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Original Article

# Molecular phylogeny and postharvest morphology of petals in two major *Nelumbo nucifera* cultivars in Thailand

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ABSTRACT

*Nelumbo nucifera* is a symbolic flower of Buddhism and widely used for decoration in Asia. In most cases its buds are used as cut-flowers, which usually do not open and the outer petals turn black, leading to loss of visual quality and thus decreasing their economic value in markets. In Thailand, two major cultivars, Sattabongkot and Saddhabutra, are used and exported to foreign countries. To investigate the difference between these two cultivars, their molecular phylogeny and postharvest morphology were examined. Using 25 simple sequence repeat (SSR) markers, the cultivar Saddhabutra was very close to the cultivar Satabuto. These results showed a high bootstrap (BS) value of 96%. In contrast, lotus cultivars in Thailand including Sattabongkot and Saddhabutra, showed a BS value of 90%. Saddhabutra and Satabuto are classified in the same group with high similarity, whereas Sattabongkot shows relatively lower similarity. Petal blackening started earlier in Saddhabutra than Sattabongkot, indicating a vase-life difference among cultivars, with Sattabongkot and Saddhabutra having a vase life of 72 and 54 h, respectively. The epidermal cells of normal petals of Sattabongkot and Saddhabutra showed freshness and a round shape with turgid cells. The petal blackening was accompanied with a reduction of the area and perimeter in both cultivars. Sattabongkot had fewer stomata than Saddhabutra. The study provided a molecular classification of the Thailand lotus cultivars and provides a useful technique for the quantification of the postharvest quality of lotus cultivars.



## Introduction

Lotus (*Nelumbo nucifera*) is widely grown in South Asia, being a symbol of Buddhism. Its floral buds are used for decoration as cut-flowers (Fig. 1A,B). In Thailand, Sattabongkot and Saddhabutra are two major commercial cultivars. Flowers of both cultivars bear more than 50 petals, whose colors are pink and white in Sattabongkot and Saddhabutra, respectively (Fig. 1C,D; Imsabai and van Doorn, 2013). Despite their importance in the market, lotus petals readily turn black from their edge shortly after cutting (Imsabai et al., 2010). The petal blackening is accompanied by senescence and a shorter vase life, reducing the value of the cut-flower in the market. A phytohormone ethylene is involved in senescence in lotus flowers. Treatment of ethephon, a source of ethylene, accelerates the petal blackening, whereas 1-MCP, an inhibitor of the ethylene receptor, delays senescence and extends the vase life of cut lotus flowers (Imsabai et al., 2010). Petal blackening and the lack of flower opening are related to water stress and carbohydrates after harvesting accompanied by an increase in ethylene production (Netlak and Imsabai, 2016).

Anatomical and ultrastructural analyses during the blackening process in petal cells of another lotus cultivar (Sattabhud) have revealed that the blackening is accompanied with the deterioration of cells, including cell collapse, precipitation of substances in the cytoplasm and shrinkage of the protoplasm (Kunsongkeit and Sathornviriyapong, 2011). This cellular morphology is important to understand the physiological process of petal blackening.

Cultivars of the genus *Nelumbo* have been classified by their morphological characters, including flower, leaf, plant size, and

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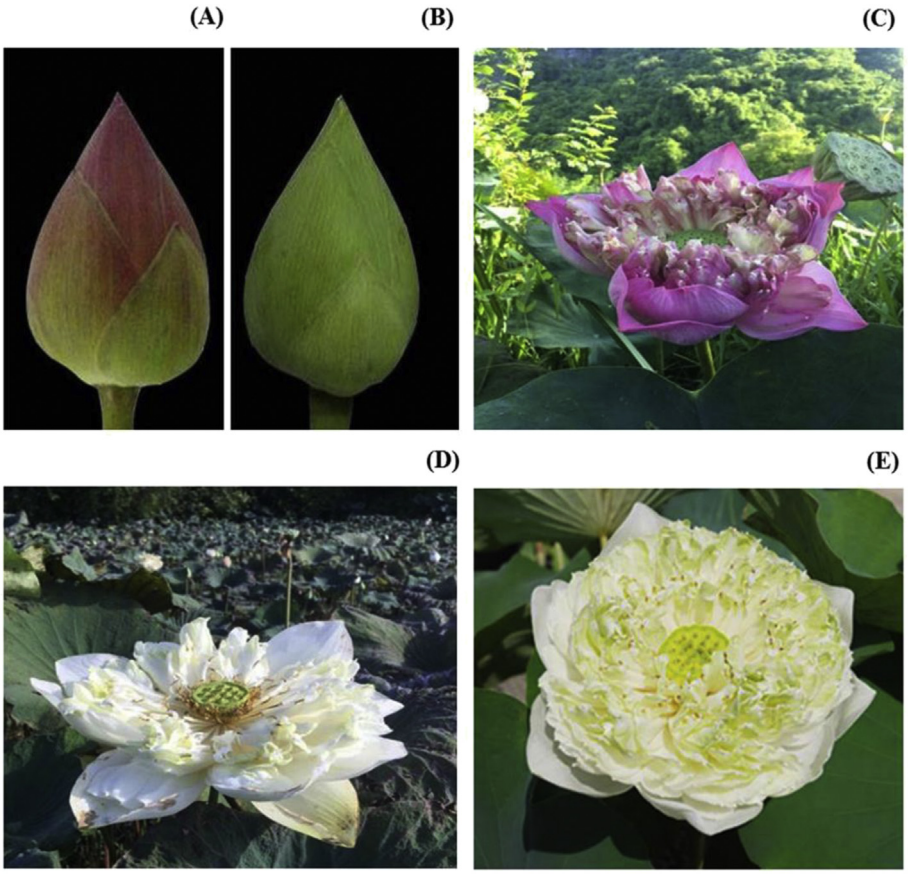


