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A seasonal strategy for pollen tube growth and ovule development to overcome winter in Japanese stone oak (*Lithocarpus edulis*)

Takenori Shagawa[1]✉, Kota Ogawa[2,3], Masahiro M. Kanaoka[4] & Akiko Satake[5]✉

Delayed fertilization is commonly observed in many acorn-producing Fagaceae trees, yet its underlying mechanisms and adaptive significance remain poorly understood. In recent years, a new hypothesis has been proposed suggesting that the nearly year-long delay in fertilization is driven by an overwintering strategy, wherein female gametophyte development is delayed, and pollen tube growth is arrested before winter. This mechanism allows ovules to be fertilized and seeds to develop during more favorable seasons while avoiding adverse winter conditions. However, empirical evidence for this overwintering strategy has been limited. To address this, we observed the seasonal progression of pollen tube growth and ovule development in *Lithocarpus edulis*, a species with spring and autumn flowering seasons. Monthly observations of pistillate flowers from both seasons were conducted using microtome techniques and scanning confocal microscopy. Our findings revealed that pollen tubes were arrested at the style joining site, and ovules remained immature in both spring and autumn flowers prior to winter. Following winter, pollen tube regrowth and ovule maturation were synchronized in the subsequent spring, regardless of the flowering season. These results support the hypothesis that ovule development is delayed, leading to delayed fertilization, until after winter. This study highlights the importance of temporally coordinating fertilization phenology with flowering and fruiting phenology in seasonal environments to avoid unfavorable winter conditions.

Keywords Evolution, Delayed fertilization, Fagaceae, *Lithocarpus*, Flowering, Seasonality, Ovule development, Phenology, Pollen tube growth, Reproductive ecology

Fertilization at the appropriate timing is crucial for the reproductive strategy of plants. In angiosperms, fertilization generally occurs within 24–48 h following pollination[1,2]. However, delays in fertilization extending beyond 4 days have been reported in various unrelated groups[1–3]. The time interval between pollination and fertilization can range from several days to more than a year, and delay in fertilization is accomplished by delay of pollen tube growth and delay of ovule development[1–3]. Although delayed fertilization was first reported over a century ago[4], the underlying mechanism and its adaptive significance remain largely unknown[3,5,6].

The sperm competition hypothesis proposed that the adaptive significance of delayed fertilization lies in enhancing competition among pollen tubes and enabling selective choice by the pistils for superior sperm[2,3,7–17]. However, this hypothesis alone does not sufficiently explain the extended delay in fertilization, which can be nearly a year[5,7]. To address this, a new hypothesis has been proposed based on a mathematical model[5]. This model, which incorporates survival and competition among pistillate flowers, predicts that in the presence of an unsuitable season for reproduction, such as winter, a strategy evolves to delay fertilization until a more favorable season, such as spring. Furthermore, the model suggests that even plants flowering in spring may evolve to delay fertilization for nearly a year rather than proceeding with immediate fertilization if seed maturation cannot be completed before the onset of an unfavorable season. This overwintering strategy of female flowers is intriguing as it provides a potential explanation for the adaptive significance of delaying fertilization for over a year. However, it has not been empirically demonstrated whether the presence of winter delays fertilization.

[1]Graduate School of Systems Life Sciences, Kyushu University, Fukuoka 819-0395, Japan. [2]Faculty of Social and Cultural Studies, Kyushu University, Fukuoka 819-0395, Japan. [3]Insect Sciences and Creative Entomology Center, Kyushu University, Fukuoka 819-0395, Japan. [4]Faculty of Life and Environmental Sciences, Prefectural University of Hiroshima, Shobara 727-0023, Japan. [5]Department of Biology, Faculty of Science, Kyushu University, Fukuoka 819-0395, Japan. ✉email: shagawa.takenori.552@s.kyushu-u.ac.jp; akiko.satake.akiko.269@m.kyushu-u.ac.jp

Similar to vernalization[18], where the floral transition begins only after experiencing winter cold, it is possible that in fertilization as well, the ovule develops, and the pollen tube grows only after exposure to winter cold.

Anatomical studies of pistillate flowers in *Fagus*, *Castanea*, *Lithocarpus*, and *Quercus* have shown that ovules are immature at the time of flowering and require several months to nearly a year to mature[3,16,17,19–21]. In *Lithocarpus dealbatus*[17] and *Quercus acutissima*[16], following pollination in spring, pollen tubes grow through the style and reach the style-joining region by or during the winter of the second year, where growth is arrested until fertilization occurs in spring. These findings highlight the influence of seasonal environmental conditions on ovule development and pollen tube growth. Further research is needed to clarify how the presence of winter affects these reproductive processes. The Fagaceae family, with species exhibiting a wide range of intervals between pollination and fertilization—from several weeks to nearly a year—provides an excellent system for studying the evolution of delayed fertilization. In *Fagus*, fertilization occurs approximately five weeks after pollination, leading to fruiting within the same year (1-year fruiting[3,5];). In *Lithocarpus*, 92% of 104 species produce fruit in the year following pollination (2-year fruiting[5];). *Quercus* includes both 1-year and 2-year fruiting species in roughly equal proportions[5].

To investigate whether ovule development and pollen tube growth occur only after experiencing winter, we compared the seasonal progression of pollen tube growth and ovule development from pollination to fertilization in flowers produced in different seasons in Japanese stone oaks, *Lithocarpus edulis* (Makino) Nakai (Fig. 1a,b). *L. edulis* exhibits two distinct flowering seasons–spring and autumn–depending on the region[6]. By comparing the pollination-to-fertilization process in pistils produced in different seasons, we aim to determine whether pollen tube growth and ovule development progress in a manner that delays fertilization until after winter, thereby avoiding winter.

Methods

Plant materials and sampling methods

Female inflorescences of *L. edulis*, identified by Takenori Shagawa, planted at the Ito Campus (33°35′45.3" N 130°12′42.2" E) of Kyushu University (Fukuoka, Japan), were collected monthly from July 2022 to October 2023. For each monthly sampling, one individual was chosen from eight mature trees arbitrarily, and at least three inflorescences were collected from the individual. At the Ito Campus, *L. edulis* exhibits two distinct flowering periods each year, with a major flowering period in June (spring flowering) and a minor flowering period from September to October (autumn flowering) (Fig. 1b)[6]. The samples collected from spring flowering were referred to as the spring samples, while those collected from autumn flowering were designated as the autumn samples. The female inflorescences for the spring samples were collected from June of the first year to June of the subsequent year, while those for the autumn samples were collected from November of the first year to June of the following year (Fig. 1c). The inflorescences were immediately fixed in FAA (70% ethanol: stock formalin:

