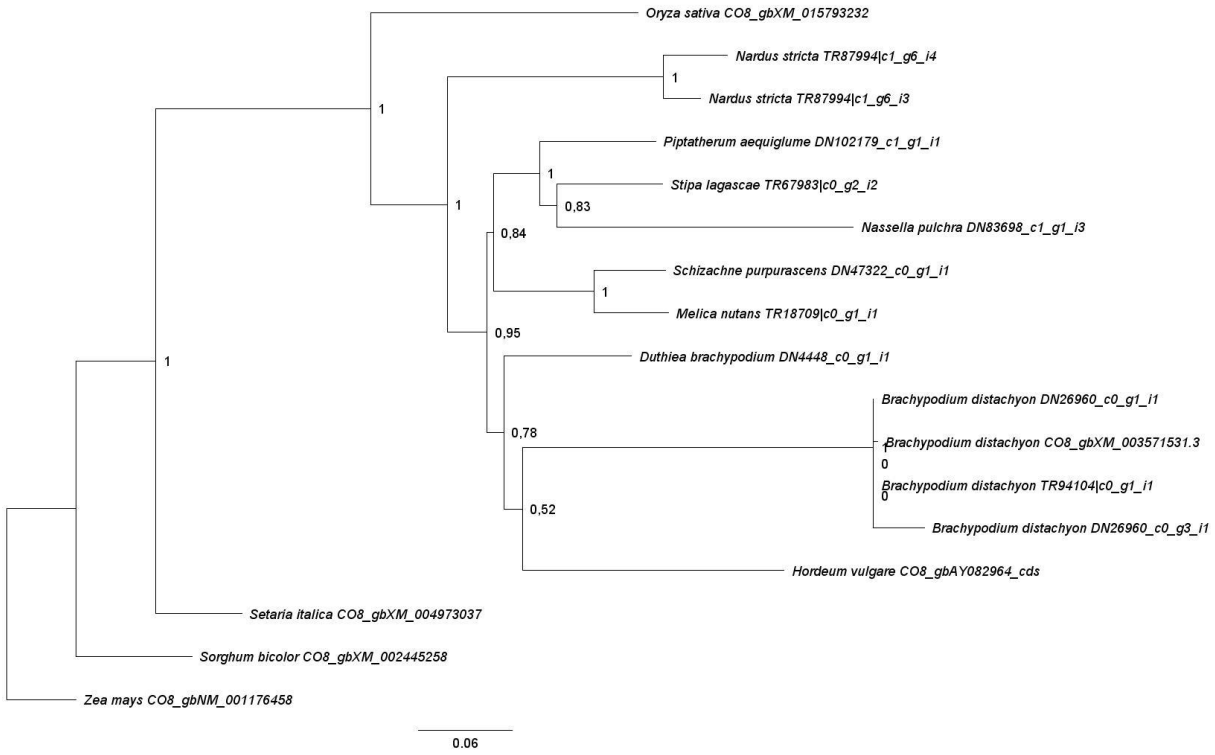
B. *CO4* Maximum likelihood tree including outgroup sequences from non-Pooideae species. Branch support is shown as node labels displaying the results of the approximate likelihood ratio test.



C. *CO5* Maximum likelihood tree including outgroup sequences from non-Pooideae species. Branch support is shown as node labels displaying the results of the approximate likelihood ratio test.



D. *CO6* Maximum likelihood tree including outgroup sequences from non-Pooideae species. Branch support is shown as node labels displaying the results of the approximate likelihood ratio test



E. *CO5* Maximum likelihood tree including outgroup sequences from non-Pooideae species. Branch support is shown as node labels displaying the results of the approximate likelihood ratio test

Norges miljø- og biovitenskapelige universitet
Noregs miljø- og biovitskapelege universitet
Norwegian University of Life Sciences

Postboks 5003
NO-1432 Ås
Norway