**Fig. 1.** Cut-floral bud in lotus flower cultivars Sattabongkot (A) and Saddhabutra (B), and floral morphology after flowering in cultivars Sattabongkot (C), Saddhabutra (D) and Satabuto (E).

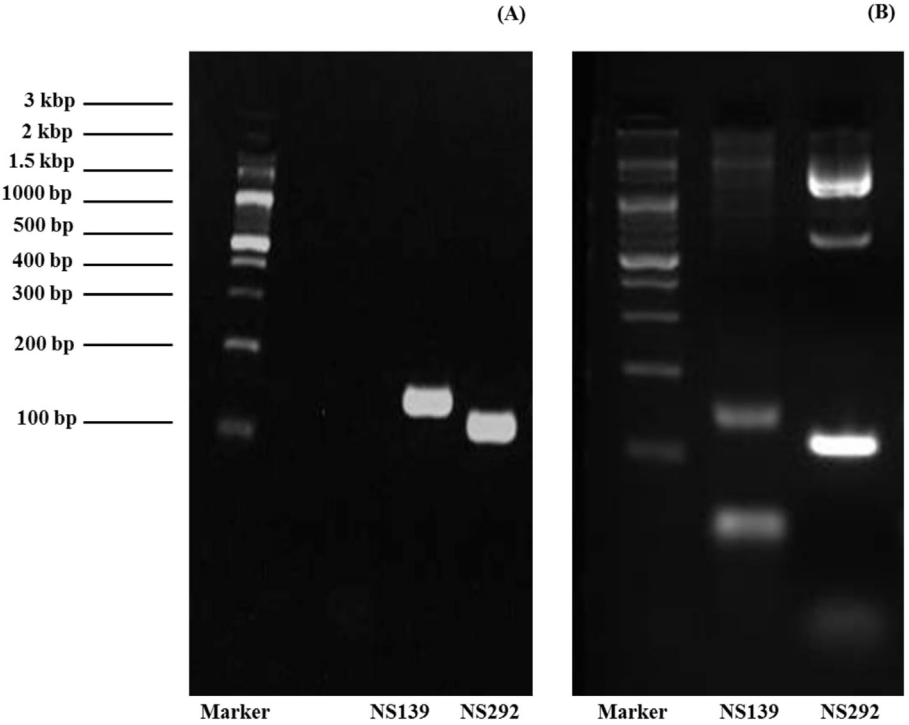


**Fig. 2.** Polymerase chain reaction amplification of two lotus cultivars Sattabongkot (A) and Saddhabutra (B) obtained by primer pairs NS-139 and NS-292, respectively. A molecular weight standard is shown at the left.