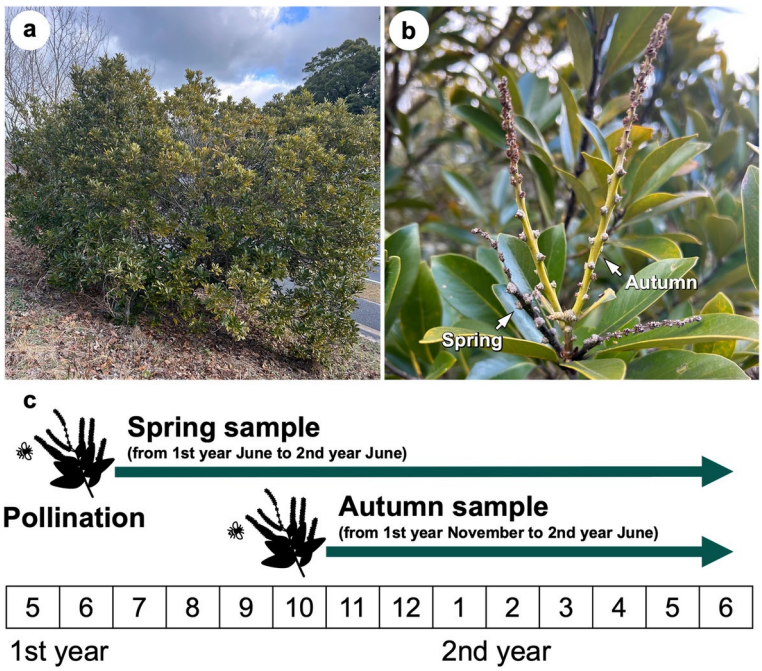

Fig. 1. Sampling scheme of *L. edulis*. (**a**) One of the target trees of *L. edulis* in our study. (**b**) A branch including both spring and autumn flowers. The height of the sampled trees was approximately 3.5–5.5 m, and the inflorescence length was approximately 3–7 cm. The photographs of (**a**, **b**) were taken on 24 January 2024. (**c**) Monthly sampling schedule. Spring samples were collected from the first year of June to the second year of June, and autumn samples were collected from the first year of November to the second year of June.

acetic acid, 90:5:5, by volume). The samples were transferred to the laboratory within 2 h after sampling and stored at 4 °C for at least 48 h until further investigation.

Observations of seasonal progression of pollen tube growth and ovule development

We employed the paraffin-embedding method to investigate the seasonal progression of the pollen tube growth and ovule development within each pistillate flower. Fixed inflorescences were dissected into each pistillate flower and then dehydrated by an ethanol series (50, 70, 80, 90, two times of 100% ethanol). After dehydration, the samples were infiltrated by the 100% xylene-ethanol series (25, 50, 75%) and then incubated twice in 100% xylene. The samples substituted for xylene were infiltrated with 100% xylene-paraffin (Paraplast plus®; Sigma-Aldrich, St. Louis, MO, USA) series (25, 50, 75%) and subsequently incubated twice in 100% paraffin. The samples were embedded in paraffin with a 57–58 °C melting point for subsequent microtome sectioning. Serial sections 6–15 µm thick were made using a rotary microtome (RX-860; Yamato Kohki Industrial Co. Ltd., Saitama, Japan). After de-waxing and rehydration, the sectioned samples were stained with 1% (w/v) toluidine blue and then stained with 0.1% aniline blue in 0.1 M K_3PO_4. The stained samples underwent dehydration using an ethanol series followed by a xylene series, and the slides were mounted with EUKITT® (ORSA-tec, Bobingen, Germany). Pollen tubes in the pistillate flowers and the developmental stage of ovules were observed with a confocal laser-scanning microscope (FV3000; Olympus, Tokyo, Japan) using excitation lasers at 405 and 448 nm wavelengths.

Figure 2 illustrates the method for observing pollen tubes. When examining the entire section, the transmitting tissue, which does not emit a fluorescent signal, is clearly distinguished from the surrounding vascular tissue, which does emit fluorescence (Fig. 2a,a',b, indicated by the dashed line in Fig. 2a'). Additionally, within the transmitting tissue, linear fluorescent signals corresponding to pollen tubes and granular fluorescent signals at the callose plugs are detected (Fig. 2c,c',d). Therefore, both the linear fluorescent signal representing the structure of the pollen tubes and the granular signal originating from the callose plugs observed within the

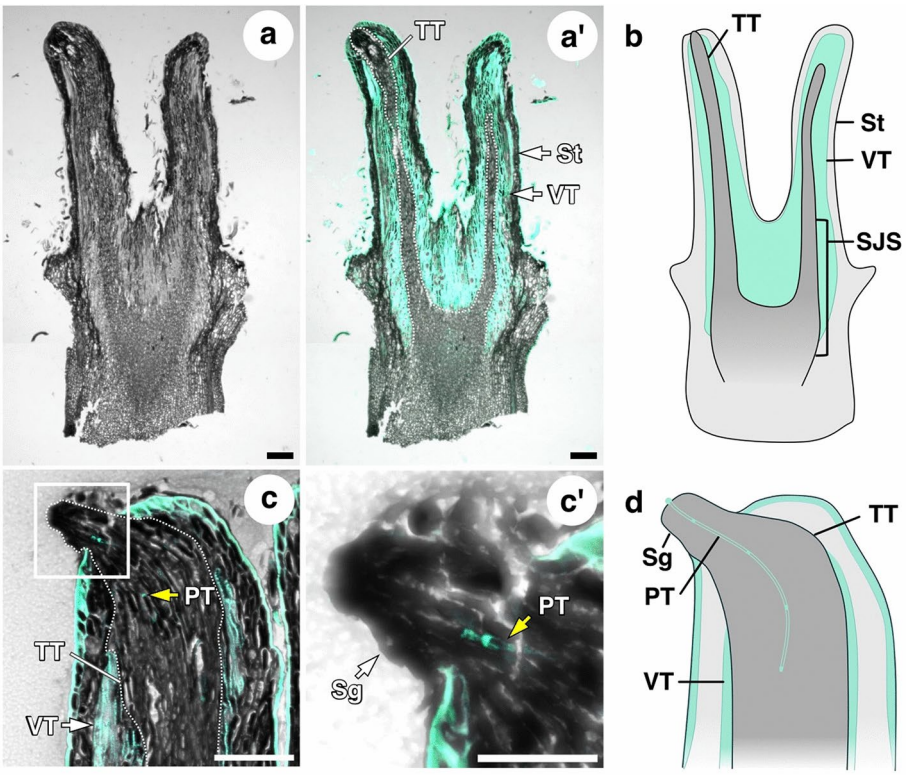

Fig. 2. Method of pollen tube observation. (**a**) Overview of the entire sample. (**a'**) Merged image of (**a**) with fluorescence signals. The area enclosed by the dashed line indicates the transmitting tissue. While the transmitting tissue does not emit fluorescence, a prominent fluorescent signal can be seen from the surrounding vascular tissue. (**b**) Schematic representation of the entire sample. The region below the style joining site (SJS), including the transmitting tissue, is shown. (**c**) The upper part of the style includes the stigma. The stigma is covered with a fluorescently emitting cell layer, and fluorescent signals from pollen tubes can be observed within the transmitting tissue. (**c'**) Enlarged view of the stigma. A callose plug can be observed within the tubular pollen tube. (**d**) Schematic representation of the upper part of the style, including the stigma. a and a' represent spring-flowering pistillate flowers collected on 12 June 2023, and c and c' represent autumn-flowering pistillate flowers collected on 10 April 2023. The yellow arrows indicate the position of the callose plugs. *PT* pollen tube, *Sg* stigma, *SJS* style joining site, *St* style, *TT* transmitting tissue, *VT* vascular tissue. Scale bars = 100 µm (**a**, **a'**, **c**), 50 µm (**c'**).