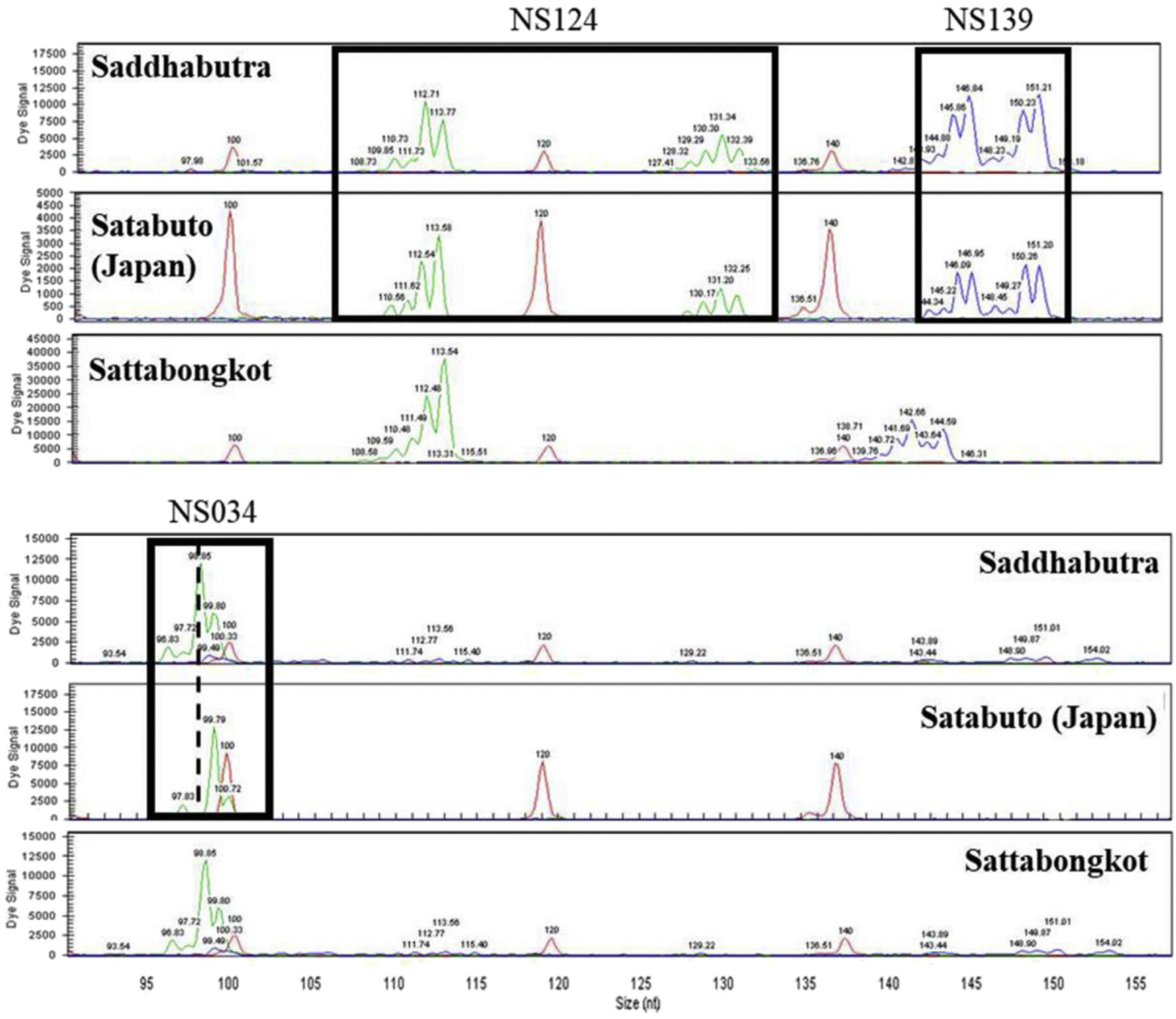


**Fig. 3.** Simple sequence repeat (SSR) polymorphisms in *Nelumbo* cultivars. Fragment analysis of the SSR markers NS124, NS139 and NS034 in three cultivars is shown as an example. The Saddhabutra and Satabuto-specific alleles of SSR markers NS124, NS139 and NS034 are enclosed by black outlines. A nucleotide difference observed in SSR marker NS034 is marked with a dashed vertical line. A molecular weight standard is shown at the bottom.

rhizome (Watanabe, 1990; Wang and Zhang, 2005). However, morphological characters are often hard to distinguish because they can change due to variation within a cultivar or environmental effects. Kubo et al. (2009) developed the simple sequence repeat (SSR) markers in *Nelumbo* and determined the genetic differences between *N. lutea* and *N. nucifera*. The SSR markers have several advantages compared to other DNA markers such as relatively high polymorphism, codominant inheritance, and abundance in eukaryotic genomes (Kalia et al., 2011). The classification of *N. nucifera* cultivars in Thailand, such as Sattabongkot and Saddhabutra, with SSR markers, has not been studied to date. Therefore, these markers are useful to classify the commercial cultivars in molecular level.

The phylogenetic relationships and morphology of two major lotus cultivars in Thailand were investigated. They were genetically separated and showed different timing for petal blackening. The morphological features found here can be used for the selection of or identifying better cultivars with a longer vase life.