transmitting tissue were used as characteristic indicators of the pollen tubes[3,16,17,19]. To confirm the presence of pollen tubes detected using the method described above, we observed the pistillate flowers with the same confocal laser-scanning microscope, utilizing a combination of excitation lasers at 405 and 640 nm wavelengths (see Supplementary Fig. 1). While fluorescence from aniline blue dye was only emitted by excitation of 405 nm laser, autofluorescence from vascular tissue was emitted by excitation of both 405 and 640 nm lasers. Therefore, we regard pollen tube signals as the former one.

Additionally, we classified four categories of pollen tube development based on the location of pollen tubes within the pistillate flower: style (St), style joining site (SJS), upper ovarian locule (UOL), and micropyle (Mic). We also classified the stages of ovule development into four categories: undifferentiated (Un), ovule primordium (Op), megaspore mother cell (MMC), and embryo sac (ES). The number of pistillate flowers available for information on developmental stages each month in pollen tubes and ovules, including spring and autumn samples are shown in Supplementary Table 1. A voucher specimen of the material used in this study has not been deposited in a publicly accessible herbarium.

Comparison of flowering and fertilization phenology of temperate Fagaceae species

To gain a comprehensive understanding on the evolution of delayed fertilization, we summarized and compared the timing of pollination and fertilization for 17 Fagaceae species from four genera reported in the studies as follows: *Q. gambelii*[22], *Q. alba*[23,24], *Q. coccinea*[25], *Q. rubra*[23], *Q. velutina*[23,24,26], *Q. acutissima*[16,21], *Q. suber*[19,27,28], *Q. schottkyana*[29], *Q. glauca*[6], *Lithocarpus dealbatus*[17], *L. edulis* (Spring)[6], *L. edulis* (Autumn), *Castanea crenata*[20,30–34], *C. henryi*[35], *C. mollissima*[36,37], *C. savita*[38], *Fagus japonica*[3], and *F. sylvatica*[39].

Results

Seasonal progression of pollen tube growth between spring and autumn pistillate flowers

In the spring samples collected right after anthesis in mid-June, most pollen tubes were observed in the transmitting tissue of the upper and middle parts of the styles (Fig. 3a,a'). In some samples, a few pollen tubes reached the lower part of the junction of the styles (the style joining site; Fig. 3a). From July of the first year to April of the following year, the tips of the pollen tubes ceased growth and arrested at the style joining site (Fig. 3b,b',c,d,d'). Pollen tube signals were only observed in the dark-gray stained tissues, not in the lighter-gray tissues below them (Fig. 3c,d,d'). In May of the second year, some pollen tubes resumed growth and penetrated the transmitting tissue in the upper ovarian locules (Fig. 3e,e'). During this period, the lower part of the pistillate flower, including the ovarian wall and ovules, developed rapidly. Samples from June of the second year showed that parts of the pollen tubes were located near the ovules, with some pollen tubes positioned close to the micropyles (Fig. 3f).

In the autumn samples, pollen tubes were arrested in the transmitting tissue of the style joining site from November of the first year to April of the second year (Fig. 4a,b,b"). In May of the second year, some pollen tubes resumed elongation from the style joining site and were located at upper ovarian locules (Fig. 4c). In June, they reached near the ovule and micropyle (Fig. 4d–d"). Our results demonstrate that fertilization is delayed by 13 months, from June to the following June, in the spring samples, whereas the delay was 9 months, from September to June, in the autumn samples.

Seasonal progression of ovule development between spring and autumn pistillate flowers

Seasonal ovule development in longitudinal and transverse sections of spring samples showed that in June of the first year, small and inconspicuous ovarian locules were observed at the base of the pistillate flower, but the ovule meristem was undifferentiated (Fig. 5a,i). From July of the first year to January of the second year, we observed that the ovarian locules enlarged, but the ovule meristem remained undeveloped (Fig. 5b,c,j,k). However, One out of seven samples collected in November showed ovule primordia (Fig. 5d). The samples from February to April of the second year showed ovule primordia (Fig. 5e,f,l,m). In May of the second year, we observed that the ovarian locules further enlarged, and the ovule primordia differentiated into megaspore mother cells with outer and inner integuments (Fig. 5g,n). From the samples collected in June of the second year, we observed further developed ovules (Fig. 5h,o,o'). They exhibited significantly larger ovules than those from May, and their outer and inner integuments were also more developed, forming a deeper micropyle (Figs. 3f, 5h).

From the autumn samples collected from November of the first year to April of the second year, ovarian locules were observed, but ovule primordium was not found (Fig. 6a,b,e,f). In May, the lower part of the pistillate flowers including the ovary began growing rapidly and reached a megaspore mother cell stage (Fig. 6c,g). From the samples obtained in June, we observed further developed ovules, which exhibited significantly larger sizes compared to those from May (Fig. 6d,d',h,h'). Similar to the spring samples, their outer and inner integuments were also more developed, forming a deeper micropyle (Figs. 4d, 6d). We also identified a single case of a more developed embryo sac in an autumn sample from June of the second year (Fig. 6d'). These results indicate that ovule development begins after winter, regardless of the flowering time.

In most ovules from the June samples of the second year, a distinguishable embryo sac was not observed, likely due to ovule abortion occurring before fertilization. However, we identified a single case of a more developed embryo sac from an autumn sample from June of the second year (Fig. 6d'). Additionally, the June samples exhibited significantly larger ovules than those from May (Figs. 5g,h, 6c,d), with more developed outer and inner integuments forming a deeper micropyle (Figs. 3f, 4d, 5h, 6d). These observations suggest that the June samples correspond to the embryo sac development and maturation stage described in previous studies[16,17,28]. Therefore, we identified the June samples from the second year as representing the embryo sac (ES) stage.