## Materials and methods

### *Plant materials*

The *Nelumbo* cultivars Sattabongkot and Saddhabutra were harvested from a commercial lotus field in Payakhan district, Phatthalung province, Thailand. Young leaves were collected and kept moist until DNA extraction for each cultivar. Floral buds at stage 5—just before flower opening with a diameter of 6.0–6.5 cm (Netlak and Imsabai, 2016)—were collected and examined. After harvest, stems were cut in water to a length of 25 cm and placed in 300 mL of distilled water in a room at ambient temperature.

*Phylogenic analysis with simple sequence repeat markers*

Total DNA was isolated from 0.1 g of leaf tissues of Sattabongkot and Saddhabutra using a DNeasy Plant Mini Kit (Qiagen; Valencia, CA, USA) as reported previously (Kubo et al., 2009). The DNA concentration was checked using a fluorometer (DyNA Quant 200, GE Healthcare Life Sciences, Pittsburgh, PA, USA) and 3% agarose gel electrophoresis. The 25 SSR markers used for phylogenetic analysis were: NSh02, NS001R, NS012, NS020, NS034, NS049, NS077, NS080, NS092, NS124, NS139, NS149, NS160, NS169, NS219, NS224, NS227, NS262, NS292, NS294, NSe01, NSe03, Nel06, Nel14 and PR09 (Pan et al., 2007; Tian et al., 2008; Kubo et al., 2009). The SSR fragments were amplified using polymerase chain reaction (PCR) with each SSR primer pair, either of which was fluorescence-labeled (Sigma-Aldrich; St. Louis, MO, USA). The PCR conditions were: initial denaturation at 94 °C for 1 min, 30 cycles at 94 °C for 30 s, 50 °C for 1 min and 72 °C for 1 min, with a final extension at 72 °C for 5 min. Thereafter, products were electrophoresed using a CEQ8000 sequencer (Backman Coulter; Fullerton, CA, USA) for determining fragment sizes and polymorphism scoring. Generating a neighbor-joining (NJ) tree was performed using the Populations ver. 1.2.32 program (Langella, 2011). Bootstrap (BS) analysis was performed with 1000 replicates. The Sattabongkot and Saddhabutra data were integrated with the phylogenetic tree that was published in Kubo et al. (2015).

*Morphological analyses of petal blackening*

Blackening of the petals of cut floral buds in a graduated cylinder containing distilled water was examined every 6 h. Each cut lotus was held at ambient temperature (28 ± 1 °C), 80–85% relative humidity and natural light (light from about 600 h to 1800 h). The degree of blackening was defined as a percentage (10% = onset of petal blackening, 25% = black patches on the edge of petals, 50% = whole edges covered by blackening, 75% = blackening region expanding and 100% = whole petals blackened), as shown in Fig. 5. For cellular analysis, petals at 50% black patches were fixed in a solution of acetic acid: ethanol of 1:9 for 1 h, hydrated in an ethanol series (90, 70, 50 and 30%) for 20 min in each step, and immersed in clearization solution (10 g of chloral hydrate, 2.5 mL of glycerol and 1.25 mL of distilled water) overnight. Epidermis cells were imaged using a light microscope BX43 (Olympus Corporation, Tokyo, Japan), and their area and perimeter were measured using the ImageJ software (Rasband, 2012). The number of stomata was counted based on the area of the petal. Data were analyzed using the General Linear Model program of Microsoft Excel 2010. Statistical data were compared using Student's *t*-test with significance tested at $p \leq 0.05$.

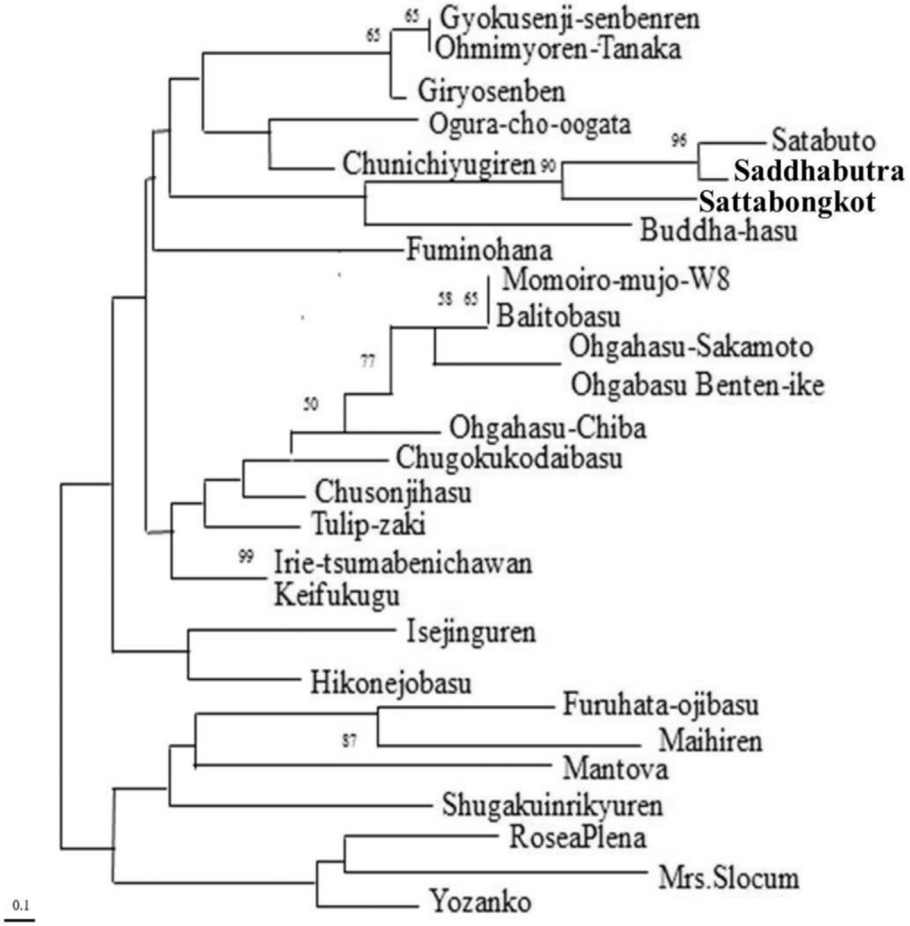


**Fig. 4.** Neighbor-joining phylogram of lotus cultivars based on 25 single sequence repeat (SSR) markers. Two Thailand-derived cultivars that were added to the data of Kubo et al. (2015) in this study are indicated with bold letters. Values at nodes are bootstrap values (≥50%) after 1000 replicates. Scale bar = genetic distance $D_A$ (Nei et al., 1983).

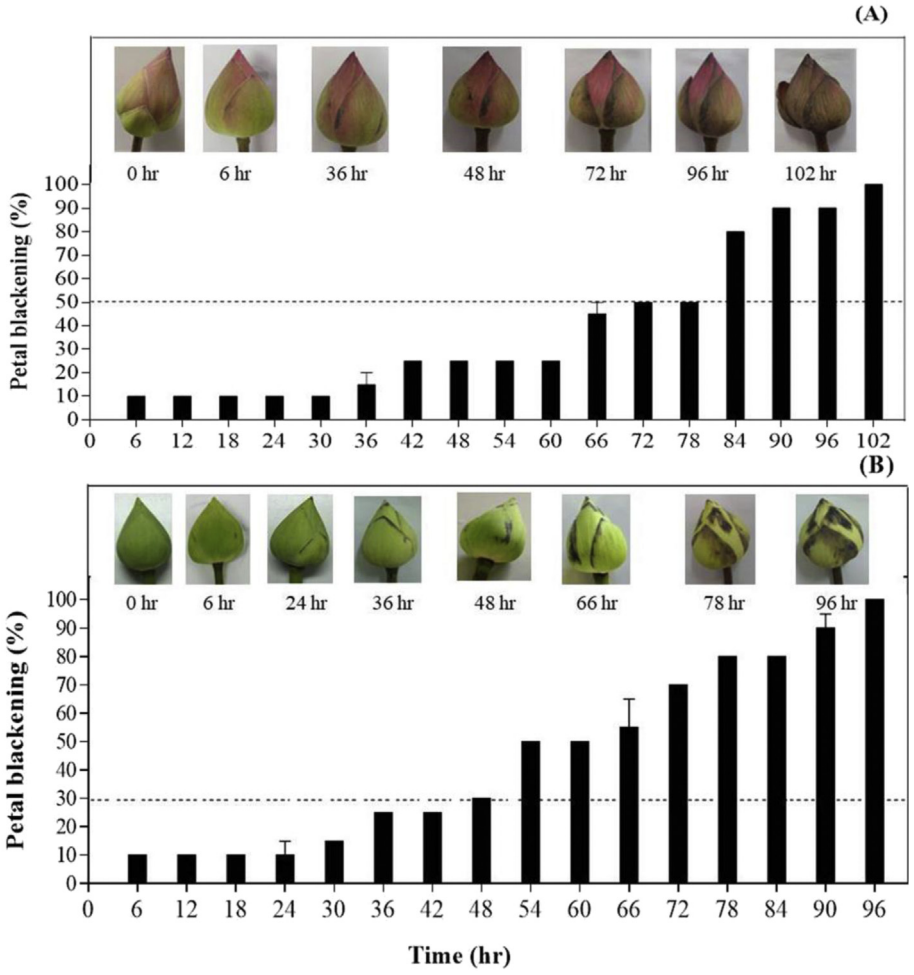


**Fig. 5.** Development in petal blackening during vase life of cut lotus cultivars Sattabongkot (A) and Saddhabutra (B). The degree of 50% blackening of the petal was used to define the end of vase life.