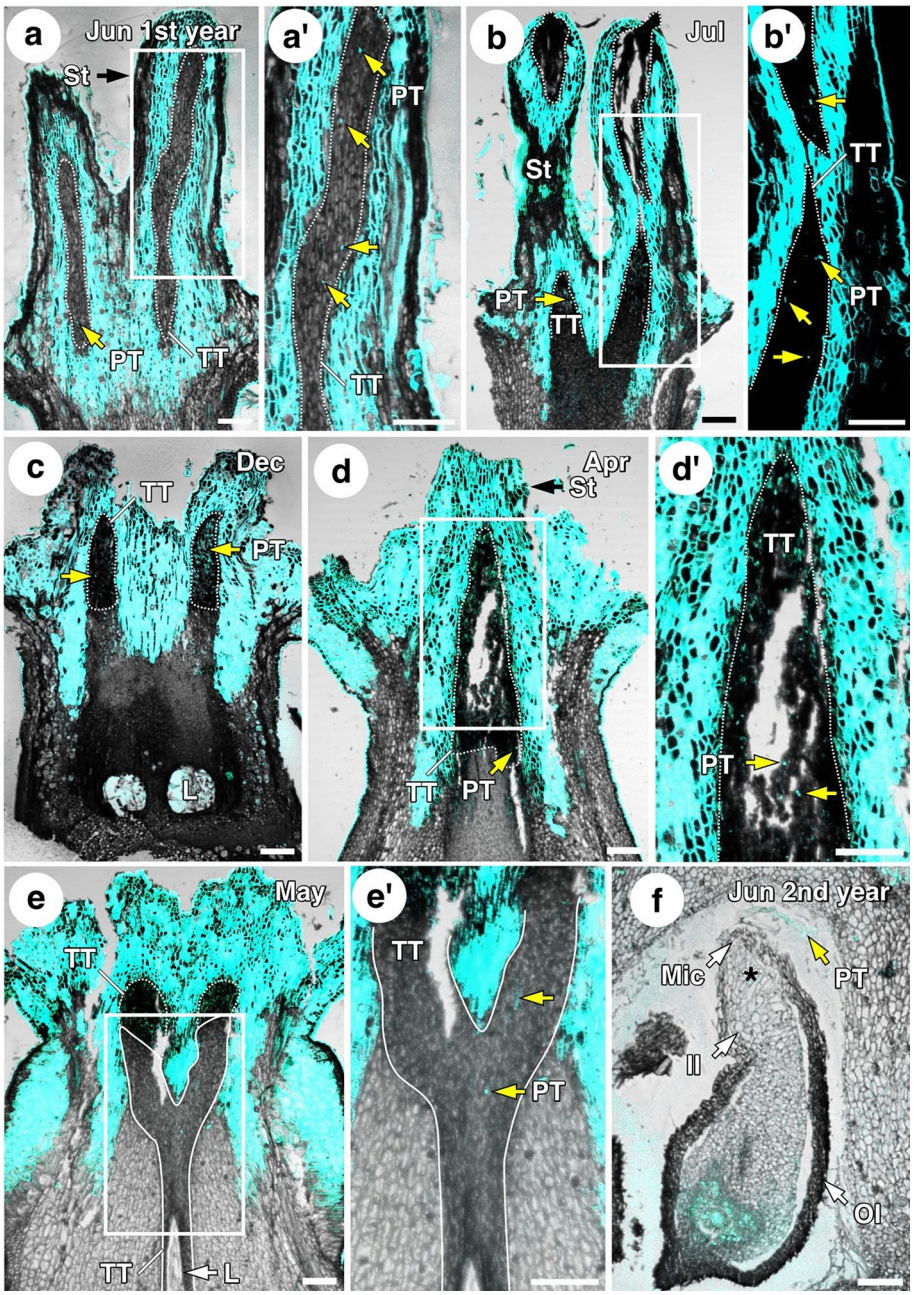

Fig. 3. Seasonal progression of pollen tube growth in spring samples. (**a**) The pistillate flower collected in June of the first year showed that some pollen tubes grew into styles (St). Some pollen tubes had reached the transmitting tissue at the base of styles. (**a'**) Magnified square region of a. (**b**, **b'**, **c**, **d**, **d'**) Pollen tubes were arrested in the transmitting tissue at the style joining site (SJS) from July of the first year to April of the second year. (**b**') Magnified square region of b, captured under 405 nm excitation laser. (**d'**) Magnified square region of d. (**e**) Some pollen tubes resumed elongation from the style joining site and reached the transmitting tissue of the upper ovarian locule (UOL). (**e'**) Magnified square region of e. (**f**) The pistillate flower of the second year showed that some parts of the pollen tubes were located near the micropyle (Mic). The area enclosed by a dotted line represents the transmitting tissue, and the area enclosed by a solid line indicates newly formed transmitting tissue observed between the style joining site and the ovarian locule after May of the second year. It encloses the upper ovarian locule (UOL) area. The granular or linear fluorescent signals observed within the transmitting tissue or ovule were identified as pollen tubes, while fluorescent signals observed in other areas were determined to originate from tissues other than pollen tubes. The longitudinal sections of the pistillate flowers were collected on the following dates: (**a**, **a'**) 12 Jun 2023; (**b**, **b'**) 11 Jul 2023; (**c**) 9 Dec 2022; (**d**, **d'**) 10 Apr 2023; (**e**, **e'**) 14 May 2023; (**f**) 12 Jun 2023. *PT* pollen tube, *St* style, *Sg* stigma, *TT* transmitting tissue, *L* ovarian locule, *OI* Outer Integument, *II* Inner Integument, *Mic* Micropyle. Scale bars = 100 μm.

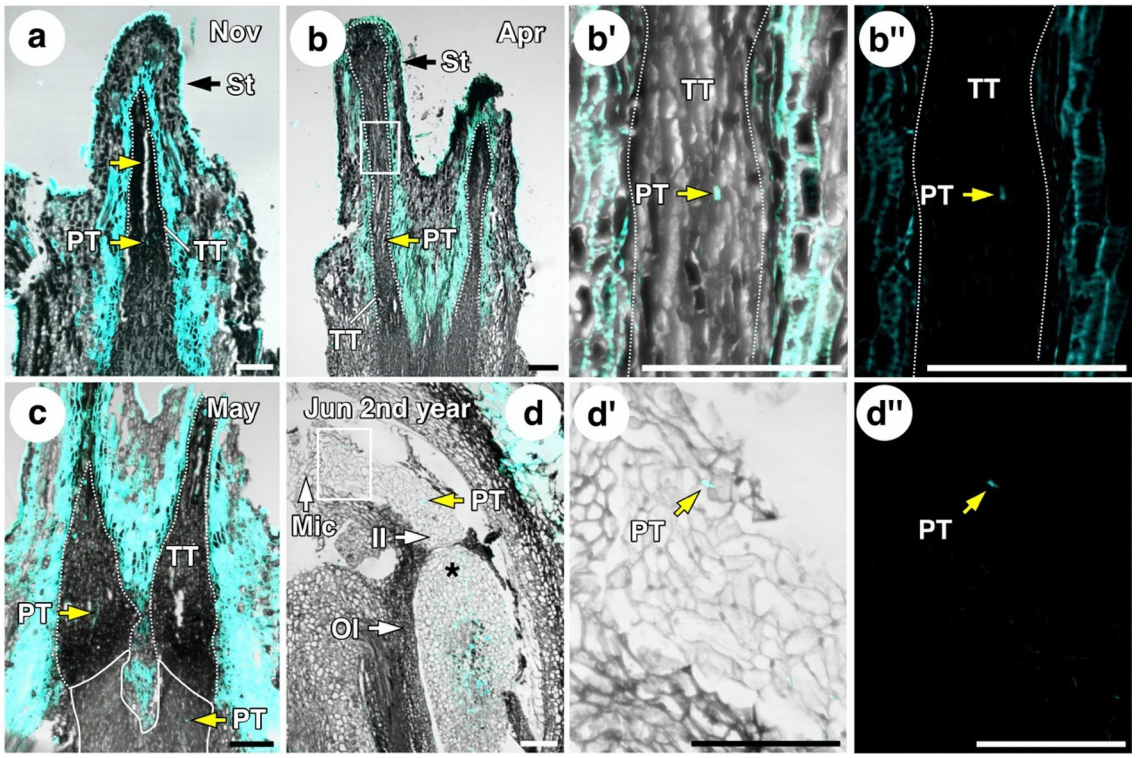

Fig. 4. Seasonal progression of pollen tube growth in autumn samples. (**a**, **b**) Pollen tubes were arrested in the transmitting tissue at the style joining site (SJS) from November of the first year to April of the second year. (**b'**, **b"**) Magnified square region of b. b' represents a merged image obtained using two excitation lasers (405 nm and 488 nm) and dia illumination (DIA), whereas b" was captured exclusively under a 405 nm excitation laser. (**c**) Some pollen tubes resumed elongation from the style joining site and reached the tissue of the upper ovarian locule (UOL). (**d**) The pistillate flower of the second year showed that some parts of the pollen tubes were located near the micropyle (Mic). (**d'**, **d"**) Magnified square region of d. d' represents a merged image obtained using two excitation lasers (405 nm and 488 nm) and dia illumination (DIA), whereas d" was captured exclusively under a 405 nm excitation laser. The area enclosed by a dotted line represents the transmitting tissue, and the area enclosed by a solid line indicates newly formed transmitting tissue observed between the style joining site and the ovarian locule after May of the second year. It encloses the upper ovarian locule (UOL) area. The granular or linear fluorescent signals observed within the transmitting tissue or ovule were identified as pollen tubes, while fluorescent signals observed in other areas were determined to originate from tissues other than pollen tubes. The longitudinal sections of the pistillate flowers were collected on the following dates: (**a**) 11 Nov 2022; (**b**, **b'**, **b"**) 10 Apr 2023; **c**, 14 May 2023; (**d**, **d'**, **d"**) 12 Jun 2023. *PT* pollen tube, *St* style, *TT* transmitting tissue, *OI* Outer Integument, *II* Inner Integument, *Mic* Micropyle. Scale bars = 100 μm.

Synchrony in the timing of pollen tube regrowth and ovule maturation

For pollen tubes, the proportion in the SJS category remained high until April of the year following flowering, while the proportion in the UOL category increased in May of the second year (Fig. 7a,b). The transition from the SJS to the UOL category in May coincided with the transition of ovules from the Un or Op category to the MMC category (Fig. 7c,d). By June, the proportion of pollen tubes in the Mic category increased (Fig. 7a,b), synchronizing with the increase of ovules in the ES stage (Fig. 7c,d).

Flowering and fertilization phenology in temperate Fagaceae species

Regardless of the genus, pollination mode (wind or insect pollinations), and fruiting patterns (1-year fruiting or 2-year fruiting), fertilization time seems to synchronize from spring to early summer. Among the species summarized in Fig. 8, all 1-year fruiting species flowered from April to June, followed by a fertilization delay of approximately two weeks to two months. On the other hand, in 2-year fruiting species, flowering occurred over a broad period from April to October, followed by a fertilization delay of 8 to 14 months, with fertilization occurring the following year, specifically in June to July of the second year. These results underscore the importance of temporally coordinating fertilization phenology with flowering and fruiting phenology in seasonal environments to avoid unfavorable winter conditions.