## Results

### *Polymorphisms of simple sequence repeats in both cultivars*

To clarify the phylogenetic relationship in the two major lotus cultivars, the genotypes were examined using 25 SSR markers. Initially, amplification used two markers, NS139 and NS292, to check the quality of extracted DNA, producing 150 bp and 100 bp amplicons of expected sizes, respectively (Fig. 2). These DNA were confirmed as suitable for subsequent analysis. The 25 SSR markers were used that had been previous successful in the classification of 98 *Nelumbo* cultivars (Kubo et al., 2009). Fig. 3 shows an example of the fragment analysis of NS124, NS139 and NS034 markers. In markers NS124 and NS139, there was no polymorphism between Saddhabutra and Satabuto (see black outlines in Fig. 3, upper panel), whereas Sattabongkot showed different band patterns compared to the other cultivars (Fig. 3, upper panel). A polymorphism was observed between Saddhabutra and Satabuto only in NS034 out of the 25 markers (see the dashed vertical line in Fig. 3, lower panel). These results portrayed the different polymorphism pattern among cultivars. The genetic distance of both cultivars was calculated from the allele data and a phylogenetic tree was constructed using the NJ method. The result suggests that the cultivar Saddhabutra was in the same group as the cultivar Satabuto (Fig. 1E) with a high BS value (96%) and the cultivar Sattabongkot was closely related to Saddhabutra with a high BS value (90%). The results were integrated with the phylogenetic tree that had been published previously (Fig. 4, Kubo et al., 2015). Saddhabutra was classified in the same group with the cultivar Satabuto, which was grown and collected in Japan, supporting the description that Satabuto was brought from Thailand (The Lotus Flower Society of Kyoto, 2012). Sattabongkot was classified to the same subgroup but with a relatively lower BS value (Fig. 4). Bhudda-hasu is in the same clade with them, suggesting that it was also brought from Southeast Asia.

### *Senescence and petals blackening of cultivars Sattabongkot and Saddhabutra*

The cut floral buds of Sattabongkot and Saddhabutra placed in distilled water showed rapid petal blackening (Fig. 5). The first sign of petal blackening appeared after 6 h in both cultivars. Sattabongkot had developed 50% blackening after 72 h of vase life, whereas Saddhabutra produced blackening after 54 h; eventually 100% blackening developed after 102 and 96 h for Sattabongkot and Saddhabutra, respectively (Fig. 5). These data indicate that the cultivar Sattabongkot, a pink petal cultivar, has a longer vase life.

### *Cellular morphology during petal blackening*

The cell shape change was investigated during petal blackening in the Sattabongkot and Saddhabutra cultivars. The epidermal cells of normal petals of Sattabongkot and Saddhabutra showed