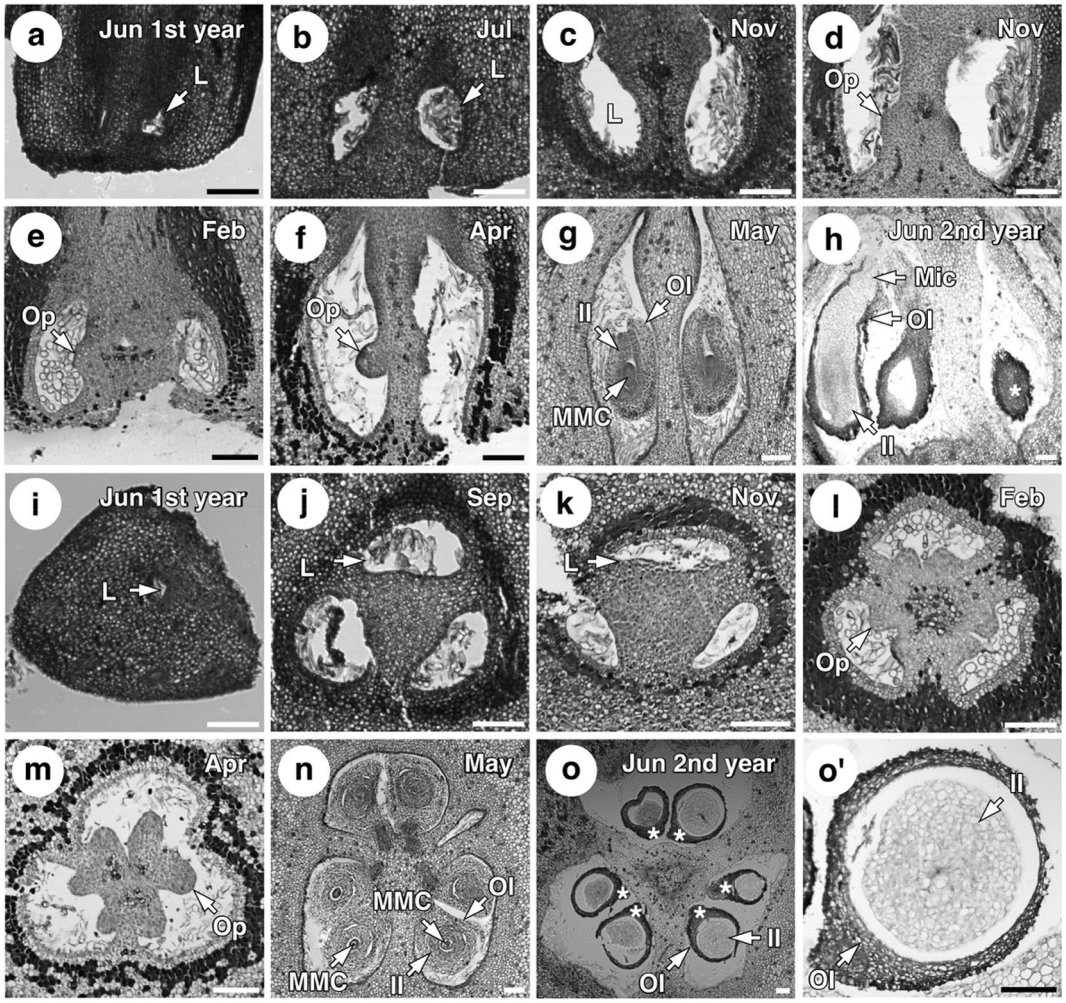

Fig. 5. Seasonal progression of ovule development of spring samples. In (**a**–**h**) and (**i**–**o**, **o'**) longitudinal and transverse sections are shown, respectively. (**a**) Undeveloped ovarian locules (Un) were observed at the base of the pistillate flower collected in June of the first year. (**b**, **c**) Ovarian locules were enlarged, but the ovarian meristem had not yet developed. (**d**) A pistillate flower collected in November showed ovule primordia (Op) in November of the first year. (**e**, **f**) Ovule primordia were observed on the placenta from February to April of the second year. (**g**) The ovule developed into the megaspore mother cell (MMC) stage. Outer integument and inner integument were also differentiated in this stage in May of the second year. (**h**) In June of the second year, the ovule developed into the embryo sac (ES) stage. (**i**) Undeveloped ovarian locules (Un) were observed from the pistillate flower collected in June of the first year. (**j**, **k**) Ovarian locules were enlarged, but the ovarian meristem had not yet developed. (**l**, **m**) Ovule primordia (Op) were observed along with the placenta. (**n**) Ovules were differentiated at the megaspore mother cell (MMC) stage in May of the second year. (**o, o'**) Ovules were developed at the embryo sac (ES) stage in June of the second year. (**o'**) Magnified view of an ovule collected in June of the second year. The sections of the pistillate flowers were collected on the following dates: (**a**, **i**) 12 Jun 2023; (**b**) 11 Jul 2023; (**j**) 20 Sep 2023; (**c**, **d**, **k**) 11 Nov 2022; (**e**, **l**) 11 Feb 2023; (**f**, **m**) 10 Apr 2023; (**g**, **n**) 14 May 2023; (**h**, **o**, **o'**) 12 Jun 2023. The locations of the ovules are marked by asterisks. *L* ovarian locule, *Op* ovule primordium, *MMC* megaspore mother cell, *OI* outer integument, *II* inner integument, *Mic* Micropyle. Scale bars = 100 μm.

Discussion

Our results demonstrate that pistils produced in both spring and autumn overwintered and exhibited synchronous ovule maturation after winter. In both spring and autumn flowers, pollen tubes were arrested at the style joining site before winter, with their regrowth synchronized the following spring. Additionally, ovule development was delayed by 10 months in spring flowers and 6 months in autumn flowers, leading to a synchronized onset of ovule maturation in both sets of flowers. These findings are consistent with previous studies[16,17] and suggest that delayed fertilization, coupled with 2-year fruiting habit, may have evolved to ensure that fertilization and seed maturation occur during the more favorable season from spring to autumn[5,6].

Previous studies in *Quercus* and *Lithocarpus* have demonstrated that the style joining site is a significant location for pollen tube cessation[16,17,19,23]. The transmitting tissue at this site was stained deeply darkish

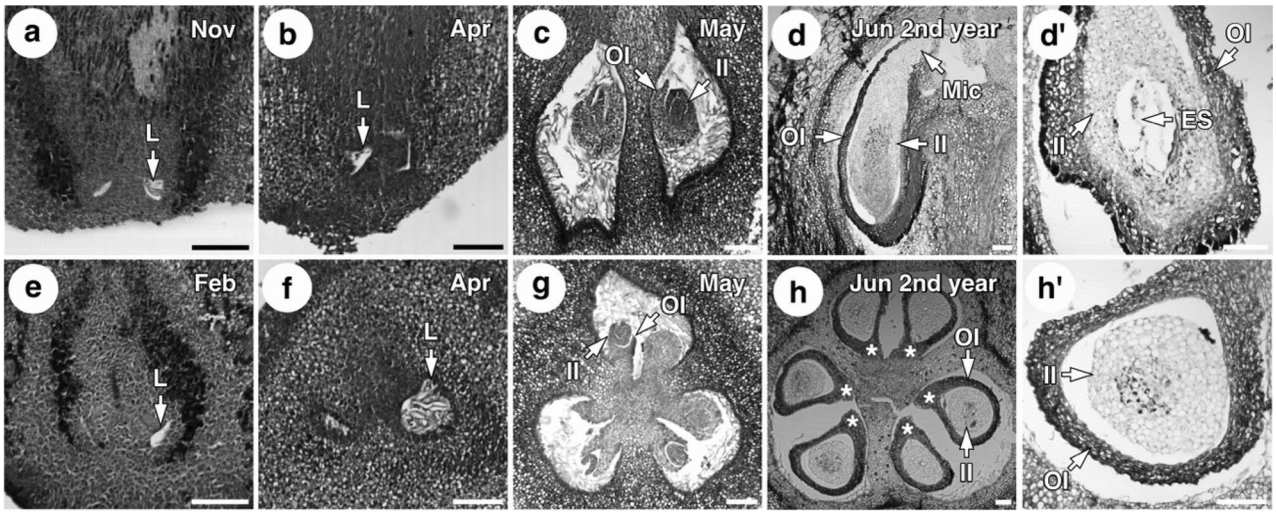

Fig. 6. Seasonal progression of ovule development of autumn samples. In (**a**–**d**, **d'**) and (**e**–**h**, **h'**) longitudinal and transverse sections are shown, respectively. (**a**, **b**) Undeveloped ovarian locules (Un) were observed at the base of the pistillate flower collected from November of the first year to April of the second year. (**c**) The ovule developed into the megaspore mother cell (MMC) stage in May of the second year. (**d**, **d'**) Ovules were developed at the embryo sac (ES) stage in June of the second year. (**d'**) Embryo sac formation was observed in June of the second year. (**e**, **f**) Undeveloped ovarian locules (Un) were observed at the base of the pistillate flower collected from November of the first year to April of the second year. (**g**) Ovules were differentiated at the megaspore mother cell (MMC) stage in May of the second year. (**h**, **h'**) Ovules were developed at the embryo sac (ES) stage in June of the second year. (**h'**) Magnified view of an ovule in June of the second year. The sections of the pistillate flowers were collected on the following dates: (**a**) 11 Nov 2022; (**e**) 11 Feb 2023; (**b**, **f**) 10 Apr 2023; (**c**, **g**) 14 May 2023; (**d**, **d'**, **h**, **h'**) 12 Jun 2023. The locations of the ovules are marked by asterisks. *L* ovarian locule, *OI* outer integument, *II* inner integument, *Mic* Micropyle. Scale bars = 100 µm.

gray, and pollen tubes were retained within this area, unable to reach the lighter, gray-stained tissue located below it[16]. Similar to previous studies, in *L. edulis*, we observed that pollen tubes stopped growth at a position corresponding to the darkly stained transmitting tissue of the style joining site, corroborating these previous findings. Therefore, the deeply dark-stained transmitting tissue within the style joining site is considered a common feature associated with the arrest of pollen tube growth in *Quercus* and *Lithocarpus*.

After the arrest of pollen tube growth during winter, we observed that some pollen tubes resumed elongation in the spring of the following year, which is also noted in *Q. acutissima*[16] and *L. dealbatus*[17]. In *Q. acutissima* and *L. dealbatus*, after ceasing elongation at the upper septum and micropyle for 1–2 weeks to one month, pollen tubes resume growth, leading to fertilization[17]. Although this study did not capture the exact moment of fertilization due to low pollination rates and a limited number of available samples, it is reasonable to infer that fertilization occurred in June, as indicated by the development of young fruits observed in July[6]. The significance of delayed fertilization has predominantly been investigated in the context of pollen tube competition and male–female interactions[2,3,7–17]. For example, in Fagales, it has been suggested that repeated pauses in pollen tube growth may enhance sperm competition, promote female selection, or prevent self-pollination, with these processes potentially contributing to delayed fertilization[3,8–11,13–17]. In addition to sperm competition, the requirement for winter cold may have evolved as a factor contributing to the nearly year-long fertilization delay observed in 2-year fruiting species of Fagaceae.

A highly resolved molecular phylogeny reconstructed from nuclear genome data supports the sequential divergence of major lineages within Fagaceae, with *Fagus* diverging first, followed by a clade containing *Castanea* and *Castanopsis*, then *Lithocarpus*, and finally *Quercus*[41–43]. Given that *Fagus* and *Castanea* consist exclusively of species with 1-year fruiting, whereas multiple species in *Castanopsis*, *Lithocarpus*, and *Quercus* exhibit 2-year fruiting[5,44], the results presented in Fig. 8 suggest that prolonged fertilization delay associated with 2-year fruiting may have evolved after the divergence of *Fagus*. Further analyses incorporating a broader range of species will be necessary to elucidate the evolutionary history of this prolonged delayed fertilization.

To explore the molecular mechanism of delayed fertilization in response to winter cold, employing genome-wide transcriptomics analyses under seasonal conditions, called molecular phenology[45–47], will be useful. Molecular phenology offers a valuable framework for studying gene–environment interactions, and a previous study identified genes differentially expressed between 1-year and 2-year fruiting species[6]. Integrated analyses of gene expression patterns related to ovule development, pollen tube growth, and fertilization, combined with the histological studies reported here, will be a promising approach to elucidate the molecular mechanisms and evolutionary aspects of delayed fertilization.