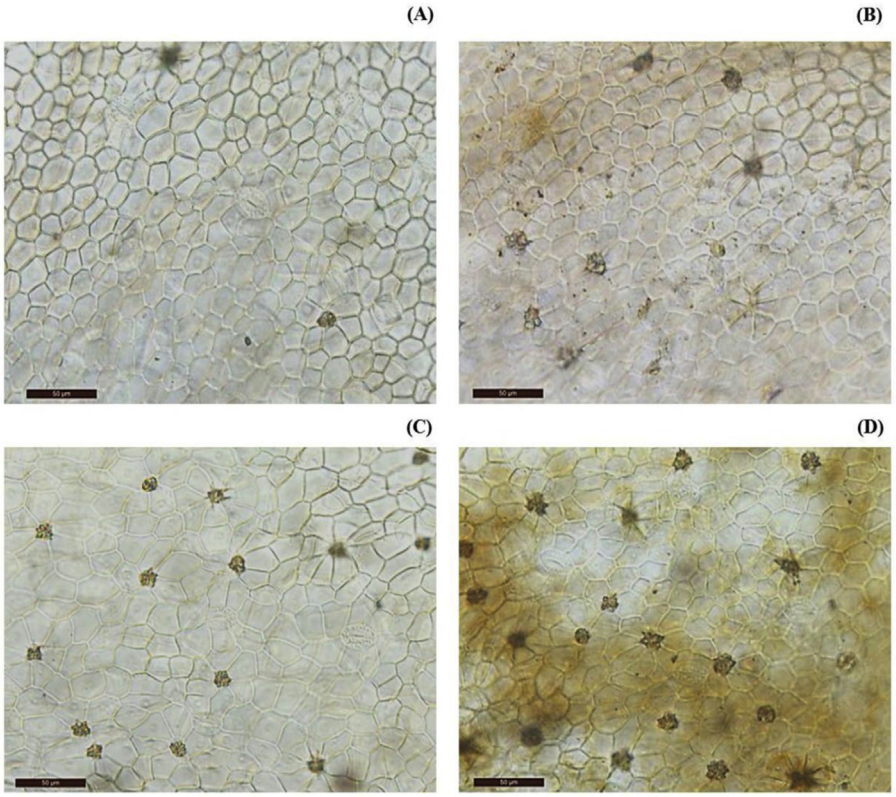


**Fig. 6.** Anatomical changes during senescence of epidermis cell in Sattabongkot, normal petals (A) and with petal blackening symptoms (B), and Saddhabutra, normal petals (C) and with petal blackening symptoms (D). Scale bar = 50 μm.

freshness and a round shape with turgid cells (Fig. 6A,C). In addition, there were turgid stomata, especially the guard cells in the normal tissue of both cultivars. The morphology of petal blackening of Sattabongkot and Saddhabutra occurred in the tissues during senescence. The petal blackening was a result of the incomplete arrangement of tissues and the size of cells being smaller than normal petals. A brown area was observed on surrounding all cells. This resulted in the shrinkage and the dark color of the petals (Fig. 6B,D).

*Quantification of cells*

Cell size measurement, the area and perimeter or length of cell were studied using the ImageJ software. The epidermis cells of both cultivars decreased less in area and perimeter than the normal cells. The area and perimeter of the normal petals of both cultivars were significantly more than that of the blackening petal (Fig. 7A,B). The cultivar Sattabongkot had a significantly larger area and perimeter of normal cells than that of the blackening cells. Because water is related to petal blackening (Imsabai et al., 2010), the stomatal number was counted in both cultivars; Sattabongkot had less stomata than Saddhabutra (Fig. 8).

## Discussion

The classification of the lotus cultivars Sattabongkot and Saddhabutra was conducted based on SSR markers developed from genomic and EST sequences. Previously, Kubo et al. (2009) developed 35 markers, while 26 markers have been developed by other researchers (Pan et al., 2007; Tian et al., 2008). The current study tested 25 SSR markers to identify which could be used as PCR markers, and some of the SSRs were also amplified products of similar size. The polymorphisms observed here can be useful for the analysis of closely-related lotus cultivars.

There was no strong overall correlation between petal characters and genetic relationships (Kubo et al., 2009). Therefore, it would be helpful to know whether or not the cultivars with different flower morphology (petal color) and postharvest life are genetically related. Phylogenetic analysis indicated that Saddhabutra and Satabuto are closely related. Their flowers have more than 50 white petals (Fig. 1B,D,E), suggesting that Satabuto is mostly identical to Saddhabutra. Satabuto was introduced from Thailand to Japan in 2011 according to records (The Lotus Flower Society of Kyoto, 2012), suggesting a mistake for pronunciation during transfer. A direct comparison of postharvest physiology between these closely related cultivars may be interesting if they could be simultaneously examined in the same place. Sattabongkot, with pink petals, was classified into another subgroup but with a 90% BS value, indicating the relatively small narrow genetic diversity of *Nelumbo* species (Xue et al., 2006).

Not only the genetic differences, but also the postharvest quality of cut flowers was different in the two cultivars. In general, senescence of cut flowers leads to the degradation of proteins, lipids and nucleic acids. This process leads to a programmed cell death (Hoeberichts et al., 2005). The timing of petal blackening was different between the two cultivars, with the white cultivar blackening earlier than the pink one. This may suggest that the pink color, due to anthocyanin, prevents blackening. Petal blackening occurs by oxidation of polyphenol oxidase (a phenolic compound accumulated in the cell cytoplasm) and by shrinkage of the protoplasm (Kunsongkeit and Sathornviriyapong, 2011). Investigation of petal blackening in many other cultivars with different petal