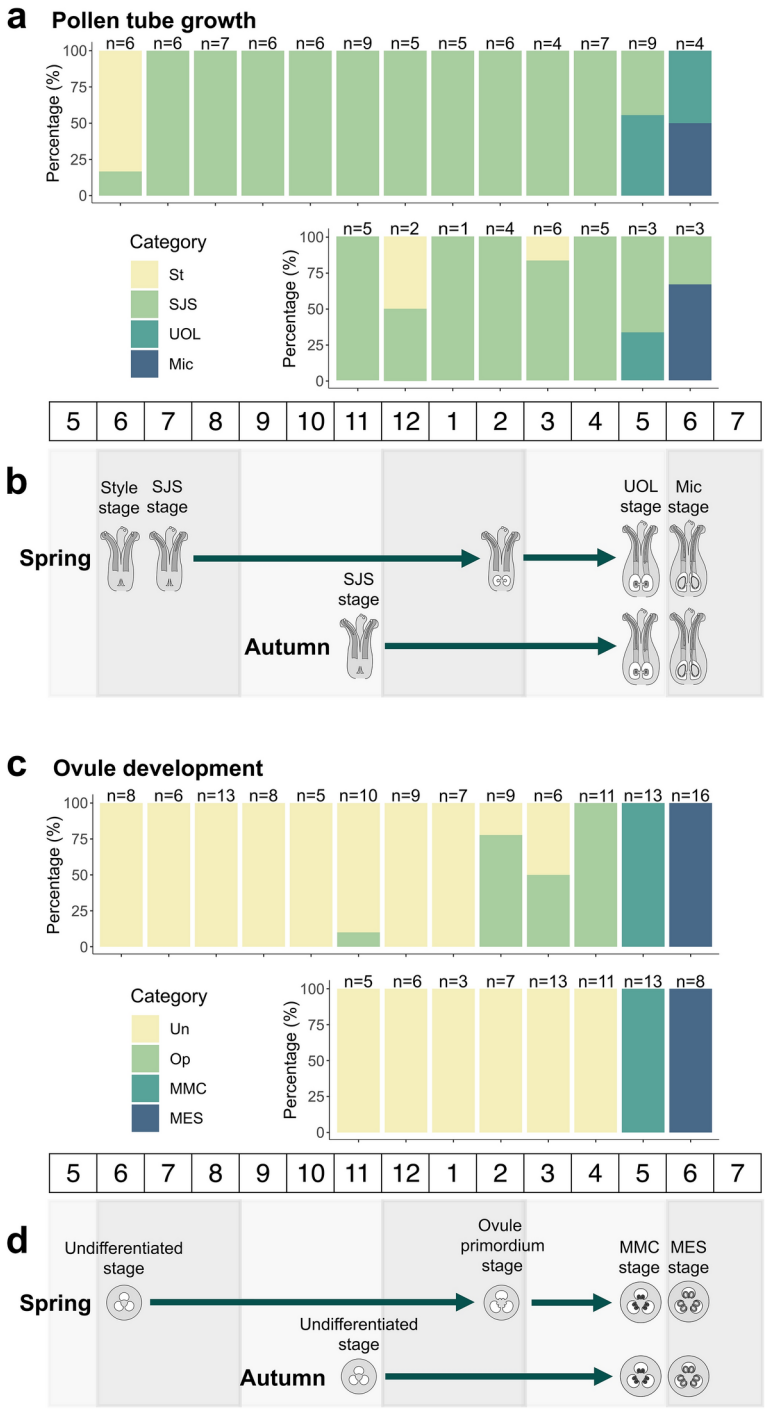

Fig. 7. Summary of seasonal progression of pollen tube growth and ovule development. (**a**) Seasonal dynamics of pollen tube growth. The state of pollen tube growth is identified as four stages, style (St), style joining site (SJS), upper ovarian locule (UOL), and the micropyle (Mic) stages. The status of each month is shown as a percentage of the above four stages. The top panel shows results for spring samples, while the bottom panel shows results for autumn samples. The number of samples referenced is indicated at the top of each bar plot. (**b**) Summary of seasonal dynamics of pollen tube growth of *L. edulis* based on the results of (**a**). (**c**) Seasonal progression of ovule development. The state of ovule development is identified as four stages, undifferentiated (Un), ovule primordium (Op), megaspore mother cell (MMC), and embryo sac (ES) stages. The status of each month is shown as a percentage of the above four stages. The top panel shows results for spring samples, while the bottom panel shows results for autumn samples. (**d**) Summary of seasonal progression of ovule development of *L. edulis* based on the results of (**c**).

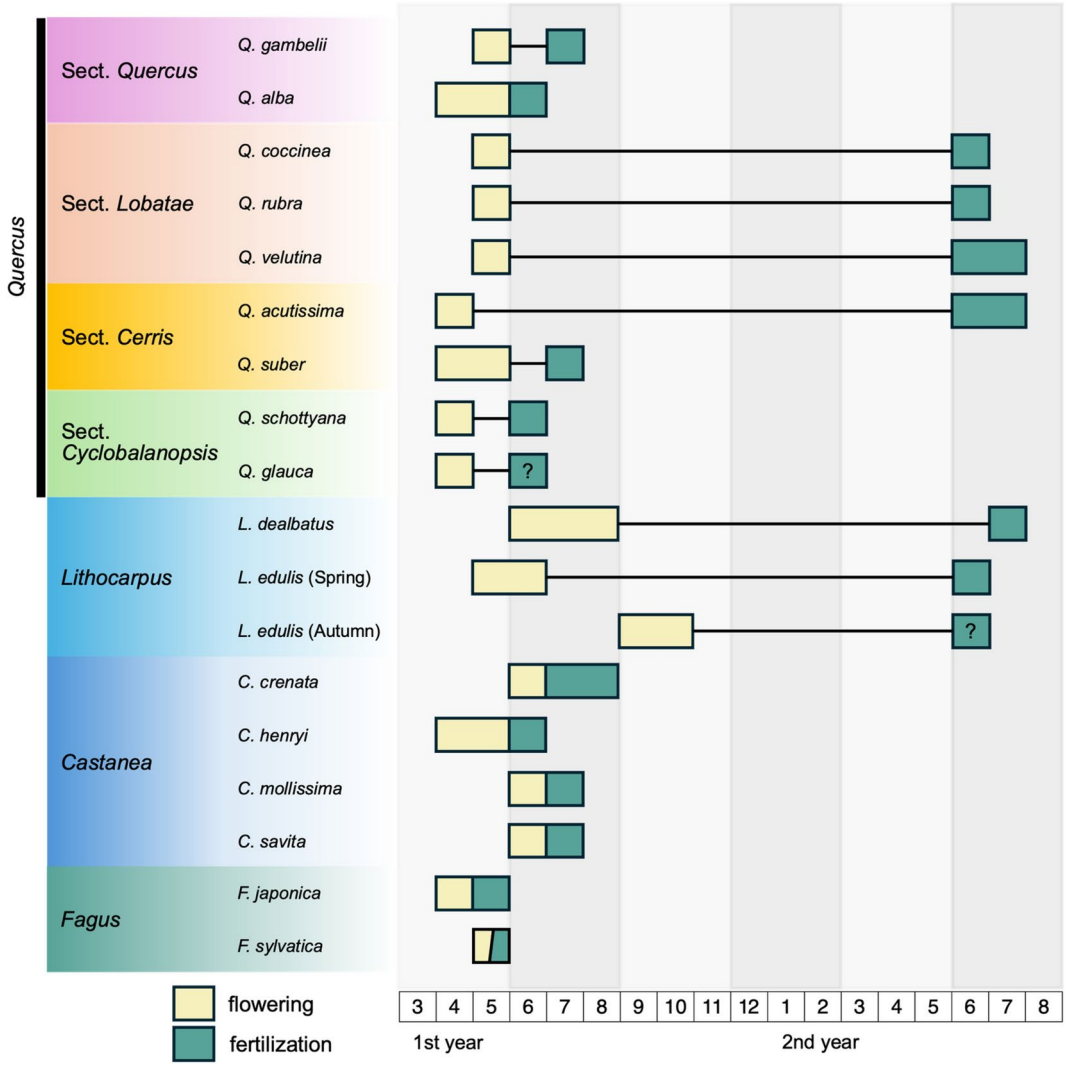

Fig. 8. Flowering and fertilization phenology of the Fagaceae species. Yellow and green indicate the month flowering and fertilization were confirmed, respectively. The month that fertilization is presumed to have occurred is marked with "?". In *Q. suber*, 1-year fruiting tree, 2-year fruiting tree, and a mixture of both types in the same individual have been reported[27,40]. In this study, only the results of the 1-year fruiting type, for which results have already been published, are shown.

Conclusion

We examined the location of pollen tubes and the developmental stage of ovules in pistillate flowers produced in spring or autumn nearly every month from pollination to fertilization in *Lithocarpus edulis*, a species exhibiting a 2-year fruiting pattern. Our findings indicate that the process leading to fertilization remains synchronous across different flowering periods, suggesting that overcoming an unfavorable winter to achieve fertilization could be significant in the delayed fertilization associated with a 2-year fruiting strategy. We observed that ovules stay undifferentiated until exposed to winter conditions and develop rapidly after winter. This suggests that cold temperatures may synchronize the development stages of ovules produced at different times. These results imply that delayed fertilization may have evolved as a strategy to ensure that fertilization and seed maturation coincide with favorable seasons, thereby avoiding the adversities of winter.

Data availability

Additional data supporting the findings are contained in the Supplementary Information.

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Author contributions
T.S. and A.S. conceived the research idea. T.S. collected the samples and carried out the experiments with the support of K.O., M.M.K., and A.S. T.S. and A.S. wrote the original draft, and all authors approved the final submission.

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Declarations

Competing interests
The authors declare no competing interests.

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Correspondence and requests for materials should be addressed to T.S. or A.S.