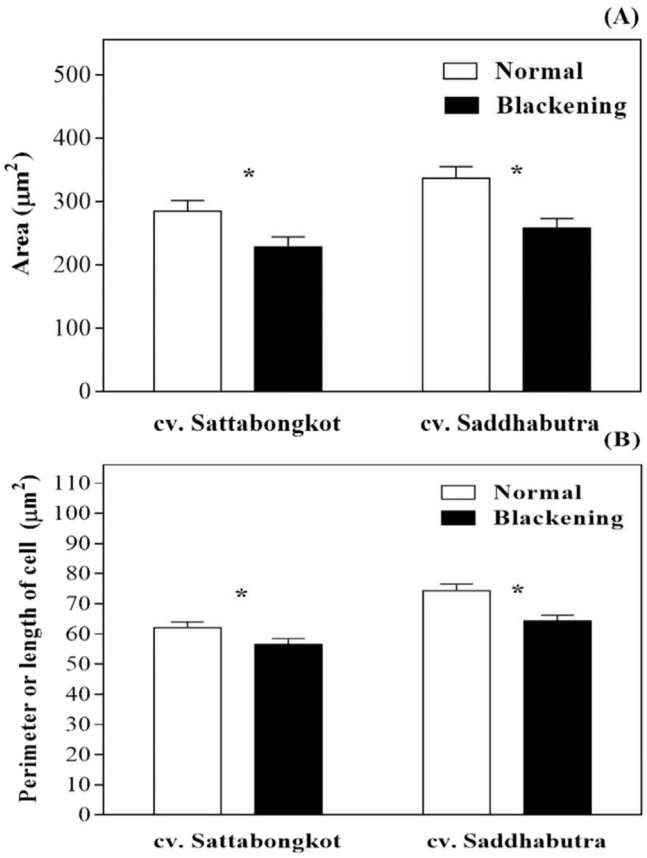


**Fig. 7.** Change of quantification of epidermis cell in cut lotus using the clearization method. Area of cell (A) and perimeter or length of cell (B). *: significant differences between Normal and Blackening at $p \leq 0.05$. Vertical bars represent the average values with SE (n = 100).

colors is required to confirm this hypothesis. Another possibility is associated with the number of stomata, as shown in Fig. 8. Sattabongkot, with fewer stomata, has a longer vase life, suggesting lower water loss. Because petal blackening can be caused by external environmental factors such as temperature, relative humidity and the amount of oxygen, it was concluded that the petal color and epidermal structure are the reasons for the longer vase life in the Sattabongkot cultivar. Still, in the market, white cultivars

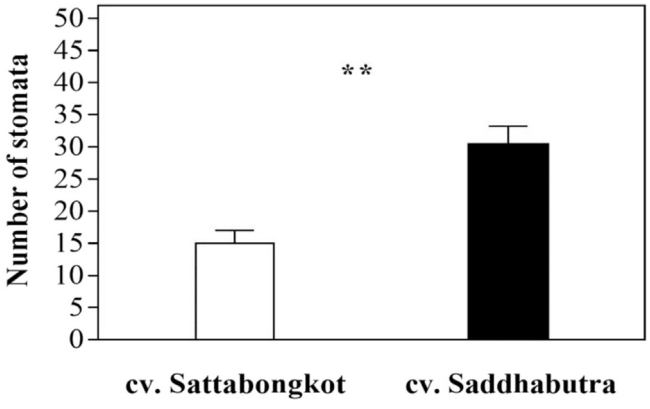


**Fig. 8.** Stomatal numbers in normal cell of cut lotus cultivars Sattabongkot and Saddhabutra using the clearization method. **: significant differences between Sattabongkot and Saddhabutra at $p \leq 0.01$. Vertical bars represent the average values with SE (n = 3).

are preferred for decoration in Thailand. Therefore, methods for prolonging the vase life and preventing petal blackening need to be developed.

Imsabai et al. (2010) reported that Saddhabutra has green outer petals and many white inner petals, with the onset of petal blackening in 24 h and a vase life of 2–3 d. The study on anatomical associate senescence and postharvest quality of the Japanese cut lotus flower cultivar Seika White lotus showed that the onset of petal blackening occurred on day 2 and developed to 50% on day 7. The Seika White lotus has a vase life of 7.0 d (Salaemae et al., 2017). These results suggest that the difference in vase life longevity is due to different genetic backgrounds, but it is possible that the growing conditions are another cause of the difference. Because lotus is a climacteric plant, it has a high respiration rate and ethylene production, which induce rapid senescence. That is why various types of physiological stresses are often accompanied by an increase in ethylene production, affecting rapid senescence (Abeles et al., 1992). Similarly, during the senescence of carnation flowers, a climacteric increase in ethylene production occurs and the evolved ethylene induces in-rolling of petals and wilting of whole flower (Satoh, 2011).

In conclusion, Saddhabutra was in the same group with Satabuto, as evidenced by a high BS value of 96%. The lotus cultivars in Thailand are close, showing BS values of 90%. Postharvest cut lotus flowers held in distilled water at ambient temperature (28 ± 1 °C) showed the onset of petal blackening at 6 h vase life. Sattabongkot and Saddhabutra had a vase life of 72 h and 54 h, respectively. Both cultivars showed cell morphological change with petal blackening.

## Conflict of interest

The authors declare no conflicts of interest associated with this manuscript.

## Acknowledgments